

## PA09 – NK CELLS AND NK CELL RECEPTORS

## PA09/1 REQUIREMENTS OF IL-15/SIGNALING FOR NK CELL DIFFERENTIATION, MATURATION AND EFFECTOR FUNCTIONS

O. D'Orlando<sup>1</sup>, Z. Orinska<sup>1</sup>, S. Bulfone-Paus<sup>1</sup><sup>1</sup>Research Center of Borstel, Department of Immunology and Cell Biology, Borstel Hamburg, Germany

Natural Killer (NK) cells are effector lymphocytes of the innate immune system that mediate cellular cytotoxicity and play an essential role in the recognition and eradication of virally infected cells and tumors. Many crucial questions were answered during the last 30 years about NK cell biology and activity nevertheless a lot of work has to be done. In this regard the role of cytokines in NK cells differentiation and survival is almost defined but the role of cytokines in NK cell activation is still unclear.

Receptors for IL-2 and IL-15 are expressed in NK cells at various developmental stages and *in vitro* studies indicate that IL-2 and IL-15 can support NK cell differentiation and survival. However how cytokines influence NK cell cytotoxicity and cytotoxic granule content has still to be elucidated.

Our results show that IL-15Ra-deficient NK cells display an impaired cytotoxic activity compared to wild type. Based on our preliminary results this is due to a lack of pre-existing pools of Granzyme A and Perforin in IL-15Ra-deficient NK cells. Indeed, even after 6 days of IL-15 stimulation *in vitro*, IL-15Ra-deficient NK cells express neither Granzyme A nor Perforin proteins. Instead Granzyme B is expressed and its expression is IL-15Ra chain independent. Conversely IL-2/15 receptor beta, a chain shared by IL-2 and IL-15 cytokines, has an important role in Granzyme B expression after IL-15 treatment. Analyzing the phenotype of IL-15Ra-deficient NK cells we found that those cells are not fully differentiated into mature NK cells.

Thus, our findings suggest that the IL-15Ra chain is required for NK cells' maturation and cytotoxic activity as well as Granzyme A and Perforin expression, but dispensable for Granzyme B induction. Moreover IL-15/IL-15R alpha-mediated signaling is required for the generation of pre-existing pools of Granzyme A and Perforin in NK cells.

These results indicate that the role of the IL-15Ra chain in the regulation of NK cell cytotoxicity might be due to an association between NK cell granula content and the expression of the IL-15Ra chain.

## PA09/2 INTEGRATION OF ACTIVATING AND INHIBITORY RECEPTOR SIGNALING BY REGULATED VAV1 PHOSPHORYLATION

D. Urlaub<sup>1</sup>, S. Mesecke<sup>1,2</sup>, H. Busch<sup>2,3</sup>, R. Eils<sup>2,4</sup>, C. Watzl<sup>1</sup>

<sup>1</sup>University Heidelberg, Institute for Immunology, Heidelberg, Germany, <sup>2</sup>German Cancer Research Center (DKFZ), Division of Theoretical Bioinformatics, Heidelberg, Germany, <sup>3</sup>Center for Biological Systems Analysis, Computational Biology, Freiburg, Germany, <sup>4</sup>BIOQUANT, University of Heidelberg, Institute of Pharmacy and Molecular Biotechnology, Heidelberg, Germany

The effector functions of NK cells are controlled by a balance of positive and negative signals that are transmitted by different surface receptors. To date our understanding about the integration of positive and negative signals and the decision-making process inside NK cells remains poor.

With the help of bioinformatic modeling we try to understand how NK cells first integrate antagonizing signals and then compute a reliable killing decision. Gradual signal input through activating and inhibitory receptors is integrated to come to a "yes or no" decision by the NK cell to kill an attached target cell. Triggering of activating receptors leads to activation of Src family kinases and Vav1 phosphorylation, whereas inhibitory receptors dephosphorylate Vav1 via the phosphatase SHP-1. Therefore, we proposed in a first hypothesis, that Vav1 is a decision making point in the signal transduction network. Based on published signaling events a simplified model of the receptor proximal events of NK cell activation was generated. Six putative signaling modules that could influence the activation can be included into the model. The predictions derived from this model family were compared with experimental data. Our experiments showed that increased triggering of activating receptors lead to a rapid switch-like increase in Vav1 phosphorylation. Similarly, titrating the engagement of inhibitory receptors resulted in switch-like dephosphorylation of Vav1. Comparing the experimental results to the predictions derived from the family of simplified models showed that kinase association with the activating receptors and the enhanced activity of SHP-1 bound to inhibitory receptors is essential to re-create such a response. Interestingly, other concepts of immune receptor signaling such as phosphatase segregation and kinase auto-phosphorylation were not necessary in our mathematical model to create a physiological response. Our current model is consistent with a central role of Vav1 in the decision making process of NK cells and enables a novel insight into the integration of positive and negative signals during lymphocyte activation.

## PA09/3 CD62L AS A MARKER FOR POLYFUNCTIONAL NK CELLS

K. Juelke<sup>1</sup>, M. Killig<sup>1</sup>, B. Morandi<sup>2</sup>, G. Ferlazzo<sup>2</sup>, A. Thiel<sup>3</sup>, C. Romagnani<sup>1</sup>

<sup>1</sup>German Rheumatism Center (DRFZ), Berlin, Germany, <sup>2</sup>University of Messina, Genova, Italy, <sup>3</sup>Berlin Brandenburg Center for Regenerative Therapies (BCRT), Berlin, Germany

**Objectives:** In the last years, several reports have elucidated that similar to T cells, NK cells do not represent a homogenous population of effectors ready to produce cytokines and to kill, but that a division of labour by different NK cell subsets is actually occurring. Human CD56bright NK cells are characterized by the ability to extensively proliferate and to produce IFN- $\gamma$  in response to cytokine stimulation, while CD56dim NK cells are highly cytotoxic. Moreover, even CD56dim NK cells represent a heterogeneous population concerning the expression of inhibitory receptors, CD27 or CD62L, a lymph node homing marker which is commonly used to identify different T cell maturation stages. The aim of this study is to analyse whether the expression of CD62L or other markers correlates with different NK cell functions and might be used to identify different stages of NK cell differentiation or maturation.

**Methods:** Human NK cells have been FACS sorted according to the expression of CD56, CD62L and/or other markers and the capacity to proliferate and to produce IFN- $\gamma$  after cytokine stimulation as well as to kill target cells has been analysed.

**Results:** We show that CD62L expression among CD56dim defines a unique population of polyfunctional NK cells which combines the ability of CD56bright NK cells to proliferate *in vivo* and *in vitro* and to produce IFN- $\gamma$  after cytokine stimulation, with a high cytotoxic potential, typical of CD56dim NK cells. This is not a consequence of differences in expression of cytokine receptors, activating receptors or cytotoxic molecules but rather results from differences in signal transduction events. Percentage of CD56dim CD62Lpos NK cells decreases with age and CD56dim CD62Lpos KIRneg NK cells display longer telomeres than CD56dim CD62Lneg KIRpos ones.

**Conclusions:** We identified a subset of polyfunctional CD56dim NK cells which express the lymph node homing marker CD62L and display an intermediate phenotype between less mature CD56bright and terminally differentiated CD56dim CD62Lneg NK cells and might be generated during differentiation or priming of CD56bright NK cells in inflamed lymph nodes.

## PA09/4 RECEPTOR-MEDIATED RECOGNITION OF MELANOMA BY NATURAL KILLER CELLS AND ITS IMPLICATIONS FOR IMMUNOTHERAPY

E. Carbone<sup>1</sup>, T.H. Almosawy<sup>1</sup>, L. Tadepally<sup>2</sup>, B. Shannon<sup>3</sup>, S. Kimpfner<sup>4</sup>, F. Ursini<sup>2</sup>, L. Ruggeri<sup>5</sup>, M. Capanni<sup>5</sup>, V. Umansky<sup>4</sup>, A. Paschen<sup>4</sup>, A. Sucker<sup>4</sup>, D. Pende<sup>6</sup>, V. Groh<sup>7</sup>, R. Biassoni<sup>8</sup>, P. Hoglund<sup>9</sup>, M. Kato<sup>10</sup>, K. Shibuya<sup>11</sup>, D. Schadendorf<sup>4</sup>, A. Anichini<sup>12</sup>, S. Ferrone<sup>13</sup>, A. Velardi<sup>13</sup>, K. Karre<sup>14</sup>, A. Shibuya<sup>11</sup>, F. Colucci<sup>3</sup>

<sup>1</sup>University of Catanzaro 'Magna Graecia', Experimental and Clinical Medicine, Catanzaro, Italy, <sup>2</sup>University of Catanzaro 'Magna Graecia', Catanzaro, Italy, <sup>3</sup>The Babraham Institute, Cambridge, United Kingdom, <sup>4</sup>Heidelberg and University Hospital Mannheim, Mannheim, Germany, <sup>5</sup>University of Perugia, Perugia, Italy, <sup>6</sup>Istituto Nazionale per la Ricerca sul Cancro, Genova, Italy, <sup>7</sup>Fred Hutchinson Cancer Research Center, Seattle, United States, <sup>8</sup>Istituto Giannina Gaslini, Genova, Italy, <sup>9</sup>Karolinska Institute, Stockholm, Sweden, <sup>10</sup>Chubu University, Aichi, Japan, <sup>11</sup>Institute of Basic Medical Sciences, Ibaraki, Japan, <sup>12</sup>Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy, <sup>13</sup>University of Pittsburgh Cancer Institute, Pittsburgh, United States, <sup>14</sup>Karolinska Institutet, Stockholm, Sweden

NK cells use a variety of receptors to detect disease. Receptors for MHC monitor the integrity of immunological "self", whereas NKG2D participates in immunosurveillance by binding stress-induced molecules. Other receptors, such as Natural cytotoxicity receptors (NCRs) and DNAM-1 are emerging as key receptors in cancer. However, the specific molecular interactions for melanoma recognition are largely unknown and it remains to be determined whether NK cells control metastatic progression and route of dissemination. Here we showed that human melanoma cell lines derived from lymph node metastases expressed ligands for NCRs and DNAM-1, but not NKG2D. Compared to metastases derived from other anatomical sites or route of dissemination, they were more susceptible to NK cell lysis and preferentially targeted by adoptively transferred NK cells in a xenogenic model of cell therapy. DNAM-1 and NCR ligands were also found on mouse spontaneous melanomas and melanoma cell lines. Interference with DNAM-1 and NCRs by antibody blockade or genetic disruption in knockout mice reduced killing of melanoma cells. These results show that DNAM-1 and NCRs are required for innate immunity to melanoma and provide a background to design NK cell-based immunotherapeutic strategies.

**PA09/5 REGULATION OF NATURAL KILLER CELL TRAFFICKING BY CD81**B. Krämer<sup>1</sup>, D. Schulte<sup>1</sup>, C. Körner<sup>1</sup>, C. Zwank<sup>1</sup>, M. Michalk<sup>1</sup>, A. Hartmann<sup>1</sup>, H.-D. Nischalke<sup>1</sup>, A. Vogt<sup>1</sup>, B. Langhans<sup>1</sup>, M. Coenen<sup>1</sup>, T. Sauerbruch<sup>1</sup>, U. Spengler<sup>1</sup>, J. Nattermann<sup>1</sup><sup>1</sup>University of Bonn, Department of Internal Medicine I, Bonn, Germany

Natural killer (NK) cells, a heterogeneous sub-population of lymphocytes, are critically involved in the regulation of both innate and adaptive immune responses in humans. Besides their participation in the control of tumors and viral infections, they also regulate inflammatory processes, mediating both beneficial and detrimental effects.

To effectively fulfil their role in immune surveillance, proper trafficking of NK cells is essential. However, the mechanisms and factors governing NK cell recruitment are only poorly dissected.

Here, we describe the functional role of tetraspanins, a family of evolutionary conserved cell-surface proteins, in modulating migration and transmigration of human NK cells.

We demonstrate expression of various tetraspanins on NK cell. Furthermore, we show that stimulation of the NK cell-expressed tetraspanin CD81 induces phosphorylation of ERM proteins in a ROCK-dependent fashion leading to NK cell polarization thereby facilitating NK cell migration towards various chemokines/cytokines. Finally, we provide evidence for a role of CD81 in promoting adhesion of NK cells to components of the extracellular matrix, a prerequisite for extravasation of lymphocytes in inflamed tissues.

Thus, our data suggest the tetraspanin CD81 to be importantly involved in the regulation of NK cell recruitment.

**PA09/6 FUNCTIONAL MATURATION AND STRUCTURAL ADAPTATION OF NK CELL REPERTOIRES IN RESPONSE TO HLA CLASS I LIGANDS DURING HUMAN DEVELOPMENT**K. Schönberg<sup>1</sup>, K. Ioannidou<sup>2</sup>, M. Hejazi<sup>1</sup>, G. Kögler<sup>1</sup>, T. Tonn<sup>3</sup>, A. Borkhardt<sup>2</sup>, J.C. Fischer<sup>1</sup>, M. Uhrberg<sup>1</sup><sup>1</sup>Heinrich-Heine-University Düsseldorf, Institute of Transplantation Diagnostics and Cell Therapeutics, Düsseldorf, Germany, <sup>2</sup>University Children's Hospital Düsseldorf, Department of Paediatric Oncology, Haematology and Immunology, Düsseldorf, Germany, <sup>3</sup>DRK Blutspendedienst Baden-Württemberg-Hessen, Institute of Transfusion Medicine and Immune Hematology, Frankfurt a.M., Germany

Natural killer (NK) cells sense changes in the levels of MHC class I on target cells through the expression of clonally-distributed inhibitory receptors. It was previously shown that the human killer cell Ig-like receptor (KIR) repertoire is shaped by interaction with specific HLA class I ligands. To unravel, when and how NK cell education takes place, a comparative analysis of NK cell repertoires was performed in peripheral blood (PB, n=154) and cord blood (CB, n=90). An 8-color flow cytometric protocol employing custom-labeled reagents was established to assess the clonal KIR and NKG2A repertoires on NK and T cells. The frequency of NKG2A-expressing NK cells declines from birth to adulthood by 50%. Reciprocally, the expression of KIR is almost doubled during the first 10 years of life. The NK cell repertoires change during development in response to HLA class I: whereas KIR expression in PB shows a significant adaptation to the respective HLA-C ligands, a similar effect was not seen in CB. Functionally, although the overall degree of degranulation was similar in CB and PB as measured by CD107 surface expression, the loading of cytotoxic granules with perforin and granzyme B was significantly lower in CB compared to adult NK cells. This translates into significantly reduced killing activity of CB derived NK cells. The present survey shows distinctive structural and functional changes in the NK cell compartment during development, which will also be relevant for stem cell transplantation. The results will be related to currently discussed models of class I-dependent NK cell education (licensing).

**PA09/7 CHARACTERIZATION OF NKP80-INDUCED NK CELL RESPONSES**S. Kutteruff<sup>1</sup>, A. Steinle<sup>1</sup><sup>1</sup>Institute for Cell Biology, Department of Immunology, Eberhard Karls University Tuebingen, Tuebingen, Germany

Natural Killer cells (NK cells) possess the ability to lyse certain virally infected cells and tumour cells. NK cells distinguish these "dangerous" cells from healthy cells by means of an arsenal of activating and inhibitory NK cell receptors. Like NKG2D, the activating NK receptor Nkp80 is a homodimeric C-type lectin-like receptor (CTLR) encoded in the human Natural Killer Gene Complex (NKC) on chromosome 12. Recently, we reported that Nkp80 interacts with the myeloid specific "orphan" CTLR AICL. Both, Nkp80 and AICL are adjacently encoded in the telomeric subregion of the human NKC. We further demonstrated that Nkp80-AICL interaction mediates an activating crosstalk between NK cells and myeloid cells and hypothesized that this interaction may critically affect the initiation of immune responses.

To further elucidate functional consequences of Nkp80 activation, we performed whole genome microarray analysis of freshly isolated human NK cells after several hours of stimulation with an immobilized anti-Nkp80 mAb or an isotype control. Following NK cell stimulation via Nkp80 transcripts of the chemokines CXCL9 and CXCL10 were strongly up regulated. The kinetics of mRNA expression of these and other chemokines/cytokines following Nkp80 triggering were analyzed by real-time PCR, and revealed a distinct expression profile when compared to stimulation of other activating NK receptors. Both, CXCL9 and CXCL10 are known to induce T cell migration to inflamed tissue and to polarise CD4 T cells towards T<sub>H</sub> responses. Apart from these chemokines, additional genes implicated in the modulation of CD4 T cell responses were differentially regulated by Nkp80-mediated stimulation of NK cells including MIP-1 $\alpha$ , MIP-1 $\beta$ , lymphotxin- $\alpha$ , OX40L, 4-1BB, Jagged 1, and IL10.

Hence, in ongoing studies we address a potential implication of Nkp80-stimulated NK cells in the modulation of T cell responses.

**PA09/8 IL-15 TRANS-PRESENTATION PROMOTES HUMAN NK CELL DEVELOPMENT AND DIFFERENTIATION IN VIVO**N. Huntington<sup>1</sup><sup>1</sup>Institut Pasteur, Paris, France

The in vivo requirements for human natural killer (NK) cell development and differentiation into cytotoxic effectors expressing inhibitory receptors for self-MHC-I (KIRs) remain undefined. Here we dissect the role of IL-15 in human NK cell development using Rag2<sup>-/-</sup> $\gamma$ C<sup>-/-</sup> mice transplanted with human hematopoietic stem cells. Human NK cell reconstitution was intrinsically low in this model due to the poor reactivity to murine IL-15. While exogenous human IL-15 alone made little improvement, IL-15 coupled to IL-15 $\alpha$  significantly augmented human NK cells. IL-15+IL-15 $\alpha$  complexes induced extensive NK cell proliferation and differentiation resulting in accumulation of CD16+KIR+ NK cells, which was not uniquely dependent on enhanced survival or preferential responsiveness of this subset to IL-15. Human NK cell differentiation in vivo required human IL-15 and progressed in a linear fashion from CD56hiCD16-KIR- to CD56loCD16+KIR- then finally CD56loCD16+KIR+. This data provides the first evidence that IL-15 trans-presentation regulates human NK cell homeostasis. Use of human IL-15 receptor agonists generates a robust humanized immune system model to study human NK cells in vivo. IL-15 receptor agonists may provide therapeutic tools to improve NK cell reconstitution following bone marrow transplants, enhance graft versus leukemia effects and increase the pool of IL-15 responsive cells during immunotherapy strategies.

**PA09/9 SEMAPHORIN 7A: A NEW MODULATOR OF NK CELL FUNCTION**C. Figueiredo<sup>1</sup>, L. Schlasha<sup>1</sup>, J. Zenk<sup>1</sup>, B. Eiz-Vesper<sup>1</sup>, A. Seltsam<sup>1,2</sup>, R. Blasczyk<sup>1</sup><sup>1</sup>Hannover Medical School, Institute for Transfusion Medicine, Hannover, Germany, <sup>2</sup>German Red Cross, Blood Services NSTOB, Institute Springe, Springe, Germany

Semaphorins are a wide family of phylogenetically conserved signalling molecules. Semaphorin 7A (Sema7A) is expressed on a variety of myeloid and lymphoid cells as well as on red blood cells and tumour cells. It has shown the ability to promote axon outgrowth and modulate T cell-mediated immune responses through integrins. In this study, we investigated the influence of Sema7A on NK cell phenotype and function.

Recombinant soluble Sema7A was expressed in HEK cells and used to stimulate primary NK cells. The NK cells were measured for activation of the mitogen-activated protein kinase (MAPK) pathway and changes in the surface expression of KIR2DL1, KIR2DL2, Nkp30, Nkp44, Nkp46, NKG2A and NKG2D receptors. NK cell proliferation assays were performed in the presence or absence of Sema7A. Cytotoxic assays using K562 cells were performed with non-stimulated or Sema7A pre-stimulated NK cells. To study whether the effect of Sema7A on NK cell function was mediated by integrins, a mutated Sema7A protein with an altered integrin-binding motif was used as negative control.

Soluble Sema7A showed the ability to bind NK cells and to induce phosphorylation of the non-receptor protein kinase focal adhesion kinase (FAK) and extracellular regulated kinases (ERK) 1 and 2. Sema7A caused a downregulation of NKG2D, Nkp30 and Nkp46 expression by up to 30%, 47% and 43%, respectively. The proliferation rate of NK cells decreased by 62% in presence of Sema7A, in comparison with non-stimulated NK cells. Pre-incubation of NK cells with Sema7A reduced the cytotoxic activity of NK cells against K562 cells by up to 60%. The mutated Sema7A protein did not show any effect on NK cell proliferation or cytotoxicity suggesting that integrin receptors are involved in Sema7A-mediated NK cell activation.

This study demonstrates for the first time that Sema7A is a potent inhibitor of NK cell function. This observation further highlights Sema7A as an important molecule involved in mechanisms of tumour cell escape.

**PA09/10 REGULATION OF NK CELL MIGRATION BY NKG2D ACTIVATION**E. Serrano-Pertierra<sup>1</sup>, E. Cernuda-Morollón<sup>1</sup>, C. Lopez-Larrea<sup>1</sup><sup>1</sup>Hospital Universitario Central de Asturias, Unidad de Histocompatibilidad y Trasplante, Oviedo, Spain

**Introduction:** NKG2D is a transmembrane receptor mainly expressed on CD8+ T cells and Natural Killer cells (NK). It has been shown that tumor cells deliver soluble NKG2D ligands as a mechanism to escape from the immune system surveillance, but the existence of alternative mechanisms has not been explored. Here we describe the regulation of NK cell migration by NKG2D activation and analyze the role of signalling cascades triggered by NKG2D cross-linking on this effect.

**Methods:** NK cells were pre-incubated with anti-NKG2D, recombinant NKG2D ligands or IgG as control. Migration was analyzed in transwell assays in the presence or absence of SDF- $\alpha$  as chemoattractant. Activation of members of the Rho GTPase family of proteins Rac and Cdc42 was analyzed by pull down assays and western blot. The implication of Rac activation was assessed by pharmacological inhibition of this protein.

**Results:** NKG2D activation inhibits NK cell random migration and chemotaxis towards SDF-1 $\alpha$  in ICAM-1-coated transwells. Rac1 and Cdc42 are activated downstream NKG2D. Moreover Op18, a protein involved in the regulation of microtubule dynamics, and MLC (Myosin Light Chain) are phosphorylated upon NKG2D cross-linking. A decrease in ERM (Ezrin-Radixin-Moesin) phosphorylation is observed. These proteins have been shown to be involved in the regulation of lymphocyte polarization. Pharmacological inhibition of Rac activation impairs Op18 phosphorylation but is not able to rescue cell migration at the different doses tested although an increase in the basal migration is observed.

**Conclusions:** NKG2D activation by specific antibodies or ligands inhibits NK cell migration. NKG2D cross-linking regulates the activity of members of the RhoGTPase and triggers signalling cascades that may be involved not only in the Immunological synapse formation but also in cell polarization and migration.

**PA09/11 ROLE OF CCR7 IN THE MIGRATION AND LOCALISATION OF CD127<sup>+</sup>THYMIC NK CELLS**V.S.G. Ribeiro<sup>1,2</sup>, L. Enault<sup>1</sup>, S. Lesjean-Pottier<sup>1</sup>, R. Förster<sup>3</sup>, J.P. Di Santo<sup>1</sup>, C.A.J. Voshenrich<sup>1</sup>

<sup>1</sup>Unité des Cytokines et Développement Lymphoïde, INSERM U668, Département d'Immunologie, Institut Pasteur, Paris, France, <sup>2</sup>PhD Programme in Experimental Biology and Biomedicine, Center for Neurosciences and Cell Biology, University of Coimbra, Portugal, <sup>3</sup>Institute of Immunology, Hannover Medical School, Hannover, Germany

The thymus is a secondary site for NK cell development and generates a distinct CD127<sup>+</sup>CD11b<sup>lo</sup>KLRG-1<sup>-</sup> NK cell population with enhanced cytokine production potential that shares functional similarities to the human CD56<sup>bright</sup>CD16<sup>+</sup> NK cell subset. We have shown that CD127<sup>+</sup> thymic NK cells are exported to the peripheral lymphoid tissues, in particular the LN, where they represent 10-20% of the total NK cells. Given the importance of chemokines in cellular trafficking, we sought to investigate whether this migration required expression of specific chemokine-receptors. The CC-chemokine receptor 7 (CCR7) is expressed by thymocytes, DCs, T and B cells and controls migration and positioning of these cells to defined functional compartments of the secondary lymphoid organs. CCR7 is also expressed by the human CD56<sup>bright</sup>CD16<sup>+</sup> NK cell subset and therefore we hypothesized that CCR7 could play a role in the homeostasis of CD127<sup>+</sup> NK cells in the thymus and in their migration to the LN.

We found selective expression of CCR7 on thymic NK cells compared to NK cells from other organs. Moreover, absolute numbers of CD127<sup>+</sup> NK cells in the thymus and LN were strongly reduced in *Ccr7*<sup>-/-</sup> mice compared to C57BL/6 mice, pointing to a role for CCR7 in the homeostasis of CD127<sup>+</sup> NK cells. After transfer of thymic NK cells from CCR7<sup>+</sup> donor mice into C57BL/6 or *plt/plt* mice (that lack CCR7 ligands), analysis of the recipient mice revealed a selective reduction of donor-derived CD127<sup>+</sup> NK cells in *plt/plt* recipients indicating that CCL19 and CCL21 influence the migration of thymic NK cells towards the LN. Furthermore CCL21 induced the migration of CD127<sup>+</sup> but not CD127<sup>-</sup> NK cells *in vitro*, in a dose-dependent manner.

Taken together, these results support the conclusion that CCR7 is involved in the homeostasis of CD127<sup>+</sup> thymic NK cells and their migration to secondary lymphoid tissue, including the LN.

**PA09/12 SIGLEC-7 DOWN-MODULATION REPRESENTS AN EARLY MARKER OF DYSFUNCTIONAL NATURAL KILLER CELL SUBSETS ASSOCIATED TO HIGH LEVELS OF HIV-1 VIREMIA**E. Brunetta<sup>1,2</sup>, M. Fogli<sup>2</sup>, S. Varchetta<sup>3</sup>, L. Bozzo<sup>1</sup>, K. Hudspeth<sup>4</sup>, E. Marcenaro<sup>5</sup>, A. Moretta<sup>5</sup>, D. Mavilio<sup>1</sup>

<sup>1</sup>IRCCS Humanitas Clinical Institute, Laboratory of Clinical and Experimental Immunology, Rozzano, Italy, <sup>2</sup>National Institutes of Health, National Institute of Allergy and Infectious Diseases, Immunoregulation, Bethesda, United States, <sup>3</sup>Policlinico San Matteo and University of Pavia, Centre for Hepatology and Infectious Diseases, Pavia, Italy, <sup>4</sup>Rush University Medical Center, Department of Immunology and Microbiology, Chicago, United States, <sup>5</sup>University of Genova, Dipartimento di Medicina Sperimentale, Genova, Italy

**Objectives:** HIV-1 has been shown to severely alter the phenotype and function of NK cells. In particular, high levels of chronic viral replication in HIV-1 infected patients leads to an aberrant NK cell surface expression of several inhibitory and activating NK cell receptors and to high frequencies of a pathologic CD56neg/CD16pos NK cell population that is rarely represented in uninfected donors. These phenotypic abnormalities have been shown to severely affect several NK cell functions. The kinetic of NK cell aberrancies in phenotype and function associated to HIV-1 viremia is still unclear, and longitudinal analyses on large cohorts of HIV-1 infected people at different stages of infection are required to characterize NK cell subset distribution in different stages of infection.

**Methods:** For this project, we analyzed the effect of HIV-1 viremia on phenotype and function of NK cells from 144 infected patients either naïve for treatment or whose ART had been discontinued at the time of the study. This cohort included 21 early, 96 chronic and 27 long term non-progressors HIV-1 infected patients. Moreover, 33 chronic viremic patients who underwent ART were followed longitudinally for 24 months.

**Results:** We found that NK cell surface distribution of Siglec-7 was significantly decreased in HIV-1 infected patients in initial phases of infection compared to healthy donors, and became even lower on NK cells from chronic HIV-1 infected viremic patients. This decrement, in association with the reduction of CD56 expression on NK cells occurring in chronic phases of HIV-1 infection, lead to the emergence of two different NK subsets: 1) Siglec-7neg/CD56pos present at high frequency only in early stages of infection and 2) Siglec-7neg/CD56neg expanded only in chronic phases of disease. Furthermore, functional analysis demonstrated that Siglec-7neg/CD56pos had an initial impairment of cytolytic responses and cytokine secretion, while Siglec-7neg/CD56neg was found to be completely anergic.

**Conclusion:** We demonstrate here that the sharp down-modulation of sialic-acid-binding-immunoglobulin-like-lectin 7 represents the earliest marker if the aberrant de-regulation of NK cell subsets preceding the down-modulation of CD56. The combined use Siglec-7 and CD56 allows the identification of new pathological and dysfunctional NK cells subsets at different stages if HIV-1 infection.

**PA09/13 HUMAN MELANOMA CELLS ARE EFFECTIVELY KILLED BY AUTOLOGOUS ACTIVATED NATURAL KILLER (NK) CELLS: ANALYSIS OF NK CELL SUBSETS AND RECEPTORS INVOLVED IN MELANOMA RECOGNITION AND LYSIS**P. Carrega<sup>1</sup>, G. Pezzino<sup>2</sup>, P. Queirolo<sup>3</sup>, M.C. Mingari<sup>4</sup>, A. Moretta<sup>4</sup>, L. Moretta<sup>1</sup>, G. Ferlazzo<sup>2</sup>

<sup>1</sup>Giannina Gaslini Institute, Genoa, Italy, <sup>2</sup>University of Messina, Department of Human Pathology, Messina, Italy, <sup>3</sup>National Cancer Institute, Genoa, Italy, <sup>4</sup>University of Genoa, Department of Experimental Medicine, Genoa, Italy

**Objectives:** In this study, by employing ten autologous primary melanoma cell cultures obtained from surgical specimens, we analyzed in detail

(a) the ability of NK cells to lyse autologous melanoma cells and

(b) the mechanisms leading to the lysis.

**Methods:** By using <sup>51</sup>Chromium release tests in the presence or absence of mAbs blocking activating NK receptors, we assessed the cytolytic activity of NK cells against autologous melanoma cells and the role of the single activating receptors in this lysis. Tumor cell lines were also analyzed for phenotypic expression of total HLA-I molecules, the various ligands for activating receptors and adhesion molecules. Furthermore, serological and genomic typing of tumor cell lines and autologous lymphocytes were performed. CD107a degranulation assay allowed the evaluation of cytotoxic ability of different NK cell subsets against autologous melanoma.

**Results:** We report that human melanoma cells are highly susceptible to the lysis by activated autologous NK cells and that a variety of NK activating receptors are involved in the lysis, although DNAM1 and Nkp46 were the most frequently engaged. Remarkably, a comparative HLA class I typing of melanoma cells and autologous lymphocytes, showed that melanoma cells presented specific allele losses in 50% of the analyzed cases. In addition, CD107a degranulation assay against autologous melanoma, evaluated on NK cell subsets expressing a single inhibitory receptor, showed that the following mechanisms are involved in melanoma cell lysis:

(1) HLA-E may be often not expressed on melanoma cell surface but retained in the cytoplasm;

(2) specific HLA class I alleles are often deleted in melanoma cells or expressed in amount insufficient to trigger inhibition in NK cells;

(3) cytokine-activated NK cell subsets lacking all inhibitory receptor exerted cytotoxic function comparable to that of NK cells expressing inhibitory receptors.

**Conclusions:** We described that NK cells are able to efficiently lyse autologous melanoma cells by recognizing ligands of activating receptors on melanoma cell surface. Melanoma susceptibility to NK-mediated lysis involves distinct defects in HLA class I expression. Remarkably, also NK cell subsets expressing no inhibitory receptor specific for self-HLA class I molecules can play a relevant role in killing autologous melanoma cells.

**PA09/14 UNDERSTANDING NATURAL KILLER CELLS IN THE PANCREAS OF NON OBESE DIABETIC MICE**H. Brauner<sup>1</sup>, M. Elemans<sup>1</sup>, S. Lemos<sup>1</sup>, C. Broberger<sup>2</sup>, D. Holmberg<sup>3</sup>, M. Flodström-Tullberg<sup>4</sup>, K. Kärre<sup>1</sup>, P. Höglund<sup>1</sup><sup>1</sup>Karolinska Institutet, MTC, Stockholm, Sweden, <sup>2</sup>Karolinska Institutet, Dept. of Neuroscience, Stockholm, Sweden, <sup>3</sup>Umeå University, Dept. of Medical Biosciences, Umeå, Sweden, <sup>4</sup>Karolinska Institutet, CIM, Stockholm, Sweden**Objectives:** The objective of this study was to investigate the presence, phenotype and function of Natural Killer (NK) cells in the autoimmune target organ of non obese diabetic (NOD) mice.**Methods:** NK cells from NOD mice pancreases were analysed by immunohistochemistry and flow cytometry. Proliferation was assessed by BrdU-incorporation. Functional responses were recorded using antibody-mediated stimulations and measurements of intracellular cytokines. Adoptive transfers were performed to investigate NK cell homing to the pancreas.**Results:** NK cells infiltrated the pancreas of NOD mice at young age, had a distinct phenotype and proliferated more compared to spleen and pancreatic lymph node NK cells. NK cells could home to the pancreas in a T and B cell-independent fashion and adoptive transfer experiments showed that the spleen contained NK cells or precursors that could traffic to the pancreas, where they displayed the typical phenotype. Despite expression of activation markers, most pancreatic NK cells showed diminished IFN- $\gamma$  secretion and degranulation compared to spleen NK cells after stimulation *in vitro*, reflecting exhaustion. However, some pancreatic NK cells produced IFN- $\gamma$  spontaneously, suggesting that the pancreas contains both NK cells mediating ongoing effector responses and NK cells rendered hyporesponsive due to chronic stimulation. Pancreases from mouse strains not prone to develop diabetes also contained NK cells. Compared to NOD mouse pancreatic NK cells, they displayed less killer cell lectin-like receptor G1 (KLRG1), a marker for mature NK cells that have undergone proliferation, and a lower rate of proliferation as measured by BrdU incorporation.**Conclusion:** In conclusion we show that NK cells are present in the pancreas of several mouse strains but show distinct properties in the NOD mouse, suggesting that pancreatic NK cells may be sentinel cells, which under inflammatory conditions could become activated, proliferate and contribute to organ-specific autoimmunity.**PA09/15 METHYLPREDNISOLONE PROMOTES PREFERENTIAL IN VITRO DIFFERENTIATION OF HUMAN CD34+ CELL PRECURSORS TOWARDS NK CELLS**E. Montaldo<sup>1</sup>, C. Vitale<sup>1</sup>, F. Cottalasso<sup>2</sup>, L. Moretta<sup>2,3,4</sup>, M. C. Mingari<sup>1,5</sup><sup>1</sup>University of Genova, Experimental Medicine Department (DIMES), Genova, Italy, <sup>2</sup>Giannina Gaslini Institute, Genova, Italy, <sup>3</sup>University of Genova, Experimental Medicine Department, Genova, Italy, <sup>4</sup>University of Genova, CEBR, Genova, Italy, <sup>5</sup>Istituto Nazionale per la Ricerca sul Cancro (IST), Genova, Italy**Objectives:** Previous studies showed that Methylprednisolone down-regulates the surface expression of activating NK receptors and abrogates the NK cytotoxicity both *in vitro* and *in vivo*. Since Methylprednisolone is administered to patients undergoing hemopoietic stem cell transplant to treat acute Graft versus Host Disease, we analyzed whether it could also inhibit the NK cell differentiation from CD34+ stem cells, thus interfering with the development of effector cells with anti-leukemic potential.**Methods:** Human CD34+ stem cells isolated from umbilical cord blood were cultured *in vitro* with IL-7, IL-15, IL-21, SCF and FLT3l in the absence or presence of Methylprednisolone. Leucocytes were then analyzed for informative cell surface markers by cytofluorimetric assays and separated by cell sorting according to CD33, CD161, CD56 and LFA-1 surface expression. Cells were tested for cytolytic activity and/or treated with different stimuli to analyze cytokine production.**Results:** Methylprednisolone accelerated the *in vitro* differentiation of CD161+CD56+/- NCR- immature NK (iNK) cells by inducing rapid expression of Nkp46, NKG2D, LFA 1 and NKG2A and cytolytic activity. Moreover, Methylprednisolone induced CD33+CD161-CD56- myeloid precursors to switch towards NK cells. It is also of note that iNK cells, when cultured in the absence of Methylprednisolone, produced high amounts of IL-8 and lower amounts of other pro-inflammatory chemokines. Since IL-8 (and MIP-1  $\alpha$ ) were shown to modulate myelopoiesis both *in vitro* and *in vivo*, it was important to characterize the receptors/soluble factors that could induce production of this cytokine. Cross-linking of CD161 on iNK cell surface induced IL-8 production while SDF-1, MIP-1  $\beta$ , TNF- $\alpha$  and IL-13 were detected only upon stimulation with cytokines or PMA. Importantly, more differentiated NK cells (CD161+CD56+LFA-1+NCR+) derived from the same cultures were not able to produce IL-8 upon stimulation via CD161 or with PMA.**Conclusions:** Methylprednisolone accelerates *in vitro* NK cell differentiation and may interfere with the development of myeloid cells. In addition, different subsets of NK cells undergoing differentiation displayed different patterns of pro-inflammatory cytokine production, suggesting that iNK cells might interfere with the differentiation of other leukocyte populations.

PA09/16 Abstract withdrawn by author

**PA09/17 HEMATOPOIETIC ZINC FINGER PROTEIN PREVENTS TUMOR CELL RECOGNITION BY NATURAL KILLER CELLS**R. La Rocca<sup>1</sup>, M. Fulcinitti<sup>1</sup>, L. Tadepally<sup>2</sup>, M. Mesuraca<sup>1</sup>, T. Hassan Ali<sup>1</sup>, V. Mazzei<sup>1</sup>, N. Amodio<sup>1</sup>, L. Catalano<sup>3</sup>, B. Rotoli<sup>3</sup>, M. Grieco<sup>3</sup>, E. Gulletta<sup>4</sup>, H. Bond<sup>5</sup>, G. Morone<sup>1</sup>, S. Ferrone<sup>5</sup><sup>1</sup>University of Catanzaro 'Magna Graecia', Experimental and Clinical Medicine, Catanzaro, Italy, <sup>2</sup>Karolinska Institutet, Stockholm, Sweden, <sup>3</sup>University Federico II, Napoli, Italy, <sup>4</sup>University 'Magna Graecia', Catanzaro, Italy, <sup>5</sup>University 'Magna Graecia', Experimental and Clinical Medicine, Catanzaro, Italy

EHZF/ZNF521 is a novel zinc finger protein expressed in hematopoietic stem and progenitor cells and is downregulated during their differentiation. Its transcript is also abundant in some hematopoietic malignancies. Analysis of the changes in the antigenic profile of cells transfected with EHZF cDNA revealed upregulation of HLA class I cell surface expression. This phenotypic change was associated with increased level of HLA class I heavy chain, in absence of detectable changes in the expression of other antigen processing machinery (APM) components. Enhanced resistance of target cells to NK cell-mediated cytotoxicity was induced by enforced expression of EHZF in the cervical carcinoma cell line HeLa and in the B-lymphoblastoid cell line IM9. Preincubation of transfected cells with HLA class I antigen-specific mAb restored target cell susceptibility to NK cell-mediated lysis, indicating a specific role for HLA class I antigen upregulation in the NK resistance induced by EHZF. A potential clinical significance of these findings is further suggested by the inverse correlation between EHZF and MHC class I expression levels, and autologous NK susceptibility of freshly explanted multiple myeloma cells.

**PA09/18 LEUKEMIC CHALLENGE UNMASKS A REQUIREMENT FOR PI3KDELTA IN NK-CELL-MEDIATED TUMOR SURVEILLANCE**E. Zebadin<sup>1</sup>, O. Simma<sup>1</sup>, C. Schuster<sup>1</sup>, E.M. Putz<sup>1</sup>, S. Fajmann<sup>1</sup>, W. Warsch<sup>1</sup>, E. Eckhart<sup>1</sup>, D. Stoiber<sup>2</sup>, E.M. Weisz<sup>2</sup>, J. Schmid<sup>2</sup>, W.F. Pickl<sup>3</sup>, C. Baumgartner<sup>4</sup>, P. Valent<sup>4</sup>, R. Piekorz<sup>5</sup>, M. Freissmuth<sup>1</sup>, V. Sexl<sup>1</sup><sup>1</sup>Centre of Biomolecular Medicine, Medical University of Vienna, Institute of Pharmacology and Toxicology, Vienna, Austria, <sup>2</sup>Ludwig Boltzmann Institute of Cancer Research, Medical University of Vienna, Vienna, Austria, <sup>3</sup>Institute of Immunology, Medical University of Vienna, Vienna, Austria, <sup>4</sup>Institute of Internal Medicine I, AKH, Medical University of Vienna, Vienna, Austria, <sup>5</sup>Heinrich-Heine Universität Düsseldorf, Institute of Biochemistry and Molecular Biology II, Düsseldorf, Germany**Objective:** Specific inhibitors of PI3K-isoforms are currently evaluated for their therapeutic potential in leukemia. Here we investigated the role of the catalytic isoform PI3Kdelta for Abelson-induced leukemia. In our experiments we considered both, the effect of loss of PI3Kdelta within the tumor cells as well as within the relevant immune cell compartments.**Methods:** We employed a broad variety of experimental methods, ranging from standard *in vitro* assays to various *in vivo* leukemia models as well as non-hematologic tumor models, followed by electrophysiologic capacitance measurements.**Results:** First of all we show that *Bcr/abl*-positive human leukemic cells express PI3Kdelta. Using PI3Kdelta-deficient mice we define a dual role of PI3Kdelta in leukemia: we observed a growth-promoting effect in tumor cells and an essential function in NK-cell-mediated tumor surveillance: Abelson-transformed PI3Kdelta-deficient cells induced leukemia in RAG2-deficient mice with an increased latency indicating that PI3Kdelta accelerated leukemia progression *in vivo*. However, the absence of PI3Kdelta also affected NK-cell-mediated tumor surveillance. PI3Kdelta-deficient NK-cells failed to lyse a large variety of target cells because of defective degranulation as also documented by capacitance recordings. Accordingly, transplanted leukemic cells killed PI3Kdelta-deficient animals more rapidly. As a net effect, no difference in disease latency *in vivo* was detected if both – leukemic cells and NK-cells – lack PI3Kdelta. Other tumor models confirmed that PI3Kdelta-deficient mice succumbed more rapidly when challenged with T- or B-lymphoid leukemic or B16 melanoma cells.**Conclusion:** The action of PI3Kdelta in the NK-compartment is as relevant to survival of the mice as the delayed tumor progression. This dual function must be taken into account when employing PI3Kdelta-inhibitors as anti-leukemic agents in clinical trials.**PA09/19 THE ADHESION PORTION OF BACTERIAL TYPE1 FIMBRIA, FIMH, DIRECTLY ACTIVATES NATURAL KILLER CELLS VIA TLR4 SIGNALING**M.F. Mian<sup>1</sup>, N.M. Lauzon<sup>1</sup>, D.W. Andrews<sup>2</sup>, B.D. Lichty<sup>1</sup>, A.A. Ashkar<sup>1</sup><sup>1</sup>McMaster University, Pathology and Molecular Medicine, Hamilton, Canada, <sup>2</sup>McMaster University, Biochemistry and Biomedical Sciences, Hamilton, Canada**Objectives:** Natural killer (NK) cells play important roles in early innate defense against microbial pathogens and tumours. However, the ability of these cells to directly recognize pathogens is not fully understood. This study was aimed to evaluate whether the sticky portion of bacterial type1 fimbria, FimH, which we recently discovered as a novel TLR4 ligand, could activate human and murine NK cells.**Methods:** FimH from uropathogenic *Escherichia coli* (UPEC) was expressed in E.coli BL21 and purified through nickel column followed by FPLC. Human NK cells were purified from peripheral blood mononuclear cells using CD56 positive selection EasySep kit, so as the murine NK cells from splenocytes by EasySep CD49b<sup>+</sup> DX5<sup>+</sup> selection kit. NK cells were then stimulated with FimH (10  $\mu$ g/ml), LPS (100 ng/ml), CpG or poly I:C (10  $\mu$ g/ml), secreted cytokines were measured by



ELISA, and cytotoxicity by chromium release assay. Finally, human NK cells were infected with FimH+ or FimH- UPEC and NK cell activation markers (CD69, Perforin) were examined by flow cytometry. Data were analyzed by GraphPad prism software and student's t test.

**Results:** We show that FimH stimulations of both human and murine NK cells substantially induced cytokines, IFN- $\gamma$  and TNF- $\alpha$ , release and increased ability to kill tumour cells. Notably, NK cell activation by FimH required TLR4-MyD88 signalling pathways, since NK cells either from TLR4<sup>-/-</sup> or MyD88<sup>-/-</sup> mice, as well as human NK92 cells (TLR4 null cells), were unresponsive to FimH stimulation. In addition, pre-treatment of purified human NK cells with anti-TLR4 neutralizing mAb abolished the response to FimH. We then confirmed that NK cell activation by FimH was independent of LPS or any other bacterial contamination. Finally, we demonstrate that NK cells can directly recognize FimH expressing pathogens as revealed by the fact that FimH+, but not FimH mutant, UPEC were able to induce human NK cell activation markers including CD69.

**Conclusion:** These data provide novel evidence that highly purified human or murine NK cells directly recognize and respond to bacterial FimH through TLR4-MyD88 signalling pathways. Thus, NK cells, similar to APCs, contribute in sensing pathogen associated molecular patterns to limit microbial infections.

**PA09/20 TRYPANOSOMA CRUZI TRYPOMASTIGOTES INDUCED INTERFERON-GAMMA PRODUCTION BY NEONATAL BLOOD NATURAL KILLER CELLS IS MONOCYTE AND IL-12P70 DEPENDENT**

A. Guilmot<sup>1</sup>, Y. Carlier<sup>1</sup>, C. Truysens<sup>1</sup>

<sup>1</sup>Free University of Brussels (ULB), Laboratory of Parasitology, Brussels, Belgium

We have previously showed that, despite their immature immune system, foetuses congenitally infected with *Trypanosoma cruzi*, the protozoan parasite agent of Chagas disease, were able to mount a strong, adult-like CD8 T cell immune response. In order to understand the subsequent mechanisms of initiation of this response, we studied interferon-gamma (IFN- $\gamma$ ) production by cord blood (CB) natural killer (NK) cells stimulated with *T. cruzi* and the possible interaction of NK cells with other cells. Using flow cytometry and ELISA, CB mononuclear cells (CBMC) of healthy newborns were incubated with live trypomastigotes of *T. cruzi* for 24h at different parasite/cell ratios (5 to 0.1), in the presence of low amounts of interleukin (IL)-15. Our results show that, in the presence of IL-15, *T. cruzi* triggers IFN- $\gamma$  production by both CD56<sup>bright</sup> and CD56<sup>dim</sup> subpopulations of NK cells, although IFN- $\gamma$  release by the former was markedly higher. The next step was to investigate the putative role of other cells in this activation. The depletion of CD14+ (by MACS magnetic beads) strongly impeded IFN- $\gamma$  production by NK cells. Furthermore, neutralization of IL-12 by blocking antibodies resulted in a dramatic inhibition of *T. cruzi* IL-15 induced IFN- $\gamma$  production by NK cells. These data suggest that monocytes and IL-12p70 are strongly involved in triggering IFN- $\gamma$  production in neonatal NK cells after stimulation with *T. cruzi* and IL-15.

**PA09/21 THE NKP80 RECEPTOR MEDIATES CYTOTOXICITY THROUGH A SYK KINASE-INDEPENDENT PATHWAY**

K. Dennehy<sup>1</sup>, A. Steinle<sup>1</sup>

<sup>1</sup>Institute for Cell Biology, University of Tuebingen, Tuebingen, Germany

The activating human NK receptor Nkp80 is a homodimeric C-type lectin-like receptor (CTLR) encoded in the Natural Killer Gene Complex. We have previously shown that Nkp80 binds to the genetically linked CTLR AI1CL (activation-induced C-type lectin). Nkp80-AI1CL interaction activates NK cytotoxicity against malignant myeloid cells and stimulates a mutual cross-talk between NK cells and monocytes under inflammatory conditions. However, the underlying signaling mechanisms of Nkp80 remain unknown.

Most activating and inhibitory NK receptors transduce signals through phosphorylation of associated tyrosine-containing sequence motifs. Given that Nkp80 contains three tyrosine residues in its cytoplasmic tail, we determined whether Nkp80 can be phosphorylated. We expressed wild-type Nkp80 and tyrosine-to-phenylalanine point mutants in NK92MI cells, a cell line that expresses no or low levels of surface Nkp80. Wild-type Nkp80, but not Nkp80/Y7F was tyrosine phosphorylated, indicating that tyrosine 7 is the only or initial site of tyrosine phosphorylation. In redirected lysis assays parental and Nkp80/Y7F NK92MI cells showed no significant cytotoxicity in contrast to wild-type Nkp80 transductants, demonstrating that phosphorylation of tyrosine 7 is required for Nkp80-mediated cytotoxicity. The sequence surrounding tyrosine 7 shows remarkable similarity to the recently described hemi-immunotyrosine-based signalling motif (hemi-ITAM), which recruits Syk kinase. However, crosslinking of Nkp80 on NK92MI cells did not induce phosphorylation of Syk or its substrate SLP-76. Furthermore, specific inhibition of Syk kinase impaired cytotoxic responses through the Nkp44 receptor, which signals through Syk, but not through Nkp80. Taken together these results indicate that Nkp80 signals through a novel hemi-ITAM-like motif, but independently of Syk kinase.

**PA09/22 VSIG9 A CD28 FAMILY-LIKE INHIBITORY RECEPTOR BINDS PVR AND INHIBITS NK CELL ACTIVITY**

N. Stanietsky<sup>1</sup>, H. Simic<sup>2</sup>, A. Toporik<sup>3</sup>, O. Levy<sup>3</sup>, A. Novik<sup>3</sup>, Z. Levine<sup>3</sup>, M. Beiman<sup>3</sup>, L. Dassa<sup>3</sup>, H. Achdout<sup>1</sup>, N. Stern-Ginossar<sup>1</sup>, P. Tsukerman<sup>1</sup>, S. Jonjic<sup>2</sup>, O. Mandelboim<sup>1</sup>

<sup>1</sup>The Hebrew University, Jerusalem, Israel, <sup>2</sup>University of Rijeka, Rijeka, Croatia, <sup>3</sup>COMPUGEN, Tel Aviv, Israel

The B7 and CD28 families of receptors play a significant role in immune cell responses against pathogens, tumors, and in autoimmune diseases. Using a computational analysis to search for CD28 family-like proteins, we identified a new receptor, containing an ITIM motif, named VSIG9. We further identified its ligand to be PVR (CD155), demonstrated that VSIG9 is expressed on CD8<sup>+</sup>, memory CD4<sup>+</sup>, Treg, NKT and NK cells and showed that the interaction between PVR and VSIG9 inhibits NK cytotoxicity through its ITIM sequence. Finally we show that VSIG9 counter inhibits the NK-mediated killing of tumor cells and protect normal cells from NK-mediated killing, thus providing an "alternative self" mechanism for MHC class I inhibition.

**PA09/23 NK CELL CHIMERISM IS A UNIQUE FEATURE OF LIVER TRANSPLANTATION AND MAY MODULATE RECIPIENT'S RESPONSE AGAINST THE GRAFT**

V. Moroso<sup>1</sup>, H.J. Metselaar<sup>1</sup>, B.M. Bosma<sup>1</sup>, S. Mancham<sup>1</sup>, L.J.W. van der Laan<sup>2</sup>, N.M. van Besouw<sup>3</sup>, H.W. Tilanus<sup>2</sup>, E.J. Kuipers<sup>1</sup>, D. Eissen<sup>4</sup>, A. van der Meer<sup>4</sup>, I. Joosten<sup>4</sup>, J. Kwekkeboom<sup>1</sup>

<sup>1</sup>Erasmus Medical Center, Gastroenterology and Hepatology, Rotterdam, Netherlands, <sup>2</sup>Erasmus Medical Center, Surgery, Rotterdam, Netherlands, <sup>3</sup>Erasmus Medical Center, Internal Medicine, Rotterdam, Netherlands, <sup>4</sup>UMC St. Radboud, Blood Transfusion and Transplantation Immunology, Nijmegen, Netherlands

**Objectives:** Liver grafts have tolerogenic properties, allowing complete discontinuation of immunosuppressive medication in about 20% of liver transplant (LTx) recipients. We hypothesized that this unique property of liver grafts may be related to their high content of organ-specific NK cells. In the present study we determined whether hepatic NK cells of donor origin migrate into recipients after clinical LTx, and characterized NK cells that detach from human liver grafts.

**Methods:** We determined the numbers of donor-derived NK cells in the circulation of LTx-recipients and renal transplant (RTx) recipients using antibodies that recognize donor HLA-alleles. We compared the immunophenotypic and cytotoxic properties of isolated liver NK cells obtained from perfusion liquid used to flush out the donor liver during the transplantation procedure, with those of NK cells from blood of healthy individuals.

**Results:** A variable percentage, ranging from 1.1% to 7.9%, of donor NK cells was detected in the LTx-recipient circulation for an average of 15 days, while no NK cell chimerism was observed in RTx-recipients. Liver graft perfusates contained 31±9% NK cells, of which 46±6% belonged to the CD56<sup>bright</sup>/CD16- subtype, while in blood only 10±3% of NK cells were CD56<sup>bright</sup>. The enriched CD56<sup>bright</sup> population in the liver expressed markers of activation (95±3% CD69+, versus 12±4% CD69+ in blood; p< 0.001), and had a higher cytotoxic capacity than its counterpart in blood, showing higher perforin/granzyme content and CD107a degranulation, and two-fold enhanced rate of killing of MHC class I devoid K562 cells, compared with blood NK cells.

**Conclusion:** After LTx, but not after RTx, donor NK cells circulate in recipients, and may play a positive role in graft acceptance by killing recipient APCs and activated T cells responsible for initiating an immune reaction that can lead to graft rejection. These results indicate that implementing the transfer of donor-derived NK cells can induce a status of chimerism that may facilitate graft acceptance.

**PA09/24 THE EXPRESSION OF CD6 DEFINES FUNCTIONAL DIFFERENT SUBSETS IN PERIPHERAL NK CELLS**

M. Braun<sup>1</sup>, C.S. Falk<sup>1</sup>

<sup>1</sup>German Cancer Research Center (DKFZ), NCT, Institute for Immunology, Heidelberg, Germany

**Introduction:** The aim of this study was to elucidate a possible correlation of function and phenotype in peripheral NK cell subsets. Peripheral NK cell subsets differ mainly in their expression of CD56 and CD16, with the majority of CD56<sup>dim</sup> CD16+ NK cells and a minority of CD56<sup>bright</sup> CD16- NK cells. In our analyses, we included the ALCAM (CD166) receptor CD6, which is expressed on a subset of CD56<sup>dim</sup> NK cells and addressed the impact of this marker on the effector functions cytotoxicity and cytokine secretion.

**Methods:** We analysed the degranulation of CD6+ and CD6- NK cells against HLA class-I negative target cells and the cytokine secretion after IL-2 activation. Furthermore different activating receptors on CD6+ and CD6- NK cells were triggered by plate-bound specific antibodies in the presence of IL-2 and the functional outcome was analysed. Degranulation was measured by CD107a assays, the secretion of 17 cytokines was analysed in the supernatant of separated NK cells using the Luminex Multiplex-technology.

**Results:** CD6+ and CD6- NK cells showed similar degranulation potential against different HLA class-I negative target cells, despite a different expression of ALCAM on these cells. The cytokine secretion pattern of IL-2 activated NK cells was comparable irrespective of the CD6 expression. However, differences were seen when individual activating receptors were triggered. Nkp30 and NKG2D for example led to strong degranulation that was accompanied by IFN- $\gamma$  and TNF- $\alpha$  secretion. NKG2C, another activating receptor, induces neither degranulation nor IFN- $\gamma$  and TNF- $\alpha$  secretion but production of IL-7, IL-10 and IL-12. The amount of secreted cytokines after receptor engagement correlated with the expression of CD6. The secretion of IFN- $\gamma$  and TNF- $\alpha$  after NKG2D activation was higher in CD6- NK cells other cytokines like IL-12 were induced predominantly in CD6+ NK cells.

**Conclusion:** We could show a functional difference between CD6+ and CD6- peripheral NK cells that appears in the cytokine secretion pattern after the activation of different receptors. These findings indicate that CD6 might serve as a new differential marker for the function of peripheral NK cells.

**PA09/25 CONDITIONAL GENE-TARGETING IN NK CELLS**V.S.G. Ribeiro<sup>1,2</sup>, S. Lesjean-Pottier<sup>1,2</sup>, O. Richard-Le Goff<sup>1,2</sup>, H. Luche<sup>3</sup>, H.-J. Fehling<sup>2</sup>, J.P. Di Santo<sup>1,2</sup>, C.A.J. Vossenhenrich<sup>1,2</sup><sup>1</sup>Institut Pasteur, Immunology, Paris, France, <sup>2</sup>U668 Inserm, Paris, France, <sup>3</sup>Uniklinik Ulm, Ulm, Germany**Objective:** Generation of a transgenic mouse allowing for the cre/loxP-mediated conditional gene deletion specifically in Nkp46+ cells.**Methods:** We generated a transgene where the proximal promoter region of the murine Ncr-1 gene drives the expression of a GFP-cre gene fusion construct. This transgene was purified and injected into fertilized B6 eggs that were then transplanted into pseudo-pregnant B6 female mice. Peripheral blood cells from transgene (Tg)+ offspring were analyzed for GFP in leukocyte subsets, including B, T, NK and myeloid cells. Tg+ mice expressing GFP+ NK cells were then crossed to ROSA-tdRFP mice that carry an inducible Red Fluorescent Protein (RFP) reporter inserted into the ROSA26-locus. RFP expression in this mouse strain is blocked due to a loxP-flanked “stopper” cassette that precedes the tdRFP gene. Upon cre-mediated deletion, RFP is strongly and stably expressed, thus allowing a) analysis of the specificity of Ncr1-Cre expression, and b) fate-mapping of cells that previously expressed Ncr1.**Results:** We identified a Ncr1-GFPcre Tg mouse line where 50-70% of NK cells from primary and secondary lymphoid organs as well as tissue-derived NK cells expressed GFP. In crosses with ROSA-tdRFP mice, GFP+ NK cells also showed RFP expression demonstrating efficient Cre activity in these tagged cells. GFP and RFP expression were only detected in NK cells and was highest in lymph node NK cells (70%), while half of the NK cells from all other organs (spleen, bone marrow, thymus) were GFP+RFP+. GFP and RFP expression were not restricted to any known NK cell subset in the tissues examined.**Conclusion:** We have generated a transgenic mouse expressing a GFP-cre fusion gene specifically in NK cells. We demonstrated that Cre activity was present and restricted to GFP+ NK cells thus allowing for the NK cell-specific gene ablation. As such, this mouse line should allow analysis of the function of loxP-flanked genes in the NK cell compartment without interference or secondary effects of specific gene loss on other cell types.**PA09/26 THE MOLECULAR BASIS OF SYNERGY AMONG RECEPTORS FOR NK CELL ACTIVATION – A SYSTEMATIC PROTEOME APPROACH**S. König<sup>1</sup>, Y.T. Bryceson<sup>2</sup>, J. Wehland<sup>1</sup>, H.-G. Ljunggren<sup>2</sup>, L. Jänsch<sup>1</sup><sup>1</sup>Helmholtz-Zentrum für Infektionsforschung, Cell and Immune Biology, Braunschweig, Germany, <sup>2</sup>Karolinska Institutet, Department of Medicine, Center for Infectious Medicine, Stockholm, Sweden**Objectives:** Natural killer (NK) cells are cytotoxic lymphocytes that constitute part of the innate immune system. Recent studies have revealed extensive co-operation among activating NK cell receptors for induction and modulation of NK cell cytotoxicity. These findings necessitate further studies of signaling by co-activation receptors, potentially elucidating molecular checkpoints specifically instigated by receptor synergies. In this project we focus on the synergy between the co-activation receptors 2B4 and DNAM1 on human NK cells. The objective is to gain insight into the molecular mechanisms of how 2B4 and DNAM1 individually or cooperatively elicit NK cell activation. Therefore, we systematically screened for phosphorylation events on protein kinases in 2B4-, DNAM1-, 2B4+DNAM1- and CD16-stimulated NK cells using high precision quantitative mass spectrometry (LTQ Orbitrap<sup>TM</sup> XL).**Methods:** Highly unspecific small molecule ATP analogues were utilized for affinity purification of kinases from total cell lysates of activated NK cells. Following tryptic protein digestion phosphorylated peptides were enriched by IMAC (Immobilized Metal Affinity Chromatography). Quantitative peptide sequencing (iTRAQ<sup>TM</sup>/SCX/nHPLC-MS/MS) was performed to reveal regulated phosphorylation events on kinases. Statistical validation and visualization of proteomic data were facilitated by a novel bioinformatics approach (Hundertmark *et al.*, Bioinformatics 2008).**Results:** This study represents a novel strategy providing comprehensive insight into the molecular processes underlying NK cell activation. Quantitative proteomics revealed 245 phosphorylation events observed on 128 kinases after 2 minutes of NK cell receptor triggering. Our approach provided access to both key components of NK cell signaling like SYK, LCK, FYN, ITK, PI3K, PKC and kinases that were not implicated in NK cell activation so far. The comparison of kinase signaling mediated by 2B4/DNAM1 and the potent activation receptor CD16 showed first hints of putative molecular checkpoints in NK cell activation.**Conclusions:** Advanced knowledge about molecular checkpoints of NK cell effector functions will help to define targets for the therapeutic manipulation of NK cells *in vivo*. In the long run this work will hopefully improve our understanding of the diverse strategies used by NK cells for specific recognition and killing of target cells.**PA09/27 INFLUENZA VIRUS INFECTION AUGMENTS NK CELL INHIBITION THROUGH REORGANIZATION OF MAJOR HISTOCOMPATIBILITY COMPLEX CLASS I PROTEINS**H. Achdout<sup>1</sup>, O. Mandelboim<sup>1</sup><sup>1</sup>Hebrew University, Immunology, Jerusalem, Israel

The killing by natural killer (NK) cells is regulated by inhibitory, costimulatory, and activating receptors. The inhibitory receptors recognize mainly major histocompatibility complex (MHC) class I molecules, while the activating NK receptors recognize stress-induced ligands and viral products. Thus, changes in the expression of the various inhibitory and activating ligands will determine whether target cells will be killed or protected. Here, we demonstrate that after influenza virus infection the binding of the two NK inhibitory receptors, KIR2DL1 and the LIR1, to the infected cells is specifically increased. The increased binding occurs shortly after the influenza virus infection, prior to the increased recognition of the infected cells by the NK activating receptor, Nkp46. We also elucidate the mechanism responsible for this effect and demonstrate that, after influenza virus infection, MHC class I proteins redistribute on the cell surface and accumulate in the lipid raft microdomains. Such redistribution allows better recognition by the NK inhibitory receptors and consequently increases resistance to NK cell attack. In contrast, T-cell activity was not influenced by the redistribution of MHC class I proteins. Thus, we present here a novel mechanism, developed by the influenza virus, of inhibition of NK cell cytotoxicity, through the reorganization of MHC class I proteins on the cell surface.

**PA09/28 THE SIGNAL PEPTIDE OF HCMV UL40 UPREGULATES CELL SURFACE EXPRESSION OF BOTH HLA-E AND THE MHC CLASS I HOMOLOGUE UL18**V. Prod'homme<sup>1</sup>, P. Tomassec<sup>1</sup>, R.J. Stanton<sup>1</sup>, B.P. McSharry<sup>1</sup>, C.R. Rickards<sup>1</sup>, V.M. Braud<sup>2</sup>, G.W. Wilkinson<sup>1</sup><sup>1</sup>Cardiff University, Medical Microbiology, Cardiff, United Kingdom, <sup>2</sup>Institut de Pharmacologie Moléculaire et Cellulaire, Centre National de la Recherche Scientifique/Université de Nice-Sophia Antipolis, Valbonne, France**Objectives:** Human cytomegalovirus (HCMV) encodes an MHC-I homologue (UL18) with high affinity for the NK cell inhibitory ligand LIR-1/ILT-2/CD85j. Furthermore, we have shown that the signal sequence of gpUL40 contains an HLA-E-binding peptide that has been implicated in the upregulation of HLA-E cell surface expression in a TAP-independent manner. In studies using an HCMV UL40 deletion mutant, we observed that UL40 was also required for elevated levels of gpUL18 surface expression. We therefore sought to investigate the mechanisms by which UL40 was able to stimulate expression of both HLA-E and gpUL18.**Methods:** Detailed expression studies (flow cytometry and western blots) were performed using HCMV deletion mutants and the high throughput AdZ system (<http://AdZ.cf.ac.uk>).**Results:** Using a panel of defined UL40 and HLA-A2 mutants, we demonstrated that the VMAPRTLIL sequence in the UL40 signal peptide was required for upregulation of HLA-E and UL18 on the cell surface. The element responsible for upregulating HLA-E in a TAP-independent manner was mapped to the N-terminal 14 residues of the UL40 signal peptide. The capacity to upregulate gpUL18 was also mapped to the sequence encoding the HLA-E-binding peptide. However, defined single amino acid substitutions within the UL40 signal sequence could selectively ablate the induction of either HLA-E or gpUL18.**Conclusion:** While it is clear that the peptide-binding specificities of HLA-E and gpUL18 are not identical, our data are consistent with VMAPRTLIL peptide binding to both HLA-E and gpUL18 to promote their expression on the cell surface.**PA09/29 PI5KI $\gamma$ -DEPENDENT PIP2 POOL PLAYS A KEY ROLE IN MUNC13-4 PRIMING FACTOR COMPARTMENTALIZATION**C. Capuano<sup>1</sup>, A. Paolini<sup>1</sup>, A. Santoni<sup>1</sup>, R. Galandrini<sup>1</sup><sup>1</sup>Sapienza, University of Rome, Dep. Experimental Medicine, Rome, Italy

In our recent reports we described a critical role for phosphatidylinositol4,5bisphosphate-5 kinase type I (PI5KI)-dependent signals in the regulation of Natural Killer (NK) cell-mediated cytotoxic function by demonstrating that PI5KI product, phosphatidylinositol4,5bisphosphate (PIP2), is critically involved in the activation of cytolytic secretory pathway at a step downstream to granule polarization.

**Aims:** Our study has been focused on the analysis of the role of PIP2 in the functional regulation of Soluble N-Ethylmaleimide Sensitive Factor Attachment Protein Receptor (SNARE) system responsible for secretory granule dynamics and exocytosis. In particular we focused our interest on lytic granule priming factor Munc13-4 whose mutation in Familial Hemophagocytic Lymphohistiocytosis (FHL3) patients results in a profound defect of NK cell-mediated cytotoxic function.**Methods:** Primary cultured human NK cells and/or YTS NK cell line has been used in our experiments. Lipid raft isolation and analysis has been performed by biochemical and confocal microscopy approaches. Degranulation and internalization has been studied by cytofluorimetric and confocal microscopy analysis, respectively. Gene silencing of PI5KI $\alpha$  and PI5KI $\gamma$  isoforms has been performed by means of GFP-bearing lentiviruses encoding shRNA specific sequences.**Results:** We observed a significant PIP2 enrichment at plasma membrane raft microdomains as demonstrated by colocalization of GM1 with PIP2 interacting domain, GFP-PH/PLC $\delta$ 1; moreover, we observed that raft integrity is critically required for target-induced lytic granule exocytosis in primary human NK cells. Our findings also show that Munc13-4 priming factor undergoes a transient membrane raft recruitment upon Fc $\gamma$ RIIIa activating receptor stimulation or phorbol ester plus ionomycin treatment. Interestingly, in PI5KI silenced YTS cells we demonstrate that the down-regulation of PI5KI $\gamma$ , but not of PI5KI $\alpha$ -dependent PIP2 pool, leads to a profound deregulation of Munc13-4 compartmentalization. Infact, we observed an increase of Munc13-4 basal levels within raft fraction associated with a lack of activation-dependent membrane raft recruitment which is possibly due to an altered PIP2-dependent clathrin-mediated recycling pathway.

**Conclusions:** Our findings suggest that PI3K $\gamma$ -dependent PIP2 pool is required to regulate Munc13-4 subcellular compartmentalization. Munc13-4 miss-localization and lipid raft retention could negatively regulate NK cells cytotoxic function.

**PA09/30 DOWN-MODULATION OF NKG2D EXPRESSION ON NK CELLS DURING IN VITRO EXPOSURE OF PBMC TO HUMAN CYTOMEGALOVIRUS**

A. Muntasell<sup>1</sup>, G. Magri<sup>1</sup>, D. Pende<sup>2</sup>, A. Angulo<sup>3</sup>, M. López-Botet<sup>1,4</sup>

<sup>1</sup>Pompeu Fabra University, Barcelona, Spain, <sup>2</sup>Istituto Nazionale per la Ricerca sul Cancro, Genova, Italy, <sup>3</sup>Institut d'Investigacions Biomèdiques Agustí Pi i Sunyer (IDIBAPS), Barcelona, Spain, <sup>4</sup>IMIM-Hospital del Mar, Barcelona, Spain

NKG2D is an activating receptor expressed on human NK cells, CD8 alpha beta, gamma delta and a subset of CD4 alpha beta T lymphocytes. NKG2D recognizes stress-induced ligands on target cells, triggering NK cell effector functions and co-stimulating TCR-mediated activation. Down-modulation of surface NKG2D has been previously described as a mechanism leading to effector function impairment during antigen-activation or recognition of tumor cells by CD8 T and NK cells, respectively.

In this work, we show that in vitro exposure of PBMC to human cytomegalovirus (HCMV, TB40/E strain) induced a marked, early and transient, selective down-modulation of surface NKG2D on NK cells, associated to their activation. This effect occurred independently of the HCMV serology of the donor, and was partially reduced when non-infective HCMV particles were used. Under these conditions, NKG2D-mediated cytotoxicity was selectively impaired, and NK cell function could be efficiently triggered through other activating receptors (i.e. Nkp46).

When permeabilized cells were analysed, NKG2D expression in HCMV-treated cells was comparable with that of control samples; moreover, NKG2D and DAP-10 transcripts were detected, thus suggesting that internalization may account for modulation of NKG2D surface expression. This effect was partially inhibited by an anti IL-12 mAb and was not observed when purified NK populations were exposed to HCMV, consistent with a complex process involving different cell types. The mechanism(s) underlying down-modulation of NKG2D expression and, in particular, the putative role of NKG2D ligands are currently being addressed.

**PA09/31 ANALYSIS OF THE NK CELL-MEDIATED RESPONSE TO HUMAN CYTOMEGALOVIRUS-INFECTED DENDRITIC CELLS**

G. Magri<sup>1</sup>, A. Muntasell<sup>1</sup>, N. Romo<sup>1</sup>, A. Sáez-Borderías<sup>1</sup>, D. Pende<sup>2</sup>, A. Angulo<sup>3</sup>, A. Moretta<sup>4</sup>, M. López-Botet<sup>1,5</sup>

<sup>1</sup>Pompeu Fabra University, Molecular Immunopathology Unit, Barcelona, Spain, <sup>2</sup>Istituto Nazionale per la Ricerca sul Cancro, Genoa, Italy, <sup>3</sup>Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Barcelona, Spain, <sup>4</sup>Università di Genova, Dipartimento di Medicina Sperimentale, Genoa, Italy, <sup>5</sup>IMIM-Hospital del Mar, Barcelona, Spain

Human cytomegalovirus (HCMV) establishes a lifelong latent infection in immunocompetent individuals, undergoing occasional reactivation episodes. Myeloid progenitor cells constitute a main reservoir for the virus. In the present study we set up an experimental system to analyse the NK cell response against HCMV-infected monocyte-derived dendritic cells (moDC).

Fresh and IL-2-activated NK cell populations from HCMV-seronegative donors were co-cultured with autologous immature moDC infected with the TB40/E HCMV strain; moDC either uninfected (mock) or treated with UV-inactivated virus preparations were used as controls. The NK cell phenotype and effector functions (cytotoxicity and cytokine production) were analysed, and assays were carried out in parallel in the presence of a panel of monoclonal antibodies (mAbs) specific for different activating NK cell receptors (NKR), including Nkp30, Nkp46, NKG2D and DNAM-1.

Infected moDC (40-90%) were detected by immunofluorescence staining with an anti IE1 mAb and surface expression of HLA class I and II molecules appeared down-regulated. Interaction with autologous HCMV-infected moDC activated fresh NK cells inducing surface expression of CD69 and CD25, stimulating IFN $\gamma$  production and triggering cytotoxicity, assessed by the CD107 mobilization assay; the latter effect became detectable upon overnight incubation in the presence of IL-2. No response was observed when fresh NK cells were co-cultured with moDC, either uninfected (mock) or treated with UV-inactivated virus preparations. The effector functions of IL-2 activated NK cell populations (7 days in the presence of IL-2) were also specifically triggered by HCMV-infected moDC, but a significant response was as well detected against non-infected immature moDC. Nkp46 and DNAM-1-specific mAbs inhibited NK cell activation in response to HCMV-infected moDC and, moreover, an antagonistic effect of mAbs specific for DNAM-1 ligands (i.e. PVRL2 and Nectin-2) was also observed.

Our results show that NK cells efficiently respond against infected HCMV-infected immature moDC, overcoming putative viral immune evasion strategies, and indicate that the Nkp46 and DNAM-1 receptors play a central role in this process.

**PA09/32 THE ROLE OF NATURAL KILLER CELLS IN RESISTANCE TO THE INTRACELLULAR BACTERIUM *LISTERIA MONOCYTOGENES* IN RATS**

H. Shegarfi<sup>1</sup>, K. Sydnese<sup>2</sup>, C. Naper<sup>1</sup>, B. Rolstad<sup>1</sup>

<sup>1</sup>University of Oslo, Dep. of Anatomy, Oslo, Norway, <sup>2</sup>University of Oslo, Oslo, Norway

We have investigated the influence of early innate immune resistance mechanisms on infection with the intracellular bacterial *Listeria Monocytogenes* (LM) infection in rats. Rats were injected iv with various numbers of LM and the number of bacterial colonies in the spleen was determined at different time points after infection. A bacterial dosage as low as  $2 \times 10^4$  cells gave reproducible infection within the spleen. Athymic nude PVG rats lacking normal T cells but with a robust NK cell repertoire for MHC antigens were more resistant to bacterial replication within the spleen than were normal littermate rats. *In vivo* depletion of NK cells or NK subpopulations expressing Ly49 receptors for MHC-I antigens with specific antibodies increased the bacterial load in the spleen, indicating that these cells were important in the initial control of *Listeria* infection. Further evidence for this was obtained by analyzing the NK cell subsets expressing Ly49 receptors. The frequency of Ly49 expressing cells was increased in LM infected rats. Since several rat strains, unlike mice, express a large repertoire of MHC-recognizing activating Ly49 receptors, these observations raise the interesting possibility that alloreactive NK cells with activating receptors may recognize alterations in the expression of MHC-I molecules on LM infected cells leading to their elimination before the adaptive immune system comes into play.

**PA09/33 CATHEPSIN INHIBITION DECREASES PERFORIN-MEDIATED KILLING OF NK CELLS AND CTLs, BUT CATHEPSIN L-DEFICIENT CTLs HAVE NORMAL PERFORIN-MEDIATED KILLING**

S. Konjari<sup>1</sup>, V.R. Sutton<sup>2</sup>, S. Hoves<sup>2</sup>, U. Repnik<sup>1</sup>, V. Turk<sup>1</sup>, B. Turk<sup>1</sup>, J.A. Trapani<sup>2</sup>, N. Kopitar-Jerala<sup>1</sup>

<sup>1</sup>Jozef Stefan Institute, Department of Biochemistry, Molecular and Structural Biology, Ljubljana, Slovenia, <sup>2</sup>Peter MacCallum Cancer Centre, Cancer Immunology Program, Melbourne, Australia

Perforin, a pore forming protein, is synthesized as an inactive precursor in NK cells and CD8<sup>+</sup> CTLs. It becomes active only when a heavily glycosylated C-terminal pro-piece of perforin is cleaved off within the cytotoxic granules. The cleavage is pH dependent and could be prevented by E-64, a cathepsin and calpain inhibitor (Uellner et al., 1997). Our results suggested that in vitro cathepsin L not only degraded but also processed the C-terminal part of perforin. In YT NK cells perforin was found partially co-localized only with cathepsin L and not with cathepsins S or B. After the addition of peptide based cathepsin inhibitors (E-64d or cathepsins L inhibitor L1) to the NK and CTL cell cultures decreased perforin processing and decreased target cell killing was determined. Contrary to our expectations, the CTL target cell killing in cathepsin L-deficient mice was comparable to killing in wild type mice; killing of NK cells prepared from cathepsin L-deficient mice was even better when compared to NK cells prepared from wild type mice. We conclude that cathepsins are essential for processing of perforin and cytotoxicity of NK cells and CTLs, but cathepsins L is not crucial and other cathepsins could compensate for the loss of cathepsin L activity.

**PA09/34 NKG2C DELETION ASSOCIATED WITH HIV-1 INFECTION AND DISEASE PROGRESSION**

R. Thomas<sup>1</sup>, R.E. Schmidt<sup>1</sup>, T. Witte<sup>1</sup>

<sup>1</sup>Medizinische Hochschule Hannover, Clinic for Immunology and Rheumatology, Hannover, Germany

**Background:** NKG2C is an activating C-type lectin-like receptor expressed on NK cells, as well as some CD8<sup>+</sup>  $\alpha\beta$  and  $\gamma\delta$  T cells. NKG2C forms a heterodimer with CD94 and upon ligand binding (ligand: HLA-E, up-regulated by HIV infection) signals via DAP12 adaptor protein, resulting in cytotoxicity or cytokine production (IFN and TNF). A genomic variation of NKG2C exists in which the entire gene and some flanking areas (total 16 kb) have been completely deleted.

This study aims to investigate any association of this presence/deletion variation with HIV infection and disease progression.

**Methods:** PCR was used to determine the allelic distribution of NKG2C in 407 HIV patients and 200 healthy controls (HC). Relative expression patterns of the NKG2C variants on the PBL of 25 ART-naïve HIV and 22 HC donors were investigated via FACS analysis.

**Results:** Homozygosity for the presence variation (i.e. two alleles of NKG2C, "PP") was more common in HC than in HIV (73% vs 63%,  $p=0.041$ ). The prevalence of PP among HIV patient subgroups, according to risk factors, was the same in intravenous drug abusers as in HC (73%), though much lower among homosexuals (58%,  $p=0.022$ ). Segregation by HIV progression shows the PP variation is significantly more frequent in long term progressors (78%) as compared to short term and normal progressors (61%,  $p=0.018$ ).

NKG2C expression, both in terms of the proportion of NKG2C<sup>+</sup> NK cells and MFIs, is increased in HIV patients as compared to HC. NKG2C expression is highest in PP individuals, non-existent in homozygously deleted individuals, and intermediate in heterozygotes.

**Discussion/conclusions:** NKG2C genotype corresponds to the degree of surface expression, with heterozygotes displaying lower expression than PP. Decreased NKG2C expression is indicated as a risk factor for initial HIV infection, due to the higher prevalence of the NKG2C deletion in the HIV cohort, especially among homosexuals. The protective effect of NKG2C in disease progression is suggested by the higher proportion of PP in long term progressors. Together these data indicate that NKG2C is important in the defence against HIV, both in terms of initial infection and disease progression.



**PA09/35 REGULATION OF NATURAL KILLER CELL TISSUE DISTRIBUTION BY CX3CL1/CX3CR1 CHEMOKINE/RECEPTOR PAIR**G. Sciumè<sup>1</sup>, G. Bernardini<sup>1</sup>, A. Santoni<sup>1</sup><sup>1</sup>Sapienza University of Rome, Department of Experimental Medicine, Rome, Italy

**Objectives:** The molecules affecting natural killer (NK) cell trafficking in the bone marrow (BM) have not been completely characterized. We have recently shown that the chemokine receptor CXCR4 provides a key retention signal for NK cell subsets in the BM during steady state, and that mobilization of mature NK cells into blood is promoted by chemokines such as CCL3 that modulate CXCR4/SDF-1 receptor/ligand axis.

In the present study, we focused on the role of CX3CR1/CX3CL1 pair in the regulation of NK cell development and tissue distribution during steady state and inflammation.

**Methods:** We used a modified mouse model, in which CX3CR1 gene was replaced by a green fluorescent protein (GFP) cDNA, maintaining CX3CR1 promoter. NK cell subsets at different maturation stages were identified in BM, spleen, blood, and liver by flow cytometry analysis discriminating CD49b<sup>+</sup>, CD11b<sup>low/high</sup>, and within the CD11b<sup>high</sup> population KLRG1<sup>+/+</sup> NK cells.

**Results:** CX3CR1 gene is prevalently expressed by the more mature KLRG1<sup>+</sup> NK cell subsets, in young (1–2 week old) and adult (7–10 week old) mice as assessed by GFP expression. Moreover, our observations indicate that NK cell number is substantially increased in the BM of CX3CR1<sup>GFP/GFP</sup> mice as compared to WT and CX3CR1<sup>GFP/+</sup> mice. DX5<sup>+</sup>CD11b<sup>high</sup> cell subset is mainly affected, being their number almost doubled in BM. Our preliminary observations suggest NK cell accumulation is not due to altered CXCR4 expression, nor to differences in the proliferation rate of CX3CR1<sup>GFP/GFP</sup> as compared to normal NK cells.

A selective role of CX3CR1 in the regulation of NK cell accumulation into tissues is supported by the doubled percentage of GFP<sup>+</sup> NK cells from CX3CR1<sup>GFP/GFP</sup> as compared to CX3CR1<sup>+/GFP</sup> in all organs analyzed. We analyzed NK cell distribution following administration of the TLR3 ligand, Poly(I:C). A strong and comparable decrease in NK cell number was observed in the spleen of CX3CR1<sup>GFP/+</sup> and of CX3CR1<sup>GFP/GFP</sup> mice, while NK cell number was much less reduced in the BM of CX3CR1<sup>GFP/GFP</sup> mice.

**Conclusions:** Our data show that CX3CR1 is differently expressed on NK cell subsets during maturation and that this receptor regulates NK cell accumulation in BM during steady state and inflammation.

**PA09/36 MODULATION OF NKP80 ON HUMAN NK CELLS BY CYTOKINES**S. Klimosch<sup>1</sup>, S. Welte<sup>1</sup>, S. Kuttruff<sup>1</sup>, A. Kelp<sup>1</sup>, A. Steinle<sup>1</sup><sup>1</sup>Institute for Cell Biology, Immunology, Tübingen, Germany

Natural Killer cells (NK cells) possess the ability to lyse certain virally infected cells and tumour cells. NK cells distinguish these “dangerous” cells from healthy cells by means of an arsenal of activating and inhibitory NK cell receptors. The activating NK receptor Nkp80 is a C-type lectin-like receptor encoded by the gene KLRF1 located in the human Natural Killer Gene Complex (NKG) on chromosome 12. Nkp80 binds to the activation-induced C-type lectin (AICL) which is adjacently encoded in the NKG and specifically expressed on myeloid cells. Nkp80-AICL interaction stimulates the cross-talk between NK cells and monocytes in the presence of inflammatory cytokines. NKR-P1A (CD161), an inhibitory NK receptor distantly related to Nkp80, and NKG2D, an activating NK receptor mediating immunosurveillance of stressed cells, are also encoded in the NKG.

In our studies we investigated alterations in Nkp80 expression on NK cells in response to various cytokines and compared these to cytokine-mediated effects on expression of NKR-P1A and NKG2D, respectively. Originally, we observed that polyclonal NK cells expanded in the presence of irradiated RPMI 8866, an IL-12-producing B cell line, differentially modulate surface expression of Nkp80, NKR-P1A and NKG2D. Subsequently, we found that the two monokines Interleukin (IL)-12 and IL-18 modulated transcript levels and surface expression of these NK receptors. While the expression of NKR-P1A and NKG2D was up-regulated after several days of culture, surface expression of Nkp80 markedly diminished in presence of these monokines. Downregulation of Nkp80 expression strongly impaired Nkp80-mediated NK cell cytotoxicity. The underlying mechanisms of Nkp80 regulation in response to monokines and its physiological impact for the cross-talk between NK cells and myeloid cells are subjects of ongoing research.

**PA09/37 RE-ORGANIZATION OF MITOCHONDRIA AT THE NK CELL IMMUNE SYNAPSE**E. Abarca-Rojano<sup>1</sup>, S. Muñoz-Hernández<sup>2</sup>, R. Mondragón-Flores<sup>2</sup>, F. Enriquez-Rincón<sup>3</sup>, F.J. Sánchez-García<sup>4</sup>

<sup>1</sup>Instituto Politécnico Nacional, Esc. Sup. de Medicina, México D.F., Mexico, <sup>2</sup>Cinvestav-ipn, Bioquímica, México D.F., Mexico, <sup>3</sup>Cinvestav-ipn, Biología Celular, México D.F., Mexico, <sup>4</sup>Instituto Politécnico Nacional, Esc. Nac. Ciencias Biológicas, México D.F., Mexico

**Objectives:** Analyse the role of NK cell mitochondria during the cytotoxic process against tumor cells and upon activation of NK cell receptors.

**Methods:** NK cells were isolated from human peripheral blood by magnetic-based negative selection (Miltenyi). K562 cells were used as tumor target cells. NK cells mitochondrial membrane potential (Dy<sub>m</sub>) was assessed by flow cytometry by using the JC-1 indicator. NK cell cytotoxicity assays over K562 cells were carried out with the LDH-based non-radioactive cytotoxicity assay kit (Promega). NK and K562 cells previously labelled with Mito Tracker red 580 (Invitrogen) were cultured together at 37°C under slight agitation for different periods of time. Cell-cell interaction was stopped with paraformaldehyde and cell conjugates analysed by confocal microscopy. Adhesion substrates based on Epon resin were prepared with anti-NKG2D, anti-KIR2DL1, a 1:1 mixture of both antibodies or with irrelevant antibodies. After incubation of NK-K562 cells over the resin, samples were prepared and examined by transmission electron microscopy.

**Results:** Human NK cells underwent Dy<sub>m</sub> depolarization as early as 15 min after stimulation with K562 cells, indicating a rapid consumption of energy in the form of ATP. Dy<sub>m</sub> gradually recovered, returning to base levels at about 1h post stimulation. Likewise NK cells cytotoxic activity on K562 was severely impaired by oligomycin (ATP synthesis inhibitor) treatment, indicating a requirement for ATP in the NK cell cytotoxic process. NK cells mitochondria redistribute towards the contact site with the K562 cells and, NK cell mitochondrial movement was observed in response to NKG2D (activating receptor) but not in response to KIR2DL1 (inhibiting receptor) stimulation.

**Conclusion:** Mitochondrial activity was observed in the course of NK cell cytotoxicity, such as Dy<sub>m</sub> depolarization and mitochondrial accumulation at the NK cell immune synapse, mitochondrial movement was dependent on NKG2D but not on KIR2DL1 stimulation.

**PA09/38 DIRECT COMPARISON OF HUMAN AND MURINE NK CELL SUBSETS BASED ON CD27 AND CXCR3 COEXPRESSION PATTERNS**N. Marquardt<sup>1</sup>, E. Wilk<sup>1</sup>, R.E. Schmidt<sup>1</sup>, R. Jacobs<sup>1</sup><sup>1</sup>Hannover Medical School, Clinic for Immunology and Rheumatology, Hannover, Germany

The well-established NK cell subsets CD56<sup>dim</sup> and CD56<sup>bright</sup> in humans do not have direct correlates in mice due to the lack of CD56 expression. CD27 has been suggested to be a feasible marker to distinguish functionally distinct murine NK cell subsets. Extensive analyses in humans revealed that CXCR3 is differentially distributed among CD56<sup>dim</sup> and CD56<sup>bright</sup> NK cells. Therefore, we analyzed if CXCR3 could also serve as a suitable marker for discriminating analogous NK cell populations in mice.

PCR, cytometry, and proliferation assays of sorted murine CXCR3<sup>+</sup> and CXCR3<sup>+</sup> NK cells revealed phenotypic and functional differences of the two subsets. With reference to CXCR3 expression, CD27<sup>+</sup> NK cells can be subdivided into functionally diverse CD27<sup>dim</sup> and CD27<sup>bright</sup> NK cells, since CXCR3 is almost exclusively expressed on CD27<sup>bright</sup> NK cells. Within the CD27<sup>bright</sup> NK cell subset, CXCR3<sup>+</sup> NK cells expressed CD16 and Ly49 receptors at higher density compared to CXCR3<sup>+</sup> NK cells. Upon activation, NK cells altered both CXCR3 and CD27 expression as well as functional abilities such as degranulation. However, we could demonstrate that CXCR3<sup>+</sup>CD27<sup>dim</sup> NK cells displayed increased degranulation, whereas higher numbers of CXCR3<sup>+</sup> than CXCR3<sup>+</sup> NK cells produced IFN-γ. In addition, the density of CD27 expression correlated with the production of IFN-γ. In response to IL-21, CXCR3<sup>+</sup> NK cells exhibited a stronger proliferation as compared to CXCR3<sup>+</sup> NK cells. This is in line with data shown for human CD56<sup>bright</sup> NK cells corroborating our hypothesis that murine CXCR3<sup>+</sup> NK cells resemble the human CD56<sup>bright</sup> NK cell subset.

Although CD27<sup>bright</sup> NK cells in mice functionally correspond to human CD56<sup>bright</sup> NK cells, a further differentiation into functionally diverse CXCR3<sup>+</sup> and CXCR3<sup>+</sup> NK cells within the CD27<sup>bright</sup> NK cell subset is favourable. We suggest that concomitant analyses of CD27 and CXCR3 on murine NK cells enable the direct comparison with the human system.

In summary, we assume that mouse CXCR3<sup>+</sup>CD27<sup>dim</sup> NK cells comply with human CD56<sup>dim</sup> NK cells, whereas CXCR3<sup>+</sup>CD27<sup>bright</sup> NK cells resemble the human CD56<sup>bright</sup> NK cell subset.

Supported by DFG grants (SFB738; Priority Program SPP1110).

**PA09/39 INCREASED FREQUENCY OF T CELLS EXPRESSING THE ACTIVATING NK CELL RECEPTORS KIR2DS4 AND NKG2C IN PAROXYSMAL NOCTURNAL HEMOGLOBINURIA (PNH)**S.T.A. van Bienen<sup>1,2</sup>, M. Withaars<sup>2</sup>, T. de Witte<sup>1,2</sup>, P. Muus<sup>1</sup>, H. Dolstra<sup>2</sup>

<sup>1</sup>Radboud University Nijmegen Medical Centre, Hematology, Nijmegen, Netherlands, <sup>2</sup>Radboud University Nijmegen Medical Centre, Central Hematology Laboratory, Nijmegen, Netherlands

**Objectives:** Paroxysmal Nocturnal Hemoglobinuria (PNH) is a disease characterized by hemolysis and bone marrow failure due to an acquired mutation in the X-linked PIG-A gene in the hematopoietic stem cell (HSC). This leads to a clone of hematopoietic cells with deficient expression of glycosyl phosphatidyl inositol (GPI) anchored proteins at the cell membrane. The clinical evolution of PNH arises through clonal expansion of PIG-A mutated HSC; however, the PIG-A mutation alone insufficiently explains clonal expansion. Hypothetically, expansion of a PNH clone could result from autoreactive T cells selectively attacking normal HSC, whereas GPI deficient HSC are unharmed.



**Methods:** We have performed immunophenotyping of peripheral blood samples of 15 PNH patients to detect potentially autoreactive T populations. Mann-Whitney U test was used for comparison of T cell subset frequencies and absolute numbers.

**Results:** We have found a significant increase in T cells expressing the NK cell marker CD56, both in percentage of peripheral blood lymphocytes ( $p < 0.0001$ ) and in absolute numbers ( $p < 0.01$ ). Furthermore, PNH patients had a significantly increased percentage of T cells expressing the activating NK cell receptor KIR2DS4 (CD158i) ( $p = 0.05$ ) compared to healthy controls. In addition, a higher percentage of T cells expressed the activating C-type lectin receptor NKG2C in PNH patients ( $p < 0.01$ ). Further characterization of KIR2DS4+ and NKG2C+ T cells revealed that these are mainly highly differentiated effector memory CD45RA+ T cells ( $T_{EMRA}$ ) (KIR2DS4; median 70%, range 37–81%) (NKG2C; median 57%, range 26–80%) and the majority is CD8+ (median 60.6%, range 27–97%). Furthermore  $\gamma\delta$  T cells compose a highly variable percentage of KIR2DS4+ (median 68%, range 6–90%) and NKG2C+ T cells (median 28%, range 3–97%).

**Conclusions:** The increased frequency of T cells expressing activating NK cell receptors such as KIR2DS4 and NKG2C with a cytotoxic, effector memory phenotype suggests that these T cells are the autoimmune effectors mediating bone marrow damage in patients with PNH. To confirm their putative role in the expansion of a PNH clone by selectively damaging normal and not GPI deficient HSC, cytotoxicity studies will be performed using KIR2DS4+ and NKG2C+ T cell clones isolated from PNH patients.

#### PA09/40 IFN ALPHA-INDUCED TRAIL ON HUMAN NK CELLS CONTRIBUTES TO CONTROL OF HEPATITIS C VIRUS INFECTION

K.A. Stegmann<sup>1,2</sup>, N.K. Björkström<sup>1</sup>, H. Liermann<sup>1</sup>, S. Ciesek<sup>1,3</sup>, P. Riese<sup>4</sup>, J. Wiegand<sup>1</sup>, J. Hadem<sup>1</sup>, P.V. Suneetha<sup>1</sup>, J. Jaroszewicz<sup>1</sup>, C. Wang<sup>1</sup>, V. Schlaphoff<sup>1</sup>, P. Fytili<sup>1</sup>, M. Cornberg<sup>1</sup>, M.P. Manns<sup>1</sup>, R. Geffers<sup>1</sup>, T. Pietschmann<sup>3</sup>, C.A. Guzman<sup>5</sup>, H.-G. Junggren<sup>2</sup>, H. Wedemeyer<sup>1</sup>

<sup>1</sup>Hannover Medical School, Department of Gastroenterology, Hepatology and Endocrinology, Hannover, Germany, <sup>2</sup>Karolinska Institute, Center for Infectious Medicine, Department of Medicine, Stockholm, Sweden, <sup>3</sup>TWINCORE, Centre for Experimental and Clinical Infection Research; A Joint Venture between the Medical School Hannover (MHH) and the Helmholtz Centre for Infection Research, Department of Experimental Virology, Hannover, Germany, <sup>4</sup>Helmholtz Center for Infection Research (HZI), Department of Vaccinology and Applied Microbiology, Braunschweig, Germany, <sup>5</sup>Helmholtz Center for Infection Research (HZI), Division of Cell Biology and Immune Biology, Braunschweig, Germany

**Aims:** The basis of all therapeutic strategies to eliminate hepatitis C virus (HCV) infection is interferon alpha (IFN $\alpha$ ). So far, the mode of action of IFN $\alpha$  is not fully understood. Besides a direct antiviral effect, different immunomodulatory effects have been proposed. An important part of the immune defense against HCV is the innate immune system. Human natural killer (NK) cells may contribute to the control of HCV replication but also mediate immunopathology. This study documents a link between IFN $\alpha$  and NK cells which may be relevant for the understanding how IFN $\alpha$  mediates its antiviral effect and may further improve concepts for the treatment of HCV infection.

**Methods and results:** Using gene arrays we identified tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) among the most upregulated genes in NK cells after in vitro IFN $\alpha$  stimulation. TRAIL was shown to be upregulated on NK cells within 4 hours after IFN $\alpha$  stimulation via flowcytometric analysis. Significantly higher TRAIL expression was observed on NK<sup>dim</sup> cells of hepatitis C patients that responded to IFN $\alpha$  treatment ( $n=5$ ) compared to patients with ongoing HCV replication ( $n=14$ ). The expression of TRAIL on NK<sup>bright</sup> cells inversely correlated with HCV-RNA decline in the early phase of IFN $\alpha$  treatment ( $n=6$ ). In vitro experiments confirmed that IFN $\alpha$  stimulated NK cells can eliminate hepatoma cells via TRAIL.

**Conclusion:** IFN $\alpha$ -induced TRAIL expression on NK cells might contribute to the control of HCV replication, suggesting a novel mode-of-action of IFN $\alpha$  to explain the second phase decline in viral load during IFN $\alpha$  treatment of HCV infection.

#### PA09/41 PKC-THETA IS REQUIRED FOR NK CELL ACTIVATION AND IN VIVO CONTROL OF TUMOR PROGRESSION

J.L. Aguiló<sup>1</sup>, J. Garaude<sup>2</sup>, J. Pardo<sup>1</sup>, M. Villalba<sup>2</sup>, A. Anel<sup>1</sup>

<sup>1</sup>University of Zaragoza, Biochemistry, Zaragoza, Spain, <sup>2</sup>IGMM, Montpellier, France

Protein kinase C-theta (PKC $\theta$ ) was initially isolated as an important PKC isoform expressed in T cells, although its expression is not restricted to these cells. Despite the central function of PKC $\theta$  in several immune responses, its role in the antitumor response against MHC class I (MHC-I)-negative cells has not been investigated. This is an important issue because most tumor cells growing in vivo down-regulate MHC-I expression to escape the CTL-mediated response. In the present work, we show that in vivo development of a MHC-I-deficient tumor (RMA-S) is much favored in PKC $\theta$ (-/-) mice compared with wild-type mice. This is associated with a reduced recruitment of NK cells to the site of tumor development and a reduced activation status of recruited NK cells. This correlates with a reduced ex vivo and in vivo cytotoxic potential of NK cells isolated from PKC $\theta$ (-/-) mice treated with polyinosinic:polycytidylic acid. Consistently, polyinosinic:cytidylic acid treatment induces PKC $\theta$  expression and activation of its enzymatic activity in NK cells in an indirect manner. These observations underline the relevance of PKC $\theta$  as a key molecule in NK cell-mediated antitumor immune surveillance.

#### PA09/42 EVIDENCE THAT THE KIR2DS5 GENE CODES FOR A SURFACE RECEPTOR TRIGGERING NATURAL KILLER CELL FUNCTION

M. Della Chiesa<sup>1</sup>, E. Romeo<sup>2</sup>, M. Falco<sup>2</sup>, M. Balsamo<sup>3</sup>, R. Augugliaro<sup>3</sup>, L. Moretta<sup>1,2,4</sup>, C. Bottino<sup>1,2</sup>, A. Moretta<sup>1,4</sup>, M. Vitale<sup>3</sup>

<sup>1</sup>Università di Genova, Dipartimento di Medicina Sperimentale (DI.ME.S.), Genoa, Italy, <sup>2</sup>Istituto Giannina Gaslini, Genoa, Italy, <sup>3</sup>IST, Istituto Scientifico per la Ricerca sul Cancro, Genoa, Italy, <sup>4</sup>Centro di Eccellenza per la Ricerca Biomedica, Genoa, Italy

**Aims:** Due to the lack of specific reagents not all the receptors codified by known KIR genes have been demonstrated to be expressed and functional in NK cells. The goal of this study was to characterize expression and function of the receptor coded by the *KIR2DS5* gene in human NK cells.

**Methods:** Mice were immunized with a polyclonal NK cell line derived from a *KIR2DS5*<sup>+</sup> donor.

By screening on HEK-293T cell transfectants expressing different members of the KIR family, we isolated a mAb, DF200, reacting with several KIR2D receptors including KIR2DL1/L2/L3, KIR2DS1/S2 and KIR2DS5. Peripheral blood NK cells were stained with DF200 mAb in combination with appropriate anti-KIR mAbs and analyzed by one- or two-color cytofluorimetric analysis.

We generated NK cell clones expressing KIR2DS5 on their surface and tested their cytolytic activity in redirected killing assays against the Fc $\gamma$ R<sup>+</sup> P815 target cell line. We evaluated cytokine release by culturing NK cells overnight in plastic wells coated or not with appropriate mAb and analyzing the content of the supernatant by ELISA.

HEK-293T cells were cotransfected with KIR2DS5 and DAP12-FLAG to study their association. We produced soluble KIR2DS5-Fc fusion protein to assess KIR2DS5 reactivity on 721.221 transfected with different HLA-C alleles.

**Results:** By the analysis of peripheral blood NK cells and in vitro derived NK cell clones, we demonstrated that KIR2DS5 was expressed at the cell surface in discrete subsets of NK cells.

We analyzed the function of NK cell clones expressing KIR2DS5 on their surface. The engagement of KIR2DS5 receptor, mediated by DF200 mAb, induced both cytotoxicity and cytokine production in all KIR2DS5<sup>+</sup> NK cell clones.

Using co-transfection and co-immunoprecipitation, we found that KIR2DS5 associated with the DAP12 signaling polypeptide.

Finally, soluble KIR2DS5-Fc fusion protein did not bind to cell transfectants expressing different HLA-C alleles, suggesting that, if KIR2DS5 does recognize HLA-C molecules, this may only occur in the presence of certain peptides.

**Conclusion:** We have analyzed the pattern of expression and the function of KIR2DS5 molecules in human NK cells, demonstrating for the first time that *KIR2DS5* coded for a functional activating NK receptor clonally distributed within NK cell populations.

#### PA09/43 LONG-TERM NON PROGRESSING HIV-DISEASE ASSOCIATED TO COINFECTION WITH HTLV-2 IS ASSOCIATED WITH UNIQUE FEATURES OF NK CELL PHENOTYPE AND FUNCTION COMPARED TO MONOINFECTED LTNP PATIENTS

F. Bozzano<sup>1</sup>, E. Piloti<sup>2</sup>, P. Costa<sup>3,4</sup>, M. Galli<sup>5</sup>, C. Casoli<sup>6</sup>, L. Moretta<sup>3,4</sup>, A. De Maria<sup>3,7</sup>

<sup>1</sup>University of Genoa, Center of Excellence for Biomedical Research, Genoa, Italy, <sup>2</sup>Hospital Trust of Parma, Immigrant Health Centre, Parma, Italy, <sup>3</sup>Università di Genova, Center of Excellence for Biomedical Research, Genova, Italy, <sup>4</sup>Istituto Giannina Gaslini, Genova, Italy, <sup>5</sup>University of Milan, Istituto di Malattie Infettive e Tropicali, Milano, Italy, <sup>6</sup>L. Sacco Hospital, University of Milan, Unit of Infectious Diseases, Department of Clinical Sciences, Milano, Italy, <sup>7</sup>IST GE-Istituto Nazionale per la Ricerca sul Cancro, S.S. Infettivologia, Genova, Italy

The influence of human T-cell leukemia/lymphoma virus type 2 (HTLV-2) coinfection in HIV-1-infected patients has recently clarified as protective rather than potentiating HIV-1 disease progression. In this context HTLV-2 mechanisms of viral interference have been associated to increased CC-chemokine production and reduced STAT1 activation. NK cell function is usually profoundly affected during HIV infection, and relevant inhibition is also observed on CD8+CTL by de novo expression of inhibitory NKR.

Here we studied whether viral interference could possibly affect also phenotypic or functional parameters in cytolytic cells (NK cells and CD8+ T-lymphocytes) in dually infected patients with non progressing disease compared to HIV-1 monoinfected LTNP.

**Patients and methods:** PBMC were obtained from 2 cohorts of LTNP: HIV-1<sup>+</sup>HTLV-2<sup>+</sup> LTNP and HIV-1<sup>+</sup>HTLV-2<sup>-</sup> LTNP. In addition HIV-1<sup>+</sup>HTLV-2<sup>+</sup> and viremic HIV-1<sup>+</sup>HTLV-2<sup>-</sup> patients were considered for comparison. Negative separation using mAbs and magnetic beads and multiple-colour cytofluorimetry were employed: two-colour cytofluorimetry using specific monoclonal antibodies was performed on enriched or purified cell populations to analyze the expression of iNKR on TCRab<sup>+</sup> resting PBMC. Purified peripheral NK cells were analyzed to evaluate NK cell activation. In vitro activated NK cell cultures were generated in the presence of rIL-2 to evaluate cytokine production profiles.

**Results:** Data indicate that the expression of iNKR on T cells and the expression of activation markers on CD16+CD56+ NK cells is significantly different in the HIV-1<sup>+</sup>HTLV-2<sup>-</sup> patients compared to HIV-1<sup>+</sup> progressors. Both LTNP groups show limited functional iNKR inhibition on CD8+ CTLs. Peripheral NK cells display relevant differences in the two LTNP groups, with reduced "exhaustion", absent activation and higher levels of activatory receptors expression in HTLV2-coinfected

LTNP. Cytokine and chemokine production patterns for IL-6, IL-8, IL-10, IL-17, IFN $\gamma$ , TNF $\alpha$ , MIP1a, MIP1b and RANTES were concordant for the two groups with relevant differences compared to viremic patients.

Some of the effects of viral interference of HLTV-2 on the progression of HIV-1 disease positively affect cytolytic cells of the immune system including CD8+ CTLs and NK cells in HTLV2-coinfected LTNP. Differential effects on NK cells in the two LTNP groups suggest relevance of different innate mechanisms for the control of disease progression in HIV-1 infected patients.

#### PA09/44 THE EFFECT OF IFN $\alpha$ IN VITRO TREATMENT IN ENHANCEMENT OF DECREASED NK CELL ACTIVITY AND ACTIVATING RECEPTOR EXPRESSION IN METASTATIC MELANOMA PATIENTS

K. Mirjagic Martinovic<sup>1</sup>, G. Konjevic<sup>1,2</sup>

<sup>1</sup>Institute of Oncology and Radiology of Serbia, Belgrade, Serbia, <sup>2</sup>School of Medicine, University of Belgrade, Belgrade, Serbia

**Objectives:** As NK cell function is regulated by opposing, activating and inhibitory receptors, the evaluation of their expression on native, as well as on cytokine treated CD3-CD56+ NK cells is of interest in metastatic melanoma patients (MM).

**Methods:** In this study native PBL of 17 melanoma patients in clinical stage IV prior to therapy and 17 healthy controls were investigated for the expression of CD107a degranulation marker, while both native and treated PBL were analyzed for NK cytotoxicity and the expression of NKG2D, CD161, CD158a and CD158b receptors on CD3-CD56+ NK cells. In vitro treatments were performed on PBL for 18 h with medium, IFN- $\alpha$  (250U/ml) and rIL-2 (2000U/ml) and PBL were additionally evaluated for IRF-1 mRNA by RT-PCR

**Results:** We show that MM patients have significantly impaired NK cell activity, decreased expression of NKG2D activating receptor and CD107a degranulation marker together with increased expression of inhibitory KIR CD158b in comparison with healthy controls. We also give novel results of IFN $\alpha$  induced NK cell activity by increasing the expression of both activating NK cell receptors, CD161 and NKG2D, on NK cells of both healthy controls and MM patients without changes in the expression of inhibitory KIR receptors. Furthermore, we show that IL-2 has no effect on NK cell activity in MM patients in spite of increasing the expression of both activating receptors. This may be the consequence of increase in both inhibitory KIR receptors only in MM patients. We also show that the effects of IFN $\alpha$  are mediated through upregulation of transcription molecule IRF-1 in healthy controls as well as MM patients.

**Conclusion:** We give novel results for MM patients showing the association of impaired NK cell activity with decreased expression of activating NKG2D and degranulation marker CD107a and increased expression of inhibitory KIR CD158b receptor on native NK cells that may represent several clinically useful parameters of suppressed NK cell function. Furthermore, we show that only IFN $\alpha$  enhances NK cell activity and the expression of activating receptors with no effect on inhibitory KIRs so this cytokine may be the successful therapy of MM patients.

#### PA09/45 INTERPLAY BETWEEN INNATE AND ADAPTIVE IMMUNITY IN TUMOUR CONTROL

C. D. Brenner<sup>1</sup>, M. Przewoznik<sup>1</sup>, M. Naujoks<sup>1</sup>, S. King<sup>1</sup>, D. Busch<sup>2</sup>, G. W. Bornkamm<sup>3</sup>, M. Röcken<sup>4</sup>, R. Mückel<sup>1</sup>

<sup>1</sup>Helmholtz-Zentrum München, Institut für Molekulare Immunologie, München, Germany, <sup>2</sup>Technische Universität München, Institut für Medizinische Mikrobiologie, Immunologie und Hygiene, München, Germany, <sup>3</sup>Helmholtz-Zentrum München, Institut für Klinische Molekularbiologie, München, Germany, <sup>4</sup>Eberhard-Karls-Universität, Universitäts-Hautklinik, Tübingen, Germany

Natural Killer (NK) cells are an important part of the innate immune system. They play an essential role for eliminating cells with down-regulated MHC class I expression, like virus-infected or malignant cells. We showed in a transgenic mouse model of spontaneously arising lymphoma that low levels of MHC class I activate NK cells and are necessary but not sufficient for inducing target cell lysis. As a second step, "triggering" by additional signals is needed to induce cytolytic functions of NK cells. In our transgenic model, we observe phenotypic activation but functional paralysis of NK cells. Thus, NK cells from tumour-bearing mice show drastically reduced IFN $\gamma$ -production and are not capable of lysing target cells that are susceptible to lysis by normal NK cells. We additionally found elevated numbers of regulatory T (T<sub>reg</sub>) cells in the lymphoid tissues from tumour-bearing mice. Since it is known that Treg are able to regulate NK cells, we investigated the link between T cells and NK cells in this autochthonous lymphoma model. *In vivo* depletion of Treg revealed that impairment of NK cells that leads to tumour escape might not only result from direct interactions with lymphoma cells but is also dependent on regulatory CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T cells.

#### PA09/46 NETWORK OF TLR MEDIATED NK CELL ACTIVATION IN HEAD AND NECK CANCER

S. Wulff<sup>1</sup>, R. Pries<sup>1</sup>, B. Wollenberg<sup>1</sup>

<sup>1</sup>University of Schleswig-Holstein – Campus Lübeck, Department of Otorhinolaryngology, Lübeck, Germany

**Objectives:** Natural killer (NK) cells play an important role as effectors of the innate immunity against tumor cells. Tumors have developed several immune escape mechanisms that lead to significant dysfunctions of immune cells. NK-mediated host defense against tumor cells is strongly impaired in patients with Head and Neck Squamous Cell Carcinoma (HNSCC). We antagonized this progress by activating NK cells through Toll-like receptors (TLRs) via nucleotide molecules.

**Methods:** Human NK cells were isolated from peripheral blood by 'magnetic bead separation' and subsequently analyzed using flow cytometry, cytotoxicity assays, cytokine assays as well as immunohistochemical methods.

**Results:** We demonstrated that native NK cells expressed TLR1, TLR2, TLR3 and TLR7. TLR3 was predominantly expressed on the cell surface of NK cells and was internalized in response to HNSCC. Simultaneous incubation of NK cells with Poly I:C and HNSCC supernatant impaired the internalization of TLR3. Poly I:C was able to activate NK cells even in the presence of HNSCC.

An increased expression of TLR7 was shown by flow cytometry analysis in response to single stranded – immunostimulatory RNA (ss-isRNA). Ss-isRNA efficiently stimulated NK cell cytotoxicity against HNSCC cells. Stimulation with ss-isRNA also results in an increased production of interferon gamma (IFN $\gamma$ ) and effector proteins perforin and granzyme B. Supernatants of permanent HNSCC cell lines negatively affected the ss-isRNA triggered stimulation of cytolytic NK cell functions. Our investigations confirmed that the stimulation of NK cell activity requires TLR7.

Poly I:C does not only act via TLR3, but MDA5 is another receptor that is able to recognize this dsRNA homologue. We currently investigate whether NK cells prefer the Poly I:C recognition via TLR3 or MDA5 in the presence of HNSCC and we will show the progress of our investigations.

**Conclusions:** The activity of NK cells is degraded in the presence of HNSCC, but stimuli such as Poly I:C or ss-isRNA were able to counteract this process. NK cells in conjunction with Toll-like receptors could consequently be an interesting target for innovative immunotherapies that engage tumors.

#### PA09/47 MOLECULAR SIGNATURE OF TUMOR INFILTRATING NK CELLS

A. Stojanovic<sup>1</sup>, L. Li<sup>2</sup>, N. Gretz<sup>2</sup>, A. Cerwenka<sup>1</sup>

<sup>1</sup>German Cancer Research Center, Innate Immunity, Heidelberg, Germany, <sup>2</sup>Center for Medical Science, Ruprecht-Karls University Heidelberg, Faculty of Medicine, Mannheim, Germany

Natural killer (NK) cells play an important role in anti-tumor defense, being particularly efficient at eliminating metastasizing cells and small tumor grafts. However, eradication of larger solid tumors is usually not efficient, despite low expression of MHC class I molecules in many cases. Besides direct killing of target cells, NK cells release mediators that induce inflammation and exert immunoregulatory effects influencing both the innate and adaptive immune response. So far, little is known about the complex control of NK cells response during tumor progression and forces that interfere with NK cell effector function. In our study, we characterized the *in vivo* NK cell response against the MHC class I deficient mouse lymphoma. We demonstrate that after subcutaneous injection of RMA-S tumor cells, NK cells infiltrating the tumor tissue show less mature phenotype compared to blood NK cells of tumor bearing animals. In addition, gene expression profiling using whole genome microarrays, revealed a strikingly different transcription profile of tumor infiltrating NK cells. When compared to blood NK cells, NK cells isolated from tumor tissue displayed a signature of inhibitory genes. Our current study is aimed at the analysis of their role in the control of NK cell anti-tumor responses, which could be used to further improve the existing immunotherapeutic strategies by augmenting NK cell activity.

#### PA09/48 FUNCTION OF NATURAL KILLER CELLS IN A SPONTANEOUS MOUSE LYMPHOMA MODEL

M. Przewoznik<sup>1</sup>, C. D. Brenner<sup>1</sup>, M. Naujoks<sup>1</sup>, G. W. Bornkamm<sup>2</sup>, M. Röcken<sup>3</sup>, R. Mückel<sup>1</sup>

<sup>1</sup>Helmholtz-Zentrum München, Institut für Molekulare Immunologie, München, Germany, <sup>2</sup>Helmholtz-Zentrum München, Institut für Klinische Molekularbiologie, München, Germany, <sup>3</sup>Eberhard-Karls-Universität, Universitäts-Hautklinik, Tübingen, Germany

Natural Killer (NK) cells play an important role in eliminating abnormal cells by directly killing target cells through release of cytolytic granules, by production of IFN- $\gamma$  and by activating antigen-presenting cells. We studied NK – target cell interactions in an endogenous mouse lymphoma model. We used c-myc transgenic mice that spontaneously develop B-cell lymphomas by the age of about twelve weeks. NK cells from tumour-bearing mice showed an activated phenotype that correlated with MHC class I downregulation on growing lymphomas. However, effector functions of those NK cells such as cytotoxicity and IFN- $\gamma$  production have been paralysed. To address the question what are the underlying mechanisms, we extensively characterised phenotype and function of tumour-derived NK cells. In normal lymph nodes, NK cells represent about 1-2% of lymphocytes, whereas in tumorous lymph nodes their absolute numbers are elevated up to 10-fold. We show that this accumulation is due to enhanced proliferation as well as migration of NK cells to tumour sites where contact to tumour cells is established. Phenotypic characterisation showed that several activating and inhibitory receptors were elevated on NK cells from tumour-bearing mice compared to wild-type mice and that degranulation at the tumour site has taken place. Although perforin and granzyme granules could be restored after *in vitro* stimulation, NK cells from tumour-bearing animals were anergic in terms of lysing target cells even after this treatment.

**PA09/49 DRASTIC DECREASED EXPRESSION OF ACTIVATING RECEPTORS ON NK CELLS IN HUMAN LUNG TUMOR MICROENVIRONMENT IMPAIRS THEIR CYTOTOXIC FUNCTIONS**

S. Platonova<sup>1</sup>, J. Cherfils-Vicini<sup>1</sup>, L. Ghazarian<sup>1</sup>, P. Validire<sup>2</sup>, V. Vieillard<sup>3</sup>, W.-H. Fridman<sup>1</sup>, C. Sautès-Fridman<sup>1</sup>, D. Damotte<sup>1</sup>, I. Cremer<sup>1</sup>

<sup>1</sup>INSERM U872/Centre de Recherche des Cordeliers, Team 13, Paris, France, <sup>2</sup>Institut Mutualiste Montsouris, Paris, France, <sup>3</sup>INSERM U543 Hôpital Pitie Salpêtrière, Paris, France

**Objectives:** While NK cells were originally identified by their ability to kill tumor cells in vitro, only limited information is available on NK cells present in tumor microenvironment. Our objectives were

- 1) to characterize the phenotype and function of NK cells in human Non Small Cell Lung Cancers (NSCLC) patients, in tumor microenvironment, in non tumoral lung tissue, and in the blood, and
- 2) to investigate the expression of NK cell receptor ligands on tumor cells.

**Methods:** NK cell infiltration was determined by immunohistochemistry using Nkp46 marker. NK cell phenotype was characterized by flow cytometry on cell suspension obtained from tumoral, non tumoral tissue, and blood of NSCLC patients. Cytotoxic functions of intratumoral and circulating NK cells were studied by CD107a mobilisation assay.

**Results:** NK cells are present both in tumoral and non tumoral lung tissues of NSCLC patients. In the tumor, they are mainly localised in the invasive margin, but outside tertiary lymphoid structures (Ti-BALT – “Tumor-induced Bronchus Associated Lymphoid Tissues”) that are induced in the tumoral area. Intratumoral NK cells are not cytotoxic even after activation with IL-2, on the contrary to NK cells from blood of the same patient, despite an activated phenotype defined by Nkp44 and CD69 expression. Consistent with this observation, intratumoral NK cells display a highly significant decreased expression of activating receptors such as NKG2D, NKP30, NKP80, DNAM-1 and CD16. On the contrary, NK cells from non tumoral lung tissue or blood of NSCLC patients have the same phenotype than healthy donors. Analysis of NK cell receptor ligand expression revealed that inhibitory receptors ligands such as HLA-G and HLA-E are strongly expressed by tumor cells, but not by normal tissue, whereas activating receptors ligands such as MICA/B and ULBP1, 2, 3 are rarely expressed by tumor cells.

**Conclusions:** Altogether these results demonstrate for the first time that the NK cells display an altered phenotype and function specifically in the tumor microenvironment and that tumor cells express high levels of inhibitory receptors ligands. This suggests a local induction of escape mechanisms established by tumor cells and directed towards NK cells.

**PA09/50 THE CHANGE OF SUBSET AND KILLER CELL INHIBITORY RECEPTORS IN DECIDUAS NK CELLS INFECTED WITH TOXOPLASMA GONDII DURING EARLY HUMAN PREGNANCY**

X. Hu<sup>1</sup>

<sup>1</sup>Medical School of Shanghai Jiaotong University, Immunology, Shanghai, China

Infection of *Toxoplasma (T) gondii* during human pregnancy may result in abnormal pregnancy. It has been shown that the increased presence of deciduas natural killer (NK) cells in early pregnancy plays an important role in normal pregnancy and these cells constitute a powerful immune barrier against *T gondii* infection. However, there are few studies on the molecular immune mechanisms underlying abnormal pregnancy caused by *T gondii* infection. In this study, we sought to explain the possible role of deciduas NK cells in abnormal pregnancy caused by *T gondii* infection. We found that during early human pregnancy, *T gondii* infection in vitro decreased the number of CD56<sup>+</sup>/CD16<sup>+</sup> deciduas NK cells and increased CD56<sup>+</sup>/CD16<sup>+</sup> deciduas NK cells; Similarly, *T gondii* also decreased the expression of killer cell inhibitory receptors (KIRs) – KIR2DL4 and ILT-2 of deciduas NK cells both in mRNA and protein levels. These changes influenced the balances between the CD56<sup>+</sup>CD16<sup>+</sup>/CD56<sup>+</sup>CD16<sup>+</sup> subset and inhibiting/activating NKR (natural killer cell receptor), which the normal pregnancy needs, thus caused abnormal killing activity of deciduas NK cells and resulted in the failure of pregnancy. These molecular mechanisms may play an important role in abnormal pregnancy caused by *T gondii* infection during early human pregnancy. Altogether, our findings elucidate for the first time the possible role of deciduas NK cells in abnormal pregnancy caused by *T gondii* infection.

**PA09/51 HETEROGENEITY OF TLR3 MRNA TRANSCRIPTS AND RESPONSIVENESS TO POLY (I:C) IN HUMAN NK CELLS DERIVED FROM DIFFERENT DONORS**

S. Carlomagno<sup>1,2</sup>, S. Sivori<sup>1</sup>, M. Falco<sup>2</sup>, E. Romeo<sup>2</sup>, L. Moretta<sup>1,2,3</sup>, A. Moretta<sup>1,3</sup>

<sup>1</sup>Dipartimento di Medicina Sperimentale (DI.ME.S.), Università di Genova, Genoa, Italy, <sup>2</sup>Istituto Giannina Gaslini, Genoa, Italy, <sup>3</sup>Centro di Eccellenza per la Ricerca Biomedica, Genoa, Italy

**Aims:** TLR3 plays an important role in the activation of different cell types of the innate immune system. Previous studies indicated that human NK cells express TLR3 and that, upon stimulation by polyinosinic-polycytidylic acid (poly (I:C)), they release cytokines and up-regulate cytotoxicity. However the ability of NK cells to respond to poly (I:C) may greatly differ in different donors. This study analyzes a panel of human NK cell clones derived from different donors for the expression of TLR3 mRNA and for their ability to respond to poly (I:C) stimulation.

**Methods:** Levels of TLR3 mRNA were evaluated using TaqMan assay. NK cells were cultured in the absence or in the presence of poly (I:C) for 20h and then analyzed for their phenotypic and functional features. The NK-mediated cytotoxicity was assessed in 4h <sup>51</sup>Cr release assay. Cytokine analysis was performed using supernatants collected from unstimulated and poly (I:C) stimulated cell clones.

**Results:** Here we show that NK cells display heterogeneous levels of TLR3 mRNA transcript. Analysis of NK cell clones did not reveal any correlation between the levels of TLR3 mRNA transcripts and the expression of different surface NK receptors including KIR and NKG2A. On the other hand, the ability of these clones to respond to stimuli acting on TLR3 correlated with their level of TLR3 mRNA transcript and did not correlate with their levels of RIG-I and mda-5 transcript, two cytoplasmic proteins recently described as sensor for dsRNA. Thus, NK cell clones displaying higher TLR3 mRNA transcripts were characterized by higher cytokine production and cytotoxicity. Moreover the increased cytolytic activity induced by treatment of NK cells with poly (I:C) correlates with higher cell responsiveness to Nkp46 ligation and does not depend on increment of the expression of activating NK receptors and co-receptors, adhesion molecules or perforine/granzime.

**Conclusions:** Our present study provides a useful tool for both quantitative and qualitative analysis of TLR3 responses in human NK cells and contributes to explain the heterogeneous responsiveness to poly (I:C) of NK cells derived from different individuals.

**PA09/52 TUBERCULOUS PLEURISY DERIVED NK CELLS EXPRESSING HIGH ICAM-1 LEVELS EXERT CO-STIMULATORY EFFECT IN T CELLS: IN VIVO UNCONVENTIONAL LOCAL ACCESSORY CELL FUNCTION OF NK**

P.L. Schierloh<sup>1</sup>, N. Yokobori<sup>1</sup>, R.M. Musella<sup>2</sup>, J. Castagnino<sup>2</sup>, S.S. de la Barrera<sup>1</sup>, M.C. Sasiain<sup>1</sup>

<sup>1</sup>National Academy of Medicine, Cellular Immunology, Buenos Aires, Argentina, <sup>2</sup>Muñiz Hospital, Neumonology, Buenos Aires, Argentina

Tuberculous pleurisy, one of the most common manifestations of extrapulmonary tuberculosis, is characterized by a T cell mediated hypersensitivity reaction along with Th1 immune profile (1). In this study, we have investigated functional cross-talk among human T and NK cells in tuberculous pleurisy. We found that, endogenously activated, pleural fluid derived NK cells (PF-NK) (2) expressed higher ICAM-1 levels than its peripheral blood counterparts (MFI PF-NK=148±9; PB-NK=55±10, p< 0.0005, N=12). Furthermore, practically all PF-NK that express IFN-γ upon *Mycobacterium tuberculosis* (Mtb) stimulation (3) have ICAM-1<sup>high</sup> phenotype (% IFN-γ<sup>+</sup>/ICAM-1<sup>high</sup> = 92±7%; IFN-γ<sup>+</sup>/ICAM-1<sup>dim</sup> = 7±3%, p< 0.05, N=5). In order to explore functional consequences for enhanced ICAM-1 in PF-NK, we conducted co-cultures with autologous PF-T cells, and found that the presence of PF-NK increased % CD69<sup>+</sup> T and that neutralizing mAb anti-ICAM-1 inhibit this effect to basal levels (Fold increase % CD69<sup>+</sup> T cell with: PF-NK+IgG1=2.9±0.1; PF-NK+anti-ICAM-1=1.1±0.2, p< 0.05, N=5). Besides, upon in vitro stimulation with monokines, normal blood derived resting NK cells increased ICAM-1 expression (p< 0.001, N=5) leading to conjugate formation (N=3), co-stimulation (N=4) and TH1 polarization (N=3) of autologous T cells. These results provide evidence of accessory cell function of human NK (4) in a physiologically relevant inflammatory site, suggesting that NK contribute to adaptative immune response by a direct cell-contact-dependent mechanism in the context of Mtb infection.

**References:**

- 1) Mitra, D.K. et al. 2005. Eur J Immunol. 35:2367-75.
- 2) Schierloh, P. et al. 2005. J. Immunol. 175: 6852-6860.
- 3) Schierloh, P. et al. 2007. Infect. Immun. 75:5325-5337.
- 4) Hanna, J. et al. 2004. J. Clin. Invest. 114:1612-1623.

**PA09/53 ACTIVATED NK CELLS DELAYED APOPTOSIS AND ENHANCED FUNCTION OF PMN**

N. Bhatnagar<sup>1</sup>, R.E. Schmidt<sup>2</sup>, R. Jacobs<sup>2</sup>

<sup>1</sup>Medizinische Hochschule Hannover, ZIB Hannover Biomedical Research School, Hannover, Germany, <sup>2</sup>Medizinische Hochschule Hannover, Hannover, Germany

**Objectives:** Polymorphonuclear neutrophils (PMN) phagocytose infective pathogens with the subsequent killing by producing reactive oxygen species (ROS). To avoid cell and tissue damage of the host, PMN are removed by macrophages and other phagocytes once they have executed their function. However, this elimination also means loss of an important function, pathogen killing. NK cells release a number of cytokines which act on other immune cells in its vicinity upon activation. We were interested in finding out whether or not NK cells have the potential to influence PMN survival and function since they are among the first cells, along with PMN, to come into action following infection.



**Methods:** PMN were isolated from blood of healthy volunteers using discontinuous percoll gradients, and NK cells were sorted by FACS (CD3<sup>+</sup> CD14<sup>+</sup> CD56<sup>+</sup>). PMN were cultured with supernatants of unstimulated or IL-2 stimulated NK cells for 18h. Apoptosis and activation status of PMN were analysed using flow cytometry where increased expression of CD11b, and CD62L shedding indicated PMN activation. Apoptosis was measured using annexin-V and viaprobe staining.

**Results:** PMN survived better when cultured with supernatant of IL-2 stimulated NK cells compared to the medium control and unstimulated NK cells. There was an inhibition of CD62L shedding and upregulation of CD11b. PMN were functionally more potent as evident by their ROS production. Heating the supernatant abrogated this effect indicating the involvement of proteins. Supernatant harvested as early as 6h was able to delay PMN apoptosis. Cytokines such as TNF-alpha, IFN-gamma and GM-CSF seem to be playing a role as inhibitors against them diminished this beneficial effect. NK cells stimulated with other cytokines, like IL-15, IL-18, IL-12 could mimic the effect as observed with IL-2. In contrast, stimulated T cells failed to produce the same effect.

**Conclusions:** Upon stimulation with different cytokines NK cells produce a wide variety of soluble factors, TNF-alpha, IFN-gamma and GM-CSF being the ones released in huge amounts. We suggest that these cytokines (apart from some other unknown soluble factors) produced by NK cells delay PMN apoptosis thereby sustaining their function.

#### PA09/54 EXPRESSION AND SHEDDING OF LIGANDS FOR THE ACTIVATING RECEPTOR NKG2D IN MELANOMA CELL LINES

S. Morgado<sup>1</sup>, J.G. Casado<sup>1</sup>, E. Durán<sup>2</sup>, B. Sánchez-Correa<sup>1</sup>, I. Gayoso<sup>3,4</sup>, R. Solana<sup>3,4</sup>, R. Tarazona<sup>1</sup>

<sup>1</sup>University of Extremadura, Immunology Unit, Cáceres, Spain, <sup>2</sup>University of Extremadura, Department of Comparative Pathology, Cáceres, Spain, <sup>3</sup>University of Córdoba, Department of Immunology, Córdoba, Spain, <sup>4</sup>Instituto Maimónides de Investigación Biomédica de Córdoba, Córdoba, Spain

**Objectives:** Natural Killer cells (NK) are a component of the innate immune system that contributes to the immune response against tumours. NK cells can kill tumour cells without previous sensitization and their activation depends on a balance of activating and inhibitory signals transduced by surface receptors on their membrane.

The aim of this work was to characterize the expression of the human NKG2D ligands (NKG2DL) MICA/B and ULBPs on melanoma cell lines in order to determine the functional role of NKG2D in NK-mediated tumor-killing activity. Moreover, the shedding of MICA as a possible immune escape mechanism and the relationship of MICA polymorphism with NK cell activation through NKG2D was also analyzed.

**Methods:** We have studied the expression of NKG2DL in melanoma cell lines from the ESTDAB cell bank by flow cytometry. In melanoma tissues, NKG2DL were analyzed by immunohistochemistry. The shedding of soluble forms of MICA (sMICA) was tested by ELISA and to study the polymorphism of the MICA gene we have used LABtype SSO MICA kit. Finally, to study the NK cell activation against melanoma cells, we have performed degranulation assays with CD107a/b in NK cells co-cultured with melanoma cells.

**Results:** Previous results showed that 80% of melanoma cell lines analyzed expressed the stress-inducible NKG2D ligand MICA/B. Although NKG2D may act as a major receptor in NK cell-mediated lysis of MICA/B<sup>+</sup> melanoma cells, we have observed that, in the absence of inhibitory signals, some MICA/B<sup>+</sup> melanoma cells are resistant to NK cell mediated lysis. Thus, we have found that 60% of melanoma cell lines release sMICA that induces downregulation of NKG2D on NK cells. The study of the MICA allele polymorphism may indicate that the shedding of sMICA should be more frequent in those melanoma cell lines expressing certain alleles.

**Conclusion:** In conclusion, most of melanoma cells express MICA/B and NKG2D-MICA interaction plays a pivotal role in the elimination of several melanoma cell lines. However, some MICA<sup>+</sup> melanoma cell lines are resistant to NK-mediated lysis suggesting that immune escape mechanisms as shedding of sMICA or differences in NKG2D affinity for MICA alleles may influence NK cell function through NKG2D.

#### PA09/55 INTERACTION BETWEEN NATURAL KILLER AND DENDRITIC CELLS IN THE PRESENCE OF VACCINE AGENT OF UTERINE CERVICAL CANCER

V.M. Renoux<sup>1</sup>, A. Reschner<sup>1</sup>, I. Langers<sup>1</sup>, J. Willems<sup>1</sup>, E. Dortu<sup>1</sup>, J. Boniver<sup>1</sup>, P. Delvenne<sup>1</sup>, N. Jacobs<sup>1</sup>

<sup>1</sup>University of Liège, Experimental Pathology, Liège, Belgium

Cervical cancer, the second most frequent gynecological malignancy in the world, is caused by infection with high-risk human papillomaviruses (HPV). HPV16 and/or 18 are detected in more than 70% of these tumours. Prophylactic HPV-L1 virus like particle (VLP) vaccines have been tested in large clinical trials. These vaccines are highly efficacious to protect against HPV16 and HPV18 infection, but not against established infection. Since the interaction between Natural Killer (NK) cells and Dendritic cells (DC) is important for the induction of adaptive immune response, we study the effect of HPV-VLP on NK cells and in co-culture with autologous DC.

In order to know if HPV-VLP are able to enter in NK cells, we used fluorescent HPV-VLP with flow cytometry and confocal microscopy and we confirmed the results by electronic microscopy. We observed a slow entry of HPV-VLP in the NK cell line, NK92MI. In opposite, primary NK cells isolated from blood internalised HPV-VLP rapidly and HPV-VLP could be detected in the cells after 10 min of contact at 37°C. Since CD16 is described as a HPV co-receptor and is expressed on NK cells, but not on NK92 cell line, we tested the effect of CD16 blocking mAb (Clone 3G8) on VLP internalisation, but we were not able to detect significant inhibition of HPV-VLP entry.

Previous works have shown that HPV-VLP were able to activate DC. We confirmed these results and observed an increase of CD69 cell surface expression and IFNγ production by NK cells in the presence of DC activated by VLP. Interestingly, NK cells seemed to further activate DC in the presence of VLP as shown by an up-regulation of HLA-DR and CD86 on DC.

The results suggest that NK could play a role in the activation of DC induced by HPV-VLP during the vaccination against cervical cancer.

#### PA09/56 HEAT SHOCK PROTEIN 70 AND ITS PEPTIDE FRAGMENTS INCREASE IFN-GAMMA PRODUCTION AND MODIFY SURFACE MARKER EXPRESSION IN HUMAN NATURAL KILLER CELLS

L. Kanevski<sup>1</sup>, P. Vlaskin<sup>1</sup>, L. Alekseeva<sup>1</sup>, A. Nekrasov<sup>1</sup>, Y. Strelnikova<sup>1</sup>, E. Kovalenko<sup>1</sup>

<sup>1</sup>Shemyakin & Ovchinnikov Institute of Bioorganic Chemistry, Moscow, Russian Federation

**Introduction:** Heat shock protein 70 kDa (Hsp70) is a potential activator of natural killer cells (NK cells). Hsp70 stimulates proliferation, chemotaxis and cytotoxicity of NK cells. Earlier we showed that Hsp70 increased interferon-gamma (IFNγ) production in natural killer cells, activated by IL-2. In this work we investigated Hsp70 influence on an expression of NK cell surface markers (CD16, CD56 and CD94) and made an attempt to find fragments of Hsp70 molecule mediating the given effects.

**Materials and methods:** High-purified NK cell population was obtained by magnetic separation of human peripheral blood mononuclear cells. NK cell surface marker expression was analyzed by flow cytometry. IFNγ production was estimated with intracellular staining by flow cytometry.

**Results:** We found that Hsp70 alone increased the expression of CD94 and had no significant effect on CD16 or CD56. On the other hand, Hsp70 in pair with IL-2 (600 u/ml) notably decreased CD16 expression and had a small effect on CD94 and CD56. Stimulation of NK cells with IL-2 and Hsp70 in the range of concentrations 0,05-5 μg/ml resulted in significant increase of IFNγ production. A novel approach based on analysis of protein informational structure was used to discriminate the peptide fragments of the Hsp70 molecule mediating Hsp70 influence on NK cells [1]. ADD (Abnormal Distribution Density) sites of the protein sequence were detected, and six peptides were synthesized. Two peptides (399-408 и 411-424) generated the Hsp70-like increase of IFNγ production.

**Conclusion:** Thus Hsp70 was shown to modulate the expression of important NK-cell-specific surface markers CD16, CD56 and CD94. Two potential key peptide fragments which can play an important role in Hsp70 signaling were revealed.

1. Nekrasov A.N. Analysis of the information structure of protein sequences: a new method for analyzing the domain organization of proteins. *J. Biomol Struct Dyn.* 2004, 21(5), 615-24.

#### PA09/57 PROGNOSTIC EVALUATION OF NK CELL IMBALANCES BEFORE CD4-GUIDED TREATMENT INTERRUPTIONS ASSOCIATED TO THE DURATION OF ANTIRETROVIRAL TREATMENT INTERRUPTION

F. Bozzano<sup>1</sup>, M. Nasi<sup>2</sup>, S. Manzini<sup>3</sup>, P. Costa<sup>1,4</sup>, F. Marras<sup>1</sup>, C. Mussini<sup>2</sup>, L. Moretta<sup>1,4</sup>, A. Cossarizza<sup>3</sup>, A. De Maria<sup>1,5</sup>

<sup>1</sup>Università di Genova, Center of Excellence for Biomedical Research, Genova, Italy, <sup>2</sup>Azienda Ospedaliero-Universitaria Policlinico di Modena, Clinica Malattie Infettive, Modena, Italy, <sup>3</sup>Università di Modena e Reggio Emilia, Dipartimento di Scienze Biomediche, Modena, Italy, <sup>4</sup>Istituto Giannina Gaslini, Genova, Italy, <sup>5</sup>IST GE-Istituto Nazionale per la Ricerca sul Cancro, S.S. Infettivologia, Genova, Italy

The introduction of HAART has positive effects in the management of HIV-1+ patients with immune reconstitution and viremic control. Long-term metabolic side effects may represent a relevant burden when the treatment is maintained along the time. For these reasons, CD4-guided treatment interruption (TI) has been evaluated to address this point in patients with sustained successful control of viremia and immune reconstitution. TI results in a wide spectrum of time off-HAART in different patients. We studied whether differences in innate immune responses, in particular NK cells, may be associated to patterns of longer (LT) or a shorter (ST) treatment interruption period.

Clinical cohort parameters were analysed on a group of 8 LT and 8 ST patients. Cytofluorimetric analysis was performed to determine NK cell phenotype and specific function by g-IFN production. Phenotypic analysis of NK cells was performed on PBMC by four-colour cytofluorimetry using mAbs specific for activating and inhibitory NK receptors. In addition intracellular g-IFN production was evaluated after challenge with NK-sensitive target cells and/or with anti-Natural Cytotoxicity Receptors (NCR) mAbs.

LT and ST patients had significant differences in HAART-free time post-TI (27,5±11,51 /4,6±1,94 months, respectively) with comparable median CD4+ T cell count nadir (327±60,27/μl and 334±135,7/μl respectively). At TI, median CD4+ T cell counts were 568,5±373,35/733,5±190/μl, for patients respectively while CD8+ T cell proportions were 38,5±10,38% and 45,5±6,50% respectively.

In all patients persistent NK cell activation is evident despite immunoreconstitution and control of HIV replication. More importantly, persistent deficiency of NCR expression was observed in this group of patients under long-term successful HAART. With regard to the expression of some triggering surface NK cell receptors, relevant differences were observed in those patients who develop an LT course on TI vs. ST patients. Lower expression of NCRs on NK cells was evident. While this does not translate in different g-IFN production in redirected functional assays, total g-IFN production was impaired in LT. NK cell phenotype and function may represent an additional parameter useful for the prognostic identification of patients who may undergo safe prolonged treatment interruption. Additional studies to evaluate larger patient cohorts and to the underlying mechanisms are warranted.

#### PA09/58 THE FUNCTIONAL SIGNIFICANCE OF MICA POLYMORPHISM

S.A. Shafi<sup>1</sup>, M.R. Stanford<sup>2</sup>, R. Vaughan<sup>3</sup>, G.R. Wallace<sup>4</sup>, A.C. Hayday<sup>1</sup>

<sup>1</sup>King's College London, Immunobiology, London, United Kingdom, <sup>2</sup>St Thoma's Hospital, Ophthalmology, London, United Kingdom, <sup>3</sup>King's College London, Immunobiology and Clinical Transplantation, London, United Kingdom, <sup>4</sup>University of Birmingham, London, United Kingdom

**Introduction and objectives:** MICA is highly polymorphic with all polymorphisms occurring between exon 2 and 5 which collectively encode the three extracellular domains and transmembrane region. The selection pressures determining polymorphism in MICA are not known. While it might be argued that polymorphism reflects linkage to MHC, it is equally possible that pathogen challenges drive MICA variation. To resolve this, we have undertaken a functional analysis of MICA polymorphism by assessing their capacity to differentially regulate the responses of NKG2D positive lymphocytes. In particular the project makes a functional analysis of the linkage of MICA\*009 and HLA-B51 to Behcet's disease (BD).

**Methods:** Stable isogenic Chinese hamster Ovary (CHO) cell lines expressing MICA\*009, MICA\*008, MICA\*027, MICA\*004 and ULBP2 (positive control) separately were created using an FRT-Flp-In system, to permit comparison of the different MICA alleles to promote killing by NKG2D<sup>+</sup> lymphocytes from healthy controls and Behcet's patients. The capacity of killing to be regulated by HLA-B51 and \*B52 (control allele) was assessed by supre-transfection of the CHO-Frt-MICA cells.

**Results and Conclusion:** Relative to other alleles, cells expressing MICA\*009 showed no significant differences in surface expression, shedding and targeting by effector cells from controls and patients. However, major differences in surface expression were shown by MICA\*008. Nonetheless MICA\*008 positive cells were comparably targeted by peripheral blood effectors. However, of particular note, different donors showed a restriction of killing of cells expressing particular but different MICA alleles, which did not obviously correlate with their own genotypes. This wholly unexpected result refutes the notion that the targeting of cells is determined simply by the interaction of NKG2D with a ligand, but must be regulated by additional factor(s) that intriguingly differ from person to person and differentially affect different alleles of MICA.

#### PA09/59 ANALYSIS OF THE RED JUNGLE FOWL LRC LOCUS

K. Lochner<sup>1</sup>, B.C. Viertelboeck<sup>1</sup>, T.W. Göbel<sup>1</sup>

<sup>1</sup>LMU München, Dept. Vet. Sciences, Inst. f. Animal Physiology, München, Germany

**Objectives:** The chicken leukocyte receptor cluster (LRC) is located on microchromosome 31 and contains inhibitory, activating, and bifunctional Ig-like genes, known as chicken Ig-like receptors (CHIR). In this report we have analyzed bacterial artificial clones (BAC) resembling the CHIR locus of the red jungle fowl.

**Methods:** A total of eight BAC was identified carrying CHIR genes. The BAC sequences were initially analyzed using Genscan for prediction of exon intron structures. The regions of interest were further analyzed using the Lasergene software package. Putative protein sequences were characterized with the SMART module.

**Results:** The size of the BACs analyzed ranged from 27 to 214 kb. The BAC sequences had some gaps, so some BAC sequences consisted of up to 17 fragments. A total of 1282 kb could be analyzed. Within this sequence 179 CHIR were identified that were further categorized into 46 activating (CHIR-A), 26 inhibitory (CHIR-B) and 25 bifunctional CHIR (CHIR-AB). The remaining 82 CHIR apparently were pseudogenes due to sequence variations that prevented the generation of full length proteins. 14 additional CHIR could not be qualified due to their position in sequence gaps of the BAC clones. On average there was a CHIR gene every 7.4 kb. The genomic structure of all CHIR genes was similar to the prototypic CHIR spanning about 2000 bp for CHIR-B and CHIR-AB and about 1200 bp for CHIR-A. Even the sizes of the introns were remarkably conserved. A comparison of all sequences identified 3 triplets and 14 pairs of identical sequences. This could reflect overlapping regions. In addition, a comparison to the genomic sequence of LSL chickens revealed that only a single CHIR gene was identical.

**Conclusion:** The CHIR gene cluster resembling the chicken LRC has been vastly expanded and contains more than hundred receptor genes. It may cover the entire microchromosome 31. The presence of only one identical sequence pair between red jungle fowl and LSL chickens indicates large sequence diversity which may be the result of ongoing strong selection.

#### PA09/60 REGULATORY ROLE OF NKP44, NKP46, DNAM-1 AND NKG2D RECEPTORS IN THE INTERACTION BETWEEN NK CELLS AND TROPHOBLAST CELLS. EVIDENCE FOR DIVERGENT FUNCTIONAL PROFILES OF DECIDUAL VERSUS PERIPHERAL NK CELLS

P. Vacca<sup>1</sup>, C. Cantoni<sup>1,2,3</sup>, C. Prato<sup>1</sup>, F. Canegallo<sup>4</sup>, N. Ragni<sup>4</sup>, A. Moretta<sup>1,3</sup>, L. Moretta<sup>1,2,3</sup>, M.C. Mingari<sup>1,5</sup>

<sup>1</sup>University of Genova, DIMES, Genova, Italy, <sup>2</sup>Istituto Giannina Gaslini, Genova, Italy, <sup>3</sup>University of Genova, Centro di Eccellenza per la Ricerca Biomedica, Genova, Italy, <sup>4</sup>Azienda Universitaria Ospedaliera San Martino, Dip. Ginecologia e Ostetricia, Genova, Italy, <sup>5</sup>Istituto Nazionale per la Ricerca sul Cancro, Genova, Italy

**Objectives:** During the first trimester of pregnancy NK cells represent >50% of the lymphoid cells present in the human decidua where they are in close contact with trophoblast cells. Because in decidua tissue NK cell activation and function can be modulated by these cell contacts we analyzed the nature and the effects of the molecular interactions occurring between NK cells and trophoblast cells.

**Methods:** Decidua samples were obtained at 9-12 weeks of gestation from singleton pregnancies of mothers requesting termination of the pregnancy for social reasons or following spontaneous pregnancy failure (upon informed consent). NK cells were isolated from peripheral blood (pNK) or decidua (dNK) tissue using Ficoll-Hypaque density gradients and enriched by RosetteSep or magnetic selection beads. Cells were analyzed by cytofluorimetry, confocal microscopy and immunohistochemistry. NK cells were tested for cytolytic activity and/or cytokine production.

**Results:** We show that trophoblast cells express ligands for the NK activating receptors NKP44 and DNAM-1 and that interaction with the corresponding receptors results in NK cell triggering. While pNK cells lysed trophoblast cell lines, dNK cells did not. On the other hand, dNK were able to release VEGF, SDF-1, IP10 and large amounts of IL-8. Interaction with K562 target cells was exploited to induce optimal NK cell triggering, allowing a parallel, quantitative assessment of both cytolytic activity and cytokine production. While dNK cells were unable to kill K562 even at high effector:target (E:T) ratios, they released large amounts of IL-8 also at low E:T ratios, a scenario compatible with dNK trophoblast cells interaction occurring within decidua tissues.

**Conclusion:** In view of our present findings, one may speculate that lack of appropriate triggering of dNK cells by trophoblast cells might be involved in pathological events such as recurrent miscarriages. Indeed, the lack of appropriate receptor-ligand interactions may lead to reduced release of a peculiar set of cytokines useful for building/remodeling of new vessels during pregnancy. On the other hand, an excessive dNK cell triggering due to abnormally high numbers of dNK cells and/or over-expression of ligands for activating NK receptors may lead to an overwhelming growth of endometrial tissues.

#### PA09/61 NATURAL KILLER CELL RESPONSES IN BOTH ACUTE AND CHRONIC PHASES OF A MURINE MODEL OF EXPERIMENTAL COLITIS

L.J. Hall<sup>1</sup>, A. Quinlan<sup>1</sup>, F. Shanahan<sup>1</sup>, K. Nally<sup>1</sup>, S. Melgar<sup>1,2</sup>

<sup>1</sup>Alimentary Pharmabiotic Centre, University College Cork, Cork, Ireland, <sup>2</sup>GlaxoSmithKline, Immuno-Inflammation, CEDD, Stevenage, United Kingdom

**Introduction/aim:** Natural killer (NK) cells contribute to host resistance against viruses, bacteria and certain parasites as well as providing immune surveillance against the development of tumours. Recently NK cells have been implicated in the modulation of various autoimmune diseases. In this study we have investigated the recruitment and functional characteristics of NK cells in an experimental model of colitis.

**Methods:** Female C57BL/6 mice were exposed to 6 days of dextran sulphate sodium (DSS) followed by 20 days of water. Organs (spleens, mesenteric lymph nodes and colons) were harvested during both acute and chronic phases of colitis to determine percentage, distribution, activation status and cytokine/cytotoxic profiles.

**Results:** We observed that as early as 1 day post DSS, animals were found to have significant increases in both the percentage and activation status of NK cells in comparison to controls, which continued throughout the study. With regards to their cytotoxic/cytokine profile we observed significant increases of perforin<sup>+</sup> NK cells initially (D1), followed by increases in granzyme B producing NK cells. We also observed a diverse range of cytokine being produced from NK cells during colitis, including both anti-inflammatory (IL-4 and IL-10) and pro-inflammatory cytokines (IFN- $\gamma$ , TNF- $\alpha$ , IL-6 and IL-17).

**Conclusions:** Collectively, our data suggest that NK cells might play a prominent role in the establishment of immune responses in DSS-induced colitis and therefore may have a consequent impact on the pathogenesis of inflammatory bowel disease (IBD).

#### PA09/62 TWO DIFFERENT APPROACHES FOR THE STUDY OF NK CELLS RECEPTORS IN UNEXPLAINED RECURRENT SPONTANEOUS ABORTIONS

E. Konsta<sup>1</sup>, V. Kitsiou<sup>2</sup>, K. Psarra<sup>2</sup>, V. Kapsimali<sup>2</sup>, T. Athanassiades<sup>2</sup>, D. Kouniaki<sup>2</sup>, V. Stamati<sup>2</sup>, S. Dendrinos<sup>3</sup>, C. Papasteriades<sup>2</sup>

<sup>1</sup>National and Kapodistrian University of Athens, Department of Chemistry, Athens, Greece, <sup>2</sup>"Evangelismos" Hospital, Department of Immunology and Histocompatibility, Athens, Greece, <sup>3</sup>University of Athens, Second Department of Obstetrics and Gynecology, Athens, Greece

**Background:** NK cells constitute an essential component of innate immunity. They express – among others – killer cell inhibitory receptors (KIRs) which bind to self HLA-class I molecules preventing killing of autologous cells. More specifically KIR2DL1 (CD158a) recognizes an epitope shared by HLA-Cw 2, 4, 5, 6, 17, 18 alleles, KIR2DL2 and KIR2DL3 (CD158b1 and CD158b2) recognize an epitope shared by HLA-Cw 1, 3, 7, 8, 13, 14 and KIR3DL1 (CD158e1) recognizes an HLA-Bw4 epitope.

tope. The population of NK cells is intensively studied in unexplained recurrent spontaneous abortions (URSA), malignancies, as well as in hematopoietic stem cell transplantation (HSCT).

The aim of this study was to investigate NK cells receptors and respective HLA antigens in each couple with URSA in order to clarify the role of these receptors in URSA.

**Materials and methods:** In 16 couples with URSA: i) flow cytometric (four colour) analysis of peripheral blood lymphocytes labeled with monoclonal antibodies CD3, CD56, CD16, CD158a, CD158b, Nkp44, Nkp30, Nkp46, CD158e1, KARP50.3 by direct whole blood staining and ii) HLA class I and II typing by serology (CDC) and molecular (SSP, SSOP) techniques, were performed.

**Results:** Couples with URSA express NK cells receptors as determined by flow cytometry: CD158a (96.9%), CD158b (81.2%), Nkp44 (0%), Nkp30 (6.2%), Nkp46 (68.8%), CD158e1 (59.4%) and KARP50.3 (31.2%). The expected expression of NK cell receptors (CD158a, CD158b, CD158e1) according to the HLA typing was mostly confirmed.

**Conclusions:** In couples with URSA, NK cells express mainly the inhibitory receptors CD158a, CD158b and CD158e1 and the cytotoxicity receptor Nkp46. The expression of the inhibitory receptors according to the HLA typing was confirmed in most of the individuals studied by flow cytometry. This is an ongoing study and HLA typing will be compared with the expression of NK cell receptors in the total population.

#### PA09/63 LIGAND SPECIFICITY OF HUMAN KLRG1

M. Hofmann<sup>1</sup>, S. Schwartzkopff<sup>1</sup>, H. Pircher<sup>1</sup>

<sup>1</sup>Institute of Medical Microbiology and Hygiene, Freiburg, Germany

The inhibitory killer cell lectin-like receptor G1 (KLRG1) is expressed by effector, effector-memory T cells and NK cells in man and mice. KLRG1 is often used as a cell differentiation marker and is associated with replicative cell senescence. Murine KLRG1 forms monomers, dimers and higher multimeric complexes in T cells. Members of the classical cadherin family are ligands of KLRG1, namely E-, R- and N-cadherin for murine KLRG1 and E-cadherin for human KLRG1. Cadherins are multimeric adhesion molecules which form homotypic interactions in "trans" and in "cis". The binding site of E-cadherin to KLRG1 is located at the N-terminal area of E-cadherin since deletion of the first and the second extracellular domain of E-cadherin abolishes reactivity. Epithelial-mesenchymal transition in tumor development involves down-regulation of E-cadherin and up-regulation of N-cadherin. It is not yet known whether N-cadherin also serves as a ligand for human KLRG1. To address this question we used a KLRG1-reporter cell system. In this system KLRG1-ligation leads to GFP-induction which is analysed by flow cytometry. This reporter system was also used for investigations concerning the detailed ligand specificity of human KLRG1.

#### PA09/64 GLYCOCONJUGATE TRIGGERED NK CELL SIGNALING IN ANIMAL TUMOR MODEL

V. Grobarova<sup>1</sup>, V. Benson<sup>1</sup>, L. Vannucci<sup>1</sup>, A. Fišerová<sup>1</sup>

<sup>1</sup>Institute of Microbiology, Academy of Science of Czech Republic, Dept. Immunology, Prague, Czech Republic

**Objectives:** Natural killer (NK) cells effector functions are regulated by homeostatic balance between activating receptors and inhibitory receptors. C-type lectin receptor superfamily recognizes carbohydrate structures involved in signal transduction events. In our previous *in vivo* experiments *N*-acetyl- $\beta$ -D-glucosamine (GlcNAc) conjugates on polyamidoamine scaffold-PAMAM (GN8P) decreased the tumor growth, increased NK mediated cytotoxicity, prolonged the survival time of animals and induced signaling cascades in spleen lymphocytes.

**Methods:** We used syngeneic B16F10 melanoma-bearing mice treated with GN8P and the schedule consisted of three doses administered intraperitoneally every third day. Fresh spleen NK cells (DX5+CD8-) were sorted for subsequent RT-PCR analysis and cDNA arrays.

**Results:** We detected increased expression of activating NKR-P1C receptor in both healthy and tumor-bearing animals. Furthermore, the expression profile of other isoforms of NKR-P1 receptors: NKR-P1A and NKR-P1D remained unchanged. After GN8P injection, we elicited modification in expression level of cytokines; notably higher level of IFN- $\gamma$  and TNF- $\alpha$ , involved in NK cells mediated cytotoxicity. In C57Bl/6 melanoma-bearing mice, the expression of interleukins IL-1 $\beta$  and IL-4 mRNA was 5-fold higher in comparison to control animals. For deeper analysis GN8P effect in NK cells, we used gene expression cDNA arrays. In healthy mice administration of glycoconjugate caused among others, significant down-regulation of RAB30, Gpsm2, and Rgs2 involved in G-protein signaling. In melanoma-bearing mice, the genes for heat shock protein (Hspa1b), hypoxia inducible factor 1 (Hif1a) were down-regulated, and IL-4 up-regulated.

**Conclusion:** GN8P influenced gene expression profile including cytokines, C-type lectin receptors, regulatory molecules, and thus modulate NK cells recognition and effector functions.

Supported by Grant Agency of Academy of Sciences of the Czech Republic IAA500200620, IAA500200509, and the Czech Science Foundation 310/08/H077.

#### PA09/65 LYMPH NODES OF MELANOMA PATIENTS THAT ARE INVADDED WITH TUMOR CELLS HAVE HIGHER FREQUENCY OF NK CELLS EXPRESSING CD16, CD69 HLA-DR BUT SIMILAR IFN $\gamma$ LEVEL COMPARED TO LYMPH NODES WITHOUT TUMOR CELLS

A. Vuletic<sup>1</sup>, G. Konjevic<sup>1,2</sup>, I. Jovanic<sup>1</sup>, M. Inic<sup>1</sup>

<sup>1</sup>Institute for Oncology and Radiology of Serbia, Beograd, Serbia, <sup>2</sup>School of Medicine, University of Belgrade, Beograd, Serbia

**Objectives:** The first line of antitumor immune defense is mediated by natural killer (NK) cells. The aim of this study it was to investigate in melanoma patients difference in NK cell receptor distribution, activation marker expression and intracellular IFN $\gamma$  content between lymph nodes (LN) that have been invaded and those that have not been invaded with tumor cells (malignant and benign, respectively).

**Methods:** PB and regional LN were obtained from patients who underwent melanoma resection. LN were incised immediately after removal and cut into two parts, one of which was paraffin embedded to perform histology evaluation and the other was mechanically dissociated to obtain a single cell suspension. Lymphocytes were purified using Lymphoprep density gradient. After being stained with monoclonal antibodies, CD16, NKG2D, CD158a, CD158b, CD25, CD69 and HLA-DR expression was analyzed on gated CD3<sup>+</sup>CD56<sup>+</sup> NK cells by flow cytometer. For intracellular staining of IFN $\gamma$  cells were first stained for extracellular markers (CD3 and CD56) and then permeabilized.

**Results:** In LN of melanoma patients CD56<sup>bright</sup> represents more abundant NK cell subset. We detected higher intracellular level of IFN $\gamma$  in non stimulated CD3<sup>+</sup>CD56<sup>+</sup> NK cells from LN compared to PB, but similar levels of IFN $\gamma$  in both malignant and benign LN. There was no difference in percentage of NK cells expressing NKG2D and CD161 activating NK cell receptors between malignant and benign LN. Substantially higher percentage of NK cells in PB expresses one of the major cytotoxic receptors CD16 and both of the investigated inhibitory KIRs (CD158a and CD158b) whereas CD69 and HLA-DR activation markers and CD25 showed increased expression on NK cells from LN. Ln malignant LN more NK cells were positive for CD16, CD69 activation marker and CD158b KIRs, which are predominantly present on CD56<sup>dim</sup> NK cell subset.

**Conclusions:** NK cell in both malignant and benign LN predominantly belong to CD56<sup>bright</sup> subset, show low CD158a and CD158b KIRs, CD16 cytotoxic receptor expression and higher IFN $\gamma$  content and therefore are less mature than PB NK cells. Invasion of malignant cells into lymph nodes might contribute to higher expression of CD16 cytotoxic receptor and CD69 and HLA-DR activation markers.

#### PA09/66 REGULATORY PATHWAYS OF KILLER IMMUNOGLOBULIN RECEPTOR GENE EXPRESSION IN PRIMATES

C. Albrecht<sup>1</sup>, J. Gruber<sup>1</sup>, M. Brameier<sup>1</sup>, L. Walter<sup>1</sup>

<sup>1</sup>Deutsches Primatenzentrum, Primatengenetik, Göttingen, Germany

**Objectives:** The regulatory pathways of KIR gene expression on natural killer (NK) cells are not yet well characterized. Recent studies indicate that epigenetic mechanisms play a role in maintaining different killer immunoglobulin receptor (KIR) expression patterns. However, exact regulatory mechanisms and in particular their driving forces remain elusive. Recently, microRNAs are shown to play a major biological role by altering the expression levels of different genes in a sequence specific manner at post-transcriptional and presumably transcriptional level. First *in silico* predictions of target sites for human microRNAs in KIR gene sequences support the hypothesis of a possible regulatory role in their expression. The aim of this study is a better understanding of regulatory networks in particular those involving non-coding RNAs in primate cells.

**Methods:** For the identification of KIR gene regulating miRNAs a set of different prediction tools such as targetScan and miRanda was used. Sequences that are fully complementary to candidate miRNAs were cloned into a dual luciferase reporter construct to check for their activity. By initiation of the RNAi process the present miRNA leads to degradation of the reporter-gene mRNA, thereby indicating its expression and function.

**Results:** Combination of results from *in silico* prediction for three KIR genes revealed six miRNAs that are likely to target at least one of the analysed sequences. In this study we functionally detected these miRNAs in a human Natural killer-cell line. The predicted interactions of these miRNAs with their native target sites within the KIR 3'UTR will be investigated.

**Conclusion:** The results are a distinguished basis for further experiments and strengthen our assumption of a possible miRNA mediated regulation of KIR genes. For the first time we successfully performed a functional detection assay for predicted miRNAs in human NK-cells.

#### PA09/67 EFFECT OF PROLACTIN *IN VITRO* AND *IN VIVO* ON PERFORIN AND TRAIL EXPRESSION IN HUMAN NK LYMPHOCYTES

J. Hernandez-Ruiz<sup>1</sup>, B. Farfan<sup>1</sup>, J. Cordoba<sup>1</sup>, A. Parra<sup>2</sup>, J. Ramirez<sup>2</sup>, D. Kershenovich<sup>1</sup>, G. Gutierrez-Reyes<sup>1</sup>

<sup>1</sup>Universidad Nacional Autónoma de México, Medicina Experimental Facultad de Medicina, México DF, Mexico, <sup>2</sup>Instituto Nacional de Perinatología, Mexico DF, Mexico

Prolactin (PRL) is a hormone that apart from its role in the endocrine system, can enhance the proliferating response and the cytotoxic capacity of NK lymphocytes *in vitro* as well as *in vivo* animal models. In human NK, experiments has been performed *in vitro* using stable cell lines and purified NK from peripheral blood and was described that prolactin increases the triggering receptors (Nkp46, Nkp44 and Nkp30) responsible for natural killer (NK) cell-mediated cytotoxicity. Although,



the effect of prolactin over the cytotoxic effector molecules Perforin and TRAIL remains unknown *in vitro* as well as *in vivo*. Aim. To assess the effect of prolactin *in vitro* and *in vivo* in the expression of perforin and TRAIL in human NK. Methods. 5 volunteers were treated with levosulpiride and cimetidine, which have been shown to increase the systemic concentration of prolactin. Blood samples were taken before and after treatment. Perforin and TRAIL expression were analyzed by flow cytometry. Additionally, culture of purified NK with PRL, IL-2 or PRL+IL-2 was done for three days and perforin and TRAIL expression was assessed. Results. Levosulpiride and Cimetidine treatment up-regulated serum concentration of PRL (before 9.3ng/ml  $\pm$  4.5, after 41.4  $\pm$  14.1;  $p < 0.01$ ). *in vitro*, PRL down-regulated TRAIL (control= 598.3 FMI  $\pm$  124.5, PRL= 256.8 FMI  $\pm$  116.4,  $p < 0.05$ ), and PRL+IL-2 up-regulated perforin (control= 103.7 FMI  $\pm$  14.8, PRL+IL2= 135.2 FMI  $\pm$  20.6,  $p < 0.01$ ). *in vivo*, perforin expression remained stable after the treatment, and only down-regulation of TRAIL expression in CD56<sup>bright</sup> was detected (before 668.4 FMI  $\pm$  136.1, after 186 FMI  $\pm$  133.3;  $p < 0.05$ ). Conclusion. The results suggest that PRL is capable to regulate TRAIL expression mainly over CD56<sup>bright</sup> NK subpopulation. It is possible that perforin expression only increase if an IL-2 source was activated, like T lymphocytes in an active infection. Prolactin could be synergized by IL-2 in order to increase the cytotoxic capacity of NK cells. This work was supported by PAPIIT-UNAM IN216307.

#### PA09/68 DIVERSITY OF HLA CLASS I AND KIR GENES IN SYSTEMIC SCLEROSIS

P.H. Salim<sup>1,2</sup>, M. Jobim<sup>1</sup>, M. Bredemeier<sup>3</sup>, J.A. Chies<sup>4</sup>, J. Schlottfeldt<sup>1</sup>, J.C. Brenol<sup>3</sup>, L.F. Jobim<sup>1</sup>, R.M. Xavier<sup>3</sup>

<sup>1</sup>Hospital de Clínicas de Porto Alegre, Serviço de Imunologia, Porto Alegre, Brazil, <sup>2</sup>Universidade Federal do Rio Grande do Sul, Pós-Graduação em Medicina: Ciências Médicas, Porto Alegre, Brazil, <sup>3</sup>Hospital de Clínicas de Porto Alegre, Serviço de Reumatologia, Porto Alegre, Brazil, <sup>4</sup>Universidade Federal do Rio Grande do Sul, Departamento de Genética, Porto Alegre, Brazil

Systemic sclerosis (SSc) is a relatively rare disease, characterized by autoimmunity and variable degrees of fibrosis within the skin and internal organs. Its pathogenesis is not well known, but evidence suggests that there is inappropriate activation of the immune system by some environmental stimuli in individuals with a genetic background of susceptibility. NK cells play a key role in regulating autoimmune responses. They kill diverse target cells with decreased or absent expression of major histocompatibility complex (MHC) class I molecules, including virus-infected cells and normal hematopoietic cells. The objective of this study is to investigate possible associations of Killer Cell Immunoglobulin-Like Receptor (KIR) polymorphisms and KIR combinations with SSc, including the limited (LSSc) and diffuse (dSSc) forms of the disease. We analyzed 113 South Brazilian patients with SSc and 115 voluntary bone marrow donors. Typing of 15 KIR genes and HLA class I alleles were performed by Polymerase Chain Reaction with Sequence Specific Primers (PCR-SSP). PCR products were analyzed on agarose gel after electrophoresis. The frequency of inhibitory KIR2DL2 was significantly decreased among patients with SSc compared with healthy controls (28.7% versus 65.2;  $P = 0.0000009$ , odds ratio [OR] 0.21, 95% confidence interval [95% CI] 0.11–0.38). When activating and inhibitory KIR genes were analyzed in combination, the concomitant presence of KIR2DS2 and absence of KIR2DL2 (KIR2DS2+/KIR2DL2-) phenotype was more frequent in SSc patients than in the control group (26.08% versus 1.75%;  $P = 0.00000003$ , OR = 19.94, 95% CI [4.78–175.10]). On the other hand, the presence of both KIR2DS2 and KIR2DL2 was more frequent in the control group (26.96% versus 57.39%;  $P = 0.000005$ , OR = 0.27, 95% CI [0.15–0.49]). No significant difference in KIR genes polymorphisms was found between LSSc and dSSc disease subsets. Analysis of KIR2DL2, -2DL3 and -2DS2 ligand interactions stratified by HLA-C1 or -C2 did not produce significant results. The combination of KIR2DS2+/KIR2DL2- may be a risk factor for development of SSc while the higher frequency of the inhibitory KIR2DL2 gene in the control group points to a protective function. These results indicate a potential role of KIR genes in the SSc pathogenesis.

#### PA09/69 CHANGES IN MHC CLASS I EXPRESSION, MICA/B AND CD95 DURING THE PROGRESSION OF MGUS TO MULTIPLE MYELOMA: EVIDENCE OF A NK-MEDIATED CELL IMMUNOEDITION

M. Bernal<sup>1</sup>, P. Garrido<sup>2</sup>, P. Jiménez<sup>1</sup>, R. Carretero<sup>1</sup>, M. Almagro<sup>3</sup>, P. López<sup>2</sup>, P. Navarro<sup>2</sup>, F. Garrido<sup>1</sup>, F. Ruiz-Cabello<sup>1</sup>

<sup>1</sup>Hospital Universitario Virgen de las Nieves, Análisis Clínicos e Inmunología, Granada, Spain, <sup>2</sup>Hospital Universitario Virgen de las Nieves, Hematología y Hemoterapia, Granada, Spain

**Objectives:** The molecular basis of Monoclonal gammopathy of undetermined Significance (MGUS) progression to a malignant monoclonal gammopathy remains poorly understood. In this study, we analyze successive changes in MHC class I expression, MICA and CD95 during the malignant transformation of plasma cells and disease progression from MGUS to Multiple Myeloma (MM).

**Methods:** We studied bone marrow (BM) of patients diagnosed with MGUS (n=6) or MM (n=6) and 1 patient with Plasma Cell Leukemia, based on standard criteria. The patients did not receive any treatment at the day of the immunophenotype analysis. We also selected by flow cytometry and cytology 5 BM donors. We analyzed the cells by direct staining using the following monoclonal antibodies (mAb): anti-CD38, anti-CD138, anti-CD95, anti-HLA-ABC and anti MICA-MICB conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE) or allophycocyanin (APC). Statistically significant differences were found between the studied groups.

**Results:** We detected high levels of MHC class I expression in plasma cells from donors without monoclonal gammopathy, that were even higher in MGUS and reached the highest level in MM plasma cells. Therefore, we observed that malignant transformation in MM is associated with upregulation of MHC class I surface expression in plasma cells. On the contrary, expression of the activating NKG2D ligand MICA showed an opposite pattern: lower in MM, intermediate levels in MGUS, and the highest expression in donor cells. The differences found were significant between plasma cells from donor and MGUS, and between MGUS and myeloma cells. We also observed a significant reduction of expression of FAS on MM cells surface ( $p < 0.001$ ). Interestingly, we have observed a total loss of FAS and MICA expression in a patient with Plasma Cell Leukemia, which is an aggressive terminal complication of plasma cells in Multiple Myeloma.

**Conclusions:** Our results show that the phenotype MHC class I<sup>bright</sup> MICA<sup>dim/-</sup>, CD95<sup>dim/-</sup> changes successively to MHC class I<sup>bright</sup> MICA<sup>+</sup>, CD95<sup>+</sup> during the transformation of MGUS cells to MM and Plasma Cell Leukemia. Changes in the expression of these molecules could deeply affect the balance between activating and inhibitory signals that regulates NK cells activity.

#### PA09/70 EXPRESSION OF CELL BOUND AND SOLUBLE NKG2D LIGAND ULBP4/RAET1E IN CANCER CELLS

I. Jafferji<sup>1</sup>, R. Eagle<sup>2</sup>, J. Trowsdale<sup>1</sup>

<sup>1</sup>University of Cambridge, Pathology, Cambridge, United Kingdom, <sup>2</sup>Caltech, California, United States

NKG2D is an activating receptor found on Natural Killer (NK) cells, CD8+ $\alpha\beta$  T cells and  $\gamma\delta$ T cells. The ligands for NKG2D can comprise eight cellular proteins, which can be classified into two families; the MHC Class-related-chain (MIC) and UL-16 binding protein (ULBP)/ Retinoic acid early transcript 1 (RAET1). NKG2D ligands are rarely expressed in normal tissue but are frequently upregulated in tumour cells. NKG2D has been shown to recruit antitumour immune responses *in vivo*. Soluble NKG2D ligands, have been shown to downregulate NKG2D from the surface of NK and T cells and therefore counteract NKG2D mediated tumor immunosurveillance. In this study we have looked at cellular expression of the NKG2D ligand ULBP4/RAET1E and the soluble spliced variant RAET1E2 without a transmembrane domain in tissue, transformed cell lines and the serum of cancer patients. We have generated polyclonal and monoclonal antibodies which can recognise the extracellular domain of RAET1E and detect both full length and soluble forms of RAET1E. Using these antibodies we have found restricted expression of RAET1E in normal tissue mainly in the skin and colon, however staining in various cancer tissue. We have detected soluble RAET1E expression in various tumour cell line supernatant and the serum of colorectal cancer patients by sandwich ELISA.

#### PA09/71 EXPRESSION ANALYSIS OF NKC-ENCODED C-TYPE LECTIN-RELATED (CLR) MOLECULES

S. Leibelt<sup>1</sup>, S. Kutruff<sup>1</sup>, A. Steinle<sup>1</sup>

<sup>1</sup>Institute for Cell Biology, Department of Immunology, Eberhard Karls University Tuebingen, Tuebingen, Germany

Target cell recognition of NK cells involves many different inhibitory and activating receptors. Most of these NK receptors are members of two gene families encoded in distinct genomic regions: the Ig-like receptors encoded in the Leukocyte Receptor Complex (LRC), and the C-type lectin-like receptors (CTLR) encoded in the Natural Killer Gene Complex (NKC), respectively. Many of the murine and human CTLR remain insufficiently characterized and several of these are still orphan receptors. The C-type lectin-related (Clr) genes map to the mouse NKC between the Nkrp1a and CD69 genes. Most members of the Clr gene family are poorly characterized and include several presumed pseudogenes. Clr genes are physically linked to and interspersed with members of the gene family of NK receptor proteins 1 (Nkrp1) including the gene of the activating NK1.1 receptor. It has been shown that certain Nkrp1 proteins are receptors of Clr molecules, e.g. the inhibitory Nkrp1d receptor engaging Clr-b. It has been suggested that Nkrp1-Clr interactions represent another layer of control of NK activation apart from the MHC class I-centric 'missing-self' recognition systems.

Since little is known about the expression of most mouse Clr molecules (except Clr-b), we set out for a thorough characterization of Clr genes and transcripts. We identified a total of eight mouse Clr genes in the genetic region between CD69 and Nkrp1a by an *in silico* approach. To address their potential functionality, we performed RT-PCR of various tissues from different mouse strains. As previously reported we found a broad expression of Clr-b, and a restricted expression of Clr-f and Clr-g in non-hematopoietic and hematopoietic tissues, respectively. Furthermore, we obtained evidence for a tissue-specific expression of other, so far uncharacterized Clr genes such as Clr-a, Clr-c, and Clr-d. Detailed expression analyses and functional studies of these novel Clr molecules are subjects of ongoing research.

#### PA09/72 CHARACTERIZATION OF SNARE PROTEINS IN HUMAN NK CELLS

L. Delgado-Perez<sup>1</sup>, A. Campos-Caro<sup>1</sup>

<sup>1</sup>Hospital Universitario Puerta del Mar, Unidad de Investigación, Cadiz, Spain

NK cells participate in host protection by eliminating cells with altered expression of Major Histocompatibility Complex class I (MHC-I) molecules, which can result from viral infection or transformation. Even though their main cytotoxic mechanism is granule secretion, little is known about the components of the membrane fusion machinery that catalyze and regulate this kind of exocytosis. The present study explores, in human NK cells, the possible presence of one of the universal mediators of membrane fusion: the SNARE (soluble N-ethylmaleimide (NEM) sensitive factor attachment protein receptor) protein system, and examines its func-

tional role in NK cell granule exocytosis. Up to now, we have identified numerous members of this protein family (as transcripts and as proteins) in the established cellular lines of NK cells (NK-92 and NK-L) as well as in peripheral blood human NK cells.

SNARE transcripts identified in human NK cells correspond with proteins previously identified as elements of the exocytic route in other cellular types of hematopoietic origin. Members of the three SNARE subfamilies have been identified: Syntaxin (2, 3, 4, 6, and 11), VAMP (2, 3, 7 y 8) and SNAP-25 subfamilies (SNAP-23). In addition, the expression of SNARE proteins was confirmed and their subcellular localizations were determined by confocal fluorescence microscopy. It widely emphasizes the coincident location of VAMP- 7 with cytotoxic granules, in accordance with previous studies of other authors, and as other SNAREs members are vesicles associated. In conclusion, human NK cells express several protein isoforms corresponding to the three SNARE families. This serves as starting point for identifying what SNARE proteins are forming the complexes that mediate the secretion of cytotoxic granules and to see if they are different from the SNARE complexes needed for cytokine secretion. In order to evaluate the role of the identified SNARE proteins in granule exocytosis our current work focuses on optimizing inhibition assays.

#### PA09/73 FAMILY BASED STUDY ON KIR/HLA LIGAND GENE POLYMORPHISMS IN PATIENTS WITH ACUTE LYMPHOBLASTIC LEUKEMIA

V.P. Varbanova<sup>1</sup>, B. Avramova<sup>2</sup>, M. Jordanova<sup>2</sup>, M. Metodieva<sup>1</sup>, A. Mihaylova<sup>1</sup>, E. Naumova<sup>1</sup>

<sup>1</sup>University Hospital "Alexandrovska", Laboratory of Clinical Immunology, Sofia, Bulgaria, <sup>2</sup>Specialized Children's Oncohematology Hospital, Sofia, Bulgaria

Natural killer cells constitute a critical immunosurveillance barrier against neoplastic transformation. Their effector function is regulated by interaction between inhibitory and activating killer immunoglobulin-like receptors (KIRs) and their MHC class I ligands. Thus, NK cell reactivity can be predicted in part by KIR and HLA class I genotype of the individual. Associations of specific KIR and/or KIR/HLA genotypes with certain diseases have been reported but the relevance of KIR and KIR/ligand polymorphisms on the development of malignancies is still less clear. The objective of this study is to investigate at a genetic level the associations of KIRs and KIR/HLA combinations with acute lymphoblastic leukaemia (ALL). Twenty nine patients with ALL treated in Children's Oncohematology Hospital, Sofia and 26 non affected siblings were evaluated for KIR and KIR HLA ligand gene polymorphisms. Thirty nine unrelated randomly selected individuals were used as controls. KIR genotyping was performed by PCR-SSP method (PEL-FREEZE™ KIR Genotyping Kit). PCR-SSP method (Olerup SSP™ KIR HLA Ligand Kit) was used for KIR HLA ligand genotyping. Increased frequency of *KIR2DL2* without the corresponding HLA-C1 group ligands ( $p=0.03$ ) was found in ALL patients compared to controls. Additionally, a slight decrease of *KIR2DS4* and increase of *KIR2DS1*+/L+ frequencies were observed in ALL in comparison to unrelated healthy individuals. KIR profiles (AA, AB) distribution was similar among patients and controls. One of A group haplotypes (*KIR2DL1*, *2DL3*, *2DL4*, *3DL1-3*, *2DS4*) predominated in all individuals studied but it was slightly decreased in ALL compared to controls. Prevalence of activating KIR/HLA profiles (*KIR2DS1* or *2DS2* and their putative ligands) were observed in patients compared to their siblings. Further investigations in genes coding for KIRs and their ligands in families of patients with leukemia are in progress in order to provide full genotypic information for KIR cluster in patients and their relatives.

#### PA09/74 NEURO-IMMUNE MODULATION OF INFLAMMATORY RESPONSES: NATURAL KILLER CELLS AND DOPAMINE

L. Bozzo<sup>1</sup>, R.C. Maio<sup>2</sup>, E. Brunetta<sup>3</sup>, K. Hudspeth<sup>3</sup>, M. Rusmini<sup>1</sup>, F. Marino<sup>2</sup>, M. Cosentino<sup>2</sup>, D. Mavilio<sup>1</sup>

<sup>1</sup>IRCSS Humanitas Clinical Institute, Laboratory of Clinical and Experimental Immunology, Rozzano, Italy, <sup>2</sup>University of Insubria, Department of Clinical Medicine, Section of Experimental and Clinical Pharmacology, Varese, Italy, <sup>3</sup>Rush University Medical Center, Department of Immunology and Microbiology, Chicago, United States, <sup>4</sup>Gaslini Institute and University of Genoa, Genoa, Italy

**Objectives:** Several lines of evidence demonstrated that central nervous system can modulate innate immune responses and that several primary cells from immune system contain the cellular and molecular machinery required to respond to neurotransmitters. In this regard, very little is known on the ability of Natural Killer (NK) cells to respond directly to catecholamines (CA) or to synthesize any of these important neuro-hormones. Here, we characterize the NK cell surface expression of receptors for dopamine as well as the ability of NK cells to produce CAs.

**Method:** The levels of mRNA for dopamine receptors (DRs) and tyrosine hydroxylase (TH) were determined through real-time PCR from purified NK cells. Surface expression of DRs and intracellular levels of TH was also detected by flow cytometry and confocal microscopy analyses.

**Results:** Resting NK cells resulted positive (both at cellular and molecular levels) for the expression of DR-2,3,4 and 5 (but not of DR1). Activation of NK cells with pro-inflammatory cytokines induced distinct patterns of modification of DR expression. In particular, interleukin rIL-2 and rIL-12 increased levels of DR2, 3 and 4, while IL-15 resulted in higher amounts of DR2 but not 3 and 4. We also found that human NK cells express mRNA for tyrosine hydroxylase (TH), the first and rate-limiting enzyme in CA synthesis. TH mRNA increased after treatment with IL-2 or IL-12, and to a minor extent with IL-15. These results were confirmed by detecting through con-focal microscopy intracellular granules containing that stained positively for TH.

**Conclusion:** DRs are expressed on human fresh NK cells and stimulation with certain pro-inflammatory cytokines can also increase DR surface levels on NK cells. Moreover, the fact that NK cells were found positive for the presence of intracellular TH highly suggests that these cells are able to produce CAs. We are presently investigating on the ability of NK cells to respond to either exogenous (paracrine mechanisms) or endogenous (autocrine mechanisms) catecholamines, with particular regard to key functions such as migration, cytotoxicity, cytokine productions and cell activation and interaction with dendritic cells. Characterization of dopaminergic modulation of NK cells function will provide the rationale for novel pharmacologic therapeutic approaches.

#### PA09/75 POLYMORPHISM OF THE KIR GENES AND HUMAN BRUCELOSIS IN SPANISH CAUCASIAN POPULATION

M.J. Bravo<sup>1</sup>, R. Lavado-Valenzuela<sup>1</sup>, N. Fernández-Arcas<sup>1</sup>, J.M. Miranda<sup>1</sup>, J.D. Colmenero<sup>2</sup>, A. Alonso<sup>1</sup>, A. Caballero<sup>1</sup>

<sup>1</sup>Carlos Haya Hospital, Immunology Service, Málaga, Spain, <sup>2</sup>Carlos Haya Hospital, Infectious Diseases Service, Málaga, Spain

**Introduction:** Killer cell immunoglobulin-like receptors (KIRs) are a family of inhibitory and activatory receptors that are expressed by most natural killer (NK) cells. The KIR gene family is polymorphic; genomic diversity is achieved through differences in gene content and allelic polymorphism. The number of KIR loci has been reported to vary among individuals, resulting in different KIR haplotypes.

Brucellosis affects humans in areas where it is endemic, especially countries around the Mediterranean basin. The incidence of human brucellosis in Spain is still high, at 2.8 cases per 100,000 inhabitants. Previous studies have shown genetic factors are important in susceptibility to human brucellosis. Host factors already studied include HLA genes, cytokine genes and MICA genes. Thus, KIR genes are an attractive candidates for an association with human brucellosis.

**Aim:** We have studied the polymorphism of the KIR genes in relation to susceptibility to or protection against human brucellosis.

**Methods:** We have studied 35 brucellosis patients and 93 healthy controls using the multiplex KIR-SSO typing kit designed to identify KIR genes.

**Results:** Framework genes *KIR3DL3*, *KIR3DP1*, *KIR2DL4* and *KIR3DL2* were present in all individuals. We have found the frequencies of *KIR2DL2* ( $P=0.000004$ ), *KIR2DS2* ( $P=0.000035$ ) and *KIR2DS3* ( $P=0.06$ ) in the brucellosis patients was significantly lower in relation to the control group.

**Conclusion:** The Spanish Caucasian healthy population shows polymorphism of the KIR gene family like other Caucasian populations. A negative association between *KIR2DL2*, *KIR2DS2*, and *KIR2DS3* and the brucellosis patients was identified. Although a greater number of cases are required to confirm our findings, variants of the KIR genes appear to confer protection from human brucellosis in a group of patients from southern Spain.

#### PA09/76 ANALYSIS OF SELECTED FUNCTIONAL PARAMETERS OF NK CELLS IN CHILDREN AND YOUNG ADULT WITH ACUTE LYMPHOBLASTIC LEUKEMIA – PRELIMINARY REPORT

S. Koltan<sup>1</sup>, A. Eljaszewicz<sup>2</sup>, M. Wiese<sup>2</sup>, L. Gackowska<sup>2</sup>, I. Kubiszewska<sup>2</sup>, R. Dębski<sup>1</sup>, M. Urbańska<sup>2</sup>

<sup>1</sup>Collegium Medicum of Nicolaus Copernicus University, Dept. of Pediatric Hematology and Oncology, Bydgoszcz, Poland, <sup>2</sup>Collegium Medicum of Nicolaus Copernicus University, Dept. of Immunology, Bydgoszcz, Poland

Natural killer cells (NK) play important role in recognition and destruction transformed cells. NK can induce killing mechanism in the target cells via the granule exocytosis pathway. The most important components of the cytolytic granules are perforin and several granzymes. Another pathway may be secretion of interferon  $\gamma$  (INF  $\gamma$ ). The knowledge about functional NK efficiency in children with acute lymphoblastic leukemia (ALL) is still unsatisfactory.

Therefore the aim of the study was to assess expression of humane granzymes A, B, K and INF  $\gamma$  in NK.

Three groups of patients were evaluated: A – 7 pts (1 – 17 yrs, median 7) at diagnosis of ALL, B – 10 pts (2,5 – 18 yrs, median 9) at the end of intensive chemotherapy and C – 6 pts (4 – 21 yrs, median 9) at cessation of therapy according to ALL IC-BFM 2002-protocol. The control group consisted of 12 healthy children 5-25 yrs old (median 11 yrs). Expression of granzymes A, B and K in isolated NK cells and INF  $\gamma$  in non activated NK cells from whole peripheral blood were analysed. NK were isolated by SuperMACS device. Expression of granzymes A, B, K and INF  $\gamma$  in NK was done by flow cytometry.

Mean expressions of granzym A in group A, B and C were 50,1%, 56,7% and 59,7% relatively. They were lower than in control group (73,4%). Mean expressions of granzym B in group A (53,3%), C (47,7%) were lower than control (61%). In group B mean percentage of granzym B positive NK (62,3%) was similar to control. Mean expressions of granzym K in group A (13,7%) were similar, in B (19,9%) and C (19,1%) – slightly upper than in control (13,1%). Expressions of perforin were minimally lower in all analyzed group than in control (A: 68,3%; B: 60%; C: 66,5%, vs. 71.5% in control). Mean percentage INF  $\gamma$  positive NK were supreme in group C (29,9%) and B (21,8%), in A was similar to control (11,4% and 10,4% respectively).

These preliminary data suggested, that function of NK cells in children with ALL may be impaired. Consecutive investigations are necessary.

**PA09/77 EVIDENCE OF NOVEL KILLER INHIBITORY MECHANISMS BY SELF: MHC CLASS I AND CD33 AS NK CELL INHIBITORY RECEPTORS OF SPECIFIC-ACTIVATING PATHWAYS**T. Hernández-Caselles<sup>1</sup>, R. Corral-San Miguel<sup>1</sup>, A. J. Ruiz-Alcaraz<sup>1</sup>, M. Martínez-Esparza<sup>1</sup>, P. García-Peñarrubia<sup>1</sup><sup>1</sup>University of Murcia, Department of Biochemistry and Molecular Biology B and Immunology, Murcia, Spain

The control of NK cell effector functions is accomplished by a combination of inhibitory receptors that modulate NK cell activation initiated by stimulatory receptors. However, the absence of inhibitory receptor ligands (mainly MHC-I molecules) does not impair NK self-tolerance, raising the question of whether other regulatory receptor-ligand pairs are also implicated. Consequently, we analyzed the inhibitory function of CD33 and MHC Class I, two structurally and functionally unrelated molecules, over different activating receptors on human activated NK cells. Our results reveal that both, CD33 and MHC-I molecules display distinct and specific patterns of "selective" inhibition with functional differences on cytotoxicity and cytokine production, in contrast to the best known "canonical" inhibitory receptors that constitutively inhibit both functions. Our results support the existence of a new fine-tuner inhibitory receptors group that could establish self-tolerance in mature activated NK cells being also important on tumor and infected cell recognition.

**PA09/78 VERTICAL EXPOSURE TO HIV-1 DOES NOT AFFECT THE NK CELL ACTIVITY MATURATION**B. M. Abramczuk<sup>1</sup>, M. T. N. da Silva<sup>1</sup>, S. C. B. S. Lima<sup>1</sup>, T. Q. Zorzeto<sup>1</sup>, M. M. S. Vilela<sup>1</sup><sup>1</sup>State University of Campinas, Campinas, Brazil

**Introduction and objectives:** Natural killer (NK) cells play an integral role in the innate immune response. NK mediates a cell contact-dependent cytotoxicity of virally infected and transformed cells and provides help to adaptive responses through cytokine secretion. Early in life functional NK is competent. The aim of this study was to evaluate the NK activity in vertically HIV-exposed infants.

**Methods and results:** We investigated the NK cell activity in 33 HIV exposed uninfected children followed at the Pediatrics Immunodeficiency outpatients unit State University of Campinas Medical School, with ages varying from 6.6 to 10.83 months (median, 7.57) and 38 controls not exposed to HIV, with ages varying from 7.1 to 10.77 months (median, 7.63). Peripheral blood mononuclear cells (PBMC), in three different concentrations, were incubated at 37°C for 2 hours in a 5% CO<sub>2</sub> atmosphere with target cell K562 (1x10<sup>5</sup> cel/mL) labeled with PKH2 (Sigma, EUA). Propidium iodide (Sigma, EUA) was added and the cell lysis percentage was acquired in an Epics XL-MCL flow cytometer (Beckman-Coulter, USA). The NK activity medians for the HIV exposed children were 1.75% (0.0–4.3), 4.1% (0.0–7.9) and 5.9% (1.2–13.8) for 0.625x10<sup>6</sup> cel/mL, 1.25x10<sup>6</sup> cel/mL and 2.5x10<sup>6</sup> cel/mL PBMC concentrations, respectively. No differences in NK activity were found between the HIV exposed children and the control group (Mann-Whitney test, p = 0.123, p = 0.289 and p = 0.204). The relative NK cell (CD3 CD16<sup>+</sup> CD56<sup>+</sup>) population was also determined for the HIV exposed children and for nine controls. The median of the NK cell percentage for the HIV exposed group was 7.2 (2.7–16.7) and for the controls was 9.0 (5.5–16.3). There were no differences between the HIV exposed children and the controls (Mann-Whitney test, p = 0.206). There was no correlation between the percentage of NK cells and the cell activity from the HIV exposed children (Spearman's test, p = 0.307, 0.103 and 0.918).

**Conclusion:** These data suggest that the maturation of NK cell activity is not affected by the in utero exposure to HIV.

**Financial support:** FAPESP and CNPq.

**PA09/79 REDUCED CYTOTOXIC ACTIVITY IN NK CELLS AND LOW NKP30 AND NKP46 EXPRESSION FROM PATIENTS WITH CERVICAL CANCER AND PRECURSOR LESIONS**T. García-Iglesias<sup>1</sup>, A. del Toro Arreola<sup>1</sup>, B. Albarran Somoza<sup>1</sup>, S. del Toro Arreola<sup>1</sup>, P. E. Sánchez Hernández<sup>1</sup>, M. G. Ramírez Dueñas<sup>1</sup>, A. Bravo Cuellar<sup>2</sup>, P. C. Ortiz Lazareno<sup>2</sup>, A. Daneri Navarro<sup>1</sup><sup>1</sup>Universidad de Guadalajara, CUCS, Fisiología, Laboratorio de Inmunología, Guadalajara, Mexico, <sup>2</sup>Instituto Mexicano del Seguro Social, Centro de Investigación Biomédica de Occidente División de Inmunología, Guadalajara, Mexico

**Background:** High risk HPV infection can lead to cervical cancer, the second most common malignant tumor in women worldwide. NK cells play a crucial role against tumors and virus-infected cells through a fine balance between signaling of activating and inhibitory receptors. Expression of triggering receptors Nkp30, and Nkp46 on NK cells correlates with cytolytic activity against tumor cells, but these receptors have not been studied in cervical cancer and precursor lesions. The aim of the present study was to study Nkp30 and Nkp46 expression and the cytotoxic activity in NK cells from patients with cervical cancer and precursor lesions, in the context of HPV infection.

**Methods:** Nkp30 and Nkp46 expression were analyzed by flow cytometry on NK cells from 59 patients with cervical cancer and squamous intraepithelial lesions. NK cell cytotoxicity was evaluated in a 4 hours CFSE/7-AAD flow cytometry assay. HPV types were identified by PCR assays.

**Results:** We report here for the first time that NK cell-activating receptors Nkp30, Nkp46 are significantly down-regulated in cervical cancer patients and HGSIL, correlated with low cytolytic activity and HPV-16 infection.

**Conclusions:** Our results suggest that Nkp30 and Nkp46 down-regulation represent an evasion mechanism associated to low NK cell activity, HPV-16 infection and cervical cancer progression.

**PA09/80 NK CELL APOPTOSIS VIA TOLL-LIKE RECEPTORS IN ASTHMA**G. Erten<sup>1</sup>, E. Aktas Cetin<sup>1</sup>, M. Akdis<sup>2</sup>, C. Akdis<sup>2</sup>, G. Deniz<sup>1</sup><sup>1</sup>Istanbul University Institute of Experimental Medicine, Department of Immunology, Istanbul, Turkey, <sup>2</sup>Swiss Institute of Allergy and Asthma Research (SIAF), Davos, Switzerland

Natural Killer (NK) cells constitute the major component of the innate immunity to mediate defense against virally infected or malignant cells. NK cells express mRNAs for TLR1-10 and can be activated through TLRs. After activation NK cells may undergo apoptosis via death receptors expressed on their cell surface. In this study, we determined death receptor expressions and activation-induced cell death of NK cells stimulated via TLR agonists in healthy and asthmatic individuals. NK cells from peripheral blood mononuclear cells (PBMCs) of healthy (n:10) and asthmatic subjects (n:16) were isolated by magnetic cell separation and surface expression of the death receptors such as TNFR1, TNFR2, TRAILR1, TRAILR2, TRAILR3, TRAILR4, CD95 and CD95L on NK cells were determined by flow cytometry. Isolated NK cells were also stimulated with PHA and TLR3, TLR4, TLR5, TLR7, TLR8 and TLR9 agonists in the presence and absence of IL-2, and cellular apoptosis was determined by AnnexinV/7-AAD by flow cytometry. Statistical analysis was performed by Student's *t* test.

NK cells expressed higher TRAILR2, TRAILR4 and CD95L in asthmatics compared to controls (p < 0.05, p < 0.01 and p < 0.001, respectively). The expressions of TRAILR1, TRAILR3 and TNFR1 were higher in controls compared to asthmatics (p < 0.01, p < 0.01 and p < 0.001, respectively). The disease severity also did not have any role in death receptor expressions.

Isolated NK cells stimulated by TLR3, TLR4, TLR5 and TLR9 agonists did not induce significant NK cell apoptosis after 72 hrs in both groups; TLR8 stimulation resulted in significant increase of NK cell apoptosis, being low in asthmatics compared to controls (p < 0.001). Stimulation of NK cells via TLR7 did not cause any increase of NK cell death in asthmatics, but apoptosis in healthy individuals was significantly increased (p < 0.001).

NK cell numbers are known to be reduced in acute exacerbation of asthma, and NK cells showed decreased numbers in response to TLR7 and TLR8 stimulation in asthmatic patients compared to healthy individuals. This might correlate with the increased expression of death receptors on NK cells, but future studies are planned to investigate polymorphisms in these receptors in the context of hygiene hypothesis.

**PA09/81 CORRELATION OF ACTIVATING NK CELL RECEPTOR NKG2D AND CD161 ON CD3-CD16+ NK CELLS WITH NK CELL ACTIVITY IN HEALTHY INDIVIDUALS**G. Konjević<sup>1,2</sup>, K. Mirjagic Martinović<sup>1</sup>, A. Vuletić<sup>1</sup>, V. Jurisic<sup>3</sup>, I. Spuzic<sup>1,2</sup><sup>1</sup>Institute of Oncology and Radiology of Serbia, Belgrade, Serbia, <sup>2</sup>School of Medicine, University of Belgrade, Belgrade, Serbia, <sup>3</sup>School of Medicine, University of Kragujevac, Kragujevac, Serbia

**Objectives:** The aim of this study was to define in healthy individuals the correlation of expression of a newer set of activating and inhibitory receptors on CD16-defined NK cells with NK cell activity, as that CD16, unlike CD56, is one of the main receptors related to NK cell cytotoxicity.

**Methods:** Peripheral blood lymphocytes (PBL) from 43 healthy volunteers were evaluated for NK cell cytotoxic function by chromium-release assay. Expression of NKG2D, CD161, CD158a, CD158b receptors and CD107a marker on CD3-CD16+ NK cells was analyzed by flow cytometry. Expression of CD107a and formation of NK cell-K562 tumor cell conjugates was analyzed after 30 min. and 4 hour co-culture with K562.

**Results:** We find that average NK cell activity against K562 tumor cells in healthy individuals is 37.34% (E:T, 80:1) and that cytotoxic CD3-CD16<sup>bright</sup> NK cell subset constitute 78.97%, while regulatory, CD3-CD16<sup>dim</sup> constitute 21.03% of NK cells. We show the distribution of NKG2D, CD161, CD158a and CD158b receptors on CD3-CD16+ cells in PBL, on gated NK cells, as well as on CD3-CD16<sup>bright</sup> and CD3-CD16<sup>dim</sup> subsets. Contrary to CD158a and CD158b KIRs, we show significant correlation of NKG2D and CD161 expression with NK cell cytotoxicity. We also show that the early kinetics of change in CD3-CD16+ NK-K562 conjugate composition being significantly in favor of CD16<sup>bright</sup> subset proves their stronger binding capacity. Furthermore, we show after co-culture of PBL with K562, that the expression of CD107a, degranulation marker, on CD3-CD16+ NK cells and their subsets, is time-dependent and significantly higher on cytotoxic CD3-CD16<sup>bright</sup> cells.

**Conclusion:** In this study we give new data for healthy individuals regarding the percent of expression of a set of activating and inhibitory receptors on CD3-CD16+ NK cells, as well as on their CD3-CD16<sup>bright</sup> and CD3-CD16<sup>dim</sup> subsets. The activating potential of NKG2D and CD161 is confirmed by significant positive correlation with NK cell cytotoxicity. Moreover, we unambiguously show, through evaluation of CD107a degranulation marker the cytotoxic potential of the rarely investigated CD16<sup>bright</sup> NK subset. The obtained novel data for healthy individuals of the expression of certain important NK cell activating and inhibitory receptors may aid in detecting changes associated with various diseases.



**PA09/82 CELL SURFACE EXPRESSION OF HSP70 ON HEMATOPOIETIC CANCER CELLS AFTER INHIBITION OF HDAC ACTIVITY**H. Jensen<sup>1</sup>, L. Andresen<sup>1</sup>, K.A. Hansen<sup>1</sup>, S. Skov<sup>1</sup><sup>1</sup>University of Copenhagen, The Faculty of Life Sciences, Department of Veterinary Disease Biology, Frederiksberg C, Denmark

The function of Hsp70 depends on its cellular location: it has cytoprotective and anti-apoptotic functions intracellularly, whereas it exerts immunostimulatory functions extracellularly. Secreted Hsp70 is for example involved in cross-presentation of cancer-derived antigenic peptides, a function which is currently explored in immunotherapeutic approaches against cancer. Additionally, membrane-bound Hsp70 can stimulate antigen presenting cells (APCs) to release proinflammatory cytokines and provide a target structure for NK cell-mediated lysis. Human cancer cells frequently express Hsp70 on their cell surface, whereas the corresponding normal tissues do not. Additionally, several clinically applied reagents, such as alkyl-lysophospholipides, chemotherapeutic agents, and anti-inflammatory reagents, have been found to enhance Hsp70 surface expression on cancer cells.

We show that inhibition of histone deacetylase (HDAC) activity leads to surface expression of Hsp70 on various hematopoietic cancer cells, an occurrence that was not observed on naïve or activated peripheral blood cells. HDAC-inhibitor mediated Hsp70 surface expression was confined to the apoptotic Annexin V positive cells and blocked by inhibition of apoptosis. Other chemotherapeutic inducers of apoptosis such as Etoposide and Camptothecin also led to a robust induction of Hsp70 surface expression. Hsp70 expression was however not caused by induction of apoptosis *per se*, since activated CD4 T cells remained Hsp70 surface negative despite effective induction of apoptosis.

Interestingly, inhibition of endolysosomes or normal ER/Golgi transport did not affect Hsp70 surface expression. Intracellular Calcium and the transcription factor Sp1, that has previously been shown to be important for the intracellular stress mediated by HDAC-inhibitors, were not involved in Hsp70 surface expression. We also found that HDAC-inhibitors decreased cellular Plasma Membrane Electron Transport (PMET) activity and that a selective inhibition of PMET activity with extracellular NADH induced a robust Hsp70 surface expression. Our data suggest that inhibition of HDAC activity selectively induces surface expression of Hsp70 on hematopoietic cancer cells and that this may increase immunorecognition of these cells.

**PA09/83 GENETIC DISTANCES BETWEEN MACEDONIAN POPULATION AND 32 OTHER WORLDWIDE POPULATIONS BASED ON ACTIVATING AND INHIBITORY KILLER CELLS IMMUNOGLOBULIN RECEPTORS (KIR) GENE FREQUENCIES**A. Petlichovski<sup>1</sup>, D. Trajkov<sup>1</sup>, A. Strezova<sup>1</sup>, S. Hristomanova<sup>1</sup>, E. Djulejic<sup>1</sup>, M. Spiroski<sup>1</sup><sup>1</sup>Institute for Immunobiology and Human Genetics, Skopje, Macedonia, the Former Yugoslav Republic of

**Objective:** The objective of this study is to compare the KIR gene frequencies in Macedonian population to frequencies found in 32 worldwide populations and to analyze the genetic relationship and genetic distances between them.

**Methods:** The study included 214 unrelated healthy individuals, all Macedonians of Macedonian origin and nationality. Commercially available PEL-FREEZ KIR genotyping SSP kit designed to detect the presence and/or absence of 16 KIR genes and pseudogenes was used. For comparison of KIR gene frequencies and genetic distance analysis, genotypic data previously published at the Allele frequencies database (www.allelefreqs.net) has been used. Since the number of KIR genes allocated in this database differs between populations, we have included in our study and used for comparison only those having complete data for 11 different genes. According to this criterion, 33 out of total of 82 populations were selected. Total of 11 KIR genes were included in the analysis divided in two groups: activating genes (KIR 3DS1, 2DS1, 2DS2, 2DS3, 2DS4 and 2DS5) and inhibitory genes (KIR 2DL1, 2DL2, 2DL3, 2DL5 and 3DL1). The DISPAN software was used for calculation of genetic distances and construction of Neighbour-joining (NJ) dendrogram.

**Results:** The NJ phylogenetic tree shows that Macedonian population clusters together with several other European populations, such as England West Midlands, Romania/Caucasians, Basques, Reunion, Northern Ireland and Spain/Granada. Both dendrograms, constructed on the average differences in frequencies of the DS and the DL KIR genes in analyzed populations, tend to follow geographical distribution with some exceptions. These findings suggest that KIR genes share some population specificities probably evolutionary influenced and determined by population specific pathogens.

**Conclusion:** This study has been initiated in order to contribute in better understanding of KIR genetic polymorphisms and their evolution, and also to elucidate migrations and relations between different populations and ethnic groups.

**PA09/84 NEGATIVE CORRELATION BETWEEN TNF-ALPHA SERA CONCENTRATION AND NUMBER OF NK CELLS IN YOUNG PATIENTS WITH TYPE 1 DIABETES**L. Hak<sup>1</sup>, K. Zorena<sup>1</sup>, J. Wiekiewicz<sup>1</sup>, M. Myśliwiec<sup>2</sup>, A. Balcerska<sup>2</sup>, J. Myśliwska<sup>1</sup><sup>1</sup>Medical University of Gdańsk, Department of Immunology, Gdańsk, Poland, <sup>2</sup>Medical University of Gdańsk, Diabetological Department at Clinic of Pediatrics, Hematology, Oncology and Endocrinology, Gdańsk, Poland

**Background:** Natural killer cells (NK) are the first line of defense against viral and bacterial infections. NK cells may be divided into two subsets, according to the CD56 antigen expression: CD56dim, characterized by high cytotoxic activity and CD56bright involved in regulation of immune response. Type 1 diabetes is an autoimmune disease connected with higher level of proinflammatory cytokines and dysfunction of immune regulation. The aim of our work was to find out NK status in patients with type 1 diabetes.

**Material and methods:** Twenty three children with diagnosed DM1 were involved into the study, control group consisted of 21 healthy, age matched children. In all blood samples percentage within lymphocytes and absolute number of NK cells and its subsets were evaluated. Furthermore TNF-alpha sera concentration was estimated.

**Results:** Type 1 diabetes group was characterized by the lower percentage and absolute number of NK cells and the CD3-CD56dim subset in comparison to control group. Level of the CD3-CD56bright cells was also decreased in blood of DM1 patients, however the difference didn't reach significance. Sera levels of TNF-alpha were higher in DM1 group. Further analysis revealed negative correlations between TNF-alpha sera level and NK cells number in patients with DM1.

**Conclusion:** This data indicated that type 1 diabetes is connected with suppression of NK cells functions and moreover explain the reason of dysfunction. It seems that NK cells depletion is dependent on inflammatory state.

**PA09/85 LYMPHOCYTES SUBSETS AND RECEPTORS OF NK CELLS IN PATIENTS WITH POST-TREATMENT RECURRENCE OF CERVICAL INTRAEPITHELIAL NEOPLASIA II-III**A. del Toro Arreola<sup>1</sup>, A.E. Suarez Rincon<sup>2</sup>, M. Jimenez Perez<sup>3</sup>, R.A. Franco Topete<sup>4</sup>, S.F. Velasco Ramirez<sup>1</sup>, S. del Toro Arreola<sup>1</sup>, R. Robles Garcia<sup>5</sup>, T. Garcia Iglesias<sup>1</sup>, A. Daneri Navarro<sup>1</sup><sup>1</sup>Universidad de Guadalajara, Fisiología CUCS, Guadalajara, Mexico, <sup>2</sup>Instituto Mexicano del Seguro Social, Hospital General Regional No. 45, Clínica de Colposcopia, Guadalajara, Mexico, <sup>3</sup>Universidad de Guadalajara, Salud Pública, CUCS, Guadalajara, Mexico, <sup>4</sup>Hospital Civil de Guadalajara, Anatomía Patológica, Guadalajara, Mexico, <sup>5</sup>Instituto para el Fortalecimiento de las Capacidades en Salud, Centro de Estudios Especializados ARG, Mexico, Mexico

Currently, cervical cancer detection is mainly directed to detect and treat CIN II-III. Loop electrosurgical excision procedure (LEEP) is mainly used for the treatment of cervical intraepithelial neoplasia grade 2-3. Recurrence of CIN has been shown in 5-20% of treated patients. The innate immune response is considered to be the first line of defense at mucosal surfaces, while humoral and cellular immune responses are likely to play a key role in the clearance or persistence and progression of lesions. The activation of natural killer cells is dependent on the balance of signals transmitted through activating and inhibitory receptors. Factors involved in post-treatment CIN 2-3 recurrence are still unknown.

The goal of this study was to determine the percentage and distribution of the following cell surface markers within patient populations with or without recurrent lesions: CD3+, CD4+, CD8+, CD19+, CD56+, and the NK cell receptors NKG2D, NKP46, NKP30 (activating) and ILT2, CD94 (inhibitory).

Our study cohort recruited 71 patients of IMSS (Instituto Mexicano del Seguro Social). Women were enrolled in the study at the moment of CIN 2-3 diagnosis. Cervical biopsies and peripheral whole blood were taken from all women with prior written informed consent. The patients were treated by LEEP and were monitored at six and twelve months post-treatment. Cell populations were evaluated by flow cytometry and immunohistochemistry. Expression of activating/inhibitory receptors was evaluated by flow cytometry.

The frequency of CIN recurrence was relatively low and at this time we did not find significant differences between cellular populations and expression of activating/inhibitory NK cell receptors from patients with and without post-treatment recurrence. However, we observed a tendency towards an increase in the expression of activating receptors and a decrease in the expression of inhibitory receptors in patients with recurrent lesions.

To evaluate with more confidence the role of these receptors and cells it will be necessary to increase the patient cohort and examine more women with recurrent lesions.

**PA09/86 EXPRESSION OF SELECTED KILLER IMMUNOGLOBULIN-LIKE RECEPTORS ON NK CELLS IN CHILDREN AND YOUNG ADULT WITH ACUTE LYMPHOBLASTIC LEUKEMIA – PRELIMINARY REPORT**S. Koltan<sup>1</sup>, R. Dębski<sup>1</sup>, B. Kuryto<sup>1</sup>, B. Kołodziej<sup>1</sup>, M. Kubicka<sup>1</sup><sup>1</sup>Collegium Medicum of Nicolaus Copernicus University, Dept. of Pediatric Hematology and Oncology, Bydgoszcz, Poland

Natural killer cells (NK) play important role in recognition and destruction transformed cells. The activation of NK is regulated by activating and inhibitory surface receptors. Killer immunoglobulin-like receptors (KIRs) recognize HLA class I molecules. The knowledge about signification of NK in children with acute lymphoblastic leukemia (ALL) is still unsatisfactory.

The aim of the study was to assess the percentage and absolute number of NK and expression of two groups KIRs on NK in children with ALL at diagnosis, post intensive chemotherapy and at cessation of treatment.

Three groups of patients were evaluated: A – 7 pts (1 – 17 yrs, median 7) at diagnosis of ALL, B – 10 pts (2,5 – 18 yrs, median 9) at ending intensive chemotherapy and C – 6 pts (4 – 21 yrs, median 9) at cessation of therapy according to ALL IC-BFM 2002-protocol. The control group consisted of 12 healthy children 5-25 yrs old (median 11 yrs). Percentage and absolute number of NK (CD3-CD16+CD56+) in peripheral whole blood and percentage of CD158a and CD 158b positive isolated NK were analysed. NK were isolated by SuperMACS device. Percentage and absolute number of NK and an expression of CD158a and CD158b NK was done by flow cytometry.

Mean percentage of NK in whole peripheral blood was low in group A (1,8%), in B and C was slightly lower than control group (4%, 5,9% and 6,8% respectively). Absolute number of NK were very different in group A: 5 – 4188/µl; mean 850/ µl, in B and C was generally lower than control (mean 65; 102,33 vs 183/ µl). Expression of CD158a and 158b were supreme in group A (mean 37,9% and 34,4% respectively) and undermost in B (6,3% and 13,5% respectively). In control group mean expression of CD158a was 26,8% and CD158b – 23,4%. Percentage of CD158a positive NK in group C (23,3%) was similar to control, and CD158b – lower 14,2%.

These preliminary data suggested, that ALL and oncological therapy have large influence not only on percentage and number of NK cells. Their function, dependent from receptors expression, is probably incorrect.

#### PA09/87 UNUSUAL NATURAL KILLER (NK) CELL EXPANSION IN A HEART TRANSPLANT RECIPIENT WITH POST-TRANSPLANT STRONGYLOIDIASIS AND CYTOMEGALOVIRUS COINFECTION

N. Del Pozo Rodríguez<sup>1</sup>, E. Sarmiento<sup>2</sup>, N. Lanio<sup>2</sup>, A. Gallego<sup>2</sup>, E. Fernández-Cruz<sup>2</sup>, J. Carbone<sup>2</sup>

<sup>1</sup>Hospital Clínico San Carlos, Clinical Immunology, Madrid, Spain, <sup>2</sup>Hospital General Universitario 'Gregorio Marañón, Cincin Immunology, Madrid, Spain

**Background:** Strongyloides stercoralis causes chronic asymptomatic intestinal infections. Hyperinfection and dissemination may occur in immunosuppressed patients. NK cells are important effectors of the innate immune response against viral and parasitic infections.

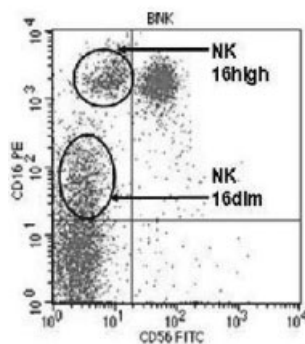
**Objective:** To characterize immunological and clinical features in a heart transplant recipient with strongyloidiasis and CMV reactivation.

**Methods:** Heart transplantation was performed in a 60-year-old immigrant woman with ischemic and valvular cardiopathy. Immunosuppression: daclizumab, steroids, tacrolimus and mycophenolate mophetil. Humoral and cellular immunity markers were performed in peripheral blood samples at different times: pre-transplant, 7 days (7d), 1 and 3-months (m) after transplantation. Lymphocyte subsets were performed by four-colour flow-cytometry.

**Results:** 1m after transplant, she developed diarrhea and pruritus, associated with Strongyloides stercoralis intestinal infection and CMV positive antigenemia. Parasitic hyperinfection/disseminated disease was excluded. Therapy with ivermectin and ganciclovir/valganciclovir was given. The patient presented C3 hypocomplementemia and low levels of CD3<sup>+</sup>CD4<sup>+</sup> T-cells (277cells/µL) at 7d and IgG hypogammaglobulinemia (641 mg/dL) at 1m. An expansion of CD16<sup>dim</sup>CD56<sup>+</sup> NK-cells was observed at 7d and 1m (5.51% and 1.67% respectively). Median percentage of CD16<sup>dim</sup>CD56<sup>+</sup> NK-cells in 10 healthy subjects was 0.82%. An atypical CD16<sup>high</sup>CD56<sup>+</sup> NK-cell population was identified (7d 0.99%, 1m 1.65%). After antimicrobial therapy, CD16<sup>dim</sup>CD56<sup>+</sup> NK-cell percentages became normal (3m 1.12%, 6m 0.54%) whereas the CD16<sup>high</sup>CD56<sup>+</sup> NK-cells still remained (3m 1.87% and 6m 0.81%). Eosinophils (cells/µL): pre-transplant 18.9(888), 7d 0.1(120), 15d 0.5(42), 1m 4.9(240), 3m 10.3(500), 6m 5.7 (300).

**Conclusion:** Symptomatic strongyloidiasis and CMV reactivation occurred after immunosuppressive therapy, associated with an expansion of CD16<sup>dim</sup>CD56<sup>+</sup> NK-cells which normalized after treatment. A similar observation has been described in HIV disease after effective antiretroviral therapy which results in restoration of normal CD56 expression. Decreased cytotoxic function of this subset has been described. The role of this expansion within parasitic and viral coinfection warrants further evaluation. An atypical CD16<sup>bright</sup>/CD56<sup>+</sup> NK cells has not been previously described in transplant patients with infections.

Figure1. Dot plot of NK cells gated on CD3 negative PBLs



[Figure1]

#### PA09/88 RELATION BETWEEN NK CELLS SUBPOPULATIONS AND TETRASPANIN MEMBRANE EXPRESSION IN MALIGNANT TUMORS

M. Surcel<sup>1</sup>, R. Huică<sup>1</sup>, D. Ciotaru<sup>1</sup>, M. Dobre<sup>1</sup>, A. Belmega<sup>1</sup>, I. Pîrvu<sup>1</sup>, G. Isvoranu<sup>1</sup>, C. Ursaciuc<sup>1</sup>

<sup>1</sup>Victor Babeş National Institute, Immunology, Bucharest, Romania

**Objectives:** Characterization of NK-CD56<sup>bright</sup> and NK-CD56<sup>dim</sup> cell subpopulations in relation with tetraspanin (TS) glycoprotein family (CD9, CD37, CD53, CD63, CD81, CD82) in peripheral blood and primary malignant tumors.

**Materials and methods:** Whole blood and tumor tissue from 13 patients with malignant tumors served for NK cell isolation. Additionally, 5 healthy donors blood samples were used as control group. The blood and tumor suspension NK cells were obtained by magnetic separation (VarioMACS, Miltenyi Biotec). NK subpopulations and TS analysis were performed by flow-cytometry (FACSCalibur, BD) and compared to lymphocyte immunophenotyping. Direct immunofluorescence and confocal microscopy on tumor sections permitted a qualitative evaluation of tumor infiltrating lymphocytes (TIL).

**Results:** Blood lymphocyte immunophenotyping showed increased NK cells values in patients group as compared to control group. Magnetic-enriched blood NK cell suspension showed a slight CD56<sup>+</sup>CD57<sup>+</sup> (NK-CD56<sup>bright</sup>) decrease and increased CD16<sup>+</sup> cells in patient group. Peripheral blood CD56<sup>+</sup>CD57<sup>+</sup> (NK-CD56<sup>dim</sup>) subpopulation was mostly CD16<sup>+</sup> and equally represented in patients and control group. TIL analysis revealed a relative NK cells increase, especially in NK-CD56<sup>dim</sup>.

TS expression on CD57<sup>+</sup> NK cells was lower in whole blood than magnetic-separated blood. In patient group CD9 and CD37 were lower expressed, while CD53, CD63 and CD81 expression was increased. Moreover, all TS presented a significant expression on CD57<sup>+</sup> cells, with lower CD9, CD37, CD63 and CD81 expression. Microscopy examination showed the presence of NK-CD56<sup>dim</sup> in all samples, alongside CD3<sup>+</sup> T lymphocytes.

**Conclusions:** Investigating NK cell subpopulations may provide useful information in evaluating anti-tumor immune response and metastasis tendency. Besides, these data may be helpful as an intermediary step in order to validate new tumor markers.

Supported by Romanian Education and Research Ministry grant PN2 41-046/2007.

#### PA09/89 FUNCTIONAL ANALYSES OF KILLER CELL IMMUNOGLOBULIN-LIKE RECEPTORS (KIR) IN RHESUS MACAQUES

M. Hermes<sup>1</sup>, L. Walter<sup>1</sup>

<sup>1</sup>German Primate Centre Göttingen, Department of Primate Genetics, Göttingen, Germany

The killer cell immunoglobulin-like receptors (KIR) are expressed in natural killer (NK) cells and subsets of T lymphocytes. These receptors acquire input via interaction with MHC class I ligands on target cells. Human KIR genes are characterised by considerable polymorphism and variegated expression pattern. In humans, certain combinations of the highly variable KIR and MHC class I genes are implicated in susceptibility/resistance to infectious, autoimmune and neoplastic diseases and impact the outcome of transplantation. Rhesus macaques (*Macaca mulatta*) are important animal models to study these diseases, yet only few data on function, expression pattern and genetic associations of KIR are known in rhesus macaques.

To investigate the function and expression pattern of KIR molecules of rhesus macaques, we have started to generate anti-KIR monoclonal antibodies. We used various KIR-Fc-fusion proteins for immunisation of mice and have obtained highly specific antisera. Currently, we are characterising the specificity and crossreactivity of the obtained monoclonal antibodies for the various KIR molecules in respective KIR gene transfectants. Additional experiments will be performed to analyse cell surface expression and clonal expression patterns of various KIR molecules in rhesus macaque lymphocytes (NK cells and T cells).

## PA09/90 KIR/HLA INTERACTIONS IN THE RUSSIAN POPULATION

E. G. Khamaganova<sup>1</sup>, Y. A. Ledniyov<sup>1</sup>, T. P. Chugreeva<sup>1</sup>, S. K. Mamaliaeva<sup>1</sup>, A. B. Sudarikov<sup>2</sup><sup>1</sup>National Research Center for Hematology, Lab of Immunotyping, Moscow, Russian Federation, <sup>2</sup>National Research Center for Hematology, Lab of Molecular Hematology, Moscow, Russian Federation

Natural killer (NK) cells eliminate infected and transformed cells while remaining tolerant to healthy cells. This function is mainly controlled by the interactions between inhibitory (i) killer immunoglobulin-like receptors (KIRs) expressed by NK cells and their ligands – HLA class I molecules. Expression of HLA class I antigens protects healthy cells from NK lysis. The independent segregation of KIR and HLA genes results in variable number of iKIR/HLA pairs. The absence or presence of HLA class I ligand in the recipient for the donor iKIR can be important for the outcome in organ and hematopoietic stem cell transplantation.

**Objective** of this work has been to establish the balance between iKIRs and their HLA-ligands in the Russian population.

**Methods:** 59 unrelated donors of blood components from Moscow region were investigated. HLA-A, -B typing was performed by serology, HLA-C typing – by PCR-SSP. KIRs were tested using the PEL-FREEZ KIR genotyping SSP kit. Direct counting was used for the estimation of observed frequencies of HLA-antigens and each particular KIR gene. The presence of the four known iKIR/HLA pairs: KIR2DL1/HLA-C2 group, KIR2DL2/3/HLA-C1 group, KIR3DL1/HLA-Bw4, KIR 3DL2/HLA-A3/A11 was analyzed.

**Results:** In our donors the inhibitory KIR genes generally have higher frequencies than the activating KIR genes, with the exception of 2DS4. The most frequent KIR genotype is: 2DL1, 2DL3, 2DL4, 3DL1, 3DL2, 3DL3, 2DS4, 2DP1, 3DP1. All investigated donors have at least one iKIR/HLA ligand combination. 6.8% of donors have all four of iKIR/HLA combinations, 35.6% possess three combinations, 45.8% – two combinations, 11.8% – only one combination. The most frequent iKIR/HLA pair is KIR2DL2/3/HLA-C1, which occurs in 78% of donors.

**Conclusion:** On the whole, the frequencies of iKIR/HLA combinations in our population are similar to those frequencies in other Caucasoid populations, although there are some peculiarities. The obtained data may be useful for the analysis of the KIR/HLA role in transplantation and diseases.

## PA09/91 MONITORING OF NK CELLS IN MISSILE ELIMINATION WORKERS

A. V. Litovskaya<sup>1</sup>, T. V. Shmakova<sup>1</sup><sup>1</sup>Research Institute for Hygiene and Occupational Pathology, Clinical Department, Nizhny Novgorod, Russian Federation

**Aim:** To study NK-cells in missile elimination workers exposed to missile fuel (MF), to investigate the cell dynamics depending on exposure intensity, length of service, worker's age.

**Methods:** 386 persons underwent clinico-immunological examination; length of service was up to 10 years. MF concentrations in neutralization area were up to 10 times higher than standard and in cutting area were at standard level. 60 persons were in control group. Lymphocyte immunophenotyping was performed by immunocytochemical method with monoclonal antibodies CD16 and visualization system Dako. Statistical treatment of results was performed with «Microsoft Excel», «Statistica 6.1».

**Results:** In an initial examination, higher NK-cell level was seen in neutralization workers ( $16,25 \pm 0,67\%$ ,  $p < 0,001$ ), but the level did not differ between cutting workers and control ( $15,08 \pm 1,46\%$  and  $12,41 \pm 0,70\%$ , respectively). In first 3 years of monitoring, NK-cell level in neutralization workers were higher than in cutting workers. Later on the increase of length of service was associated with increase of NK-cell level in cutting workers. Increase of NK-cell level in all workers was seen on 5<sup>th</sup>-year of monitoring. NK-cell level in neutralization workers was higher than in cutting worker. Increase of NK-cell level in neutralization workers was observed in 1-2 years and increase of NK-cell level in cutting workers was found in 2-3 years. Higher NK-cell level in older workers (50 and older) was observed in comparison with workers under 30 years old ( $17,00 \pm 0,69\%$  vs.  $15,06 \pm 0,68\%$ ,  $p < 0,05$ ). NK-cell functional activity was measured by levels of TNF- $\alpha$ , IFN- $\gamma$  and was higher in older workers.

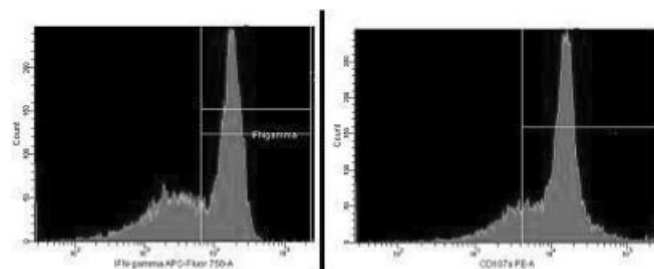
**Conclusion:** NK-cell monitoring in missile elimination workers revealed higher level of the cell in comparison with control. High concentrations of toxicants cause an earlier increase of NK-cell level with increase of length of service. Higher level of CD16<sup>+</sup>-cells in older workers was accompanied by increase of cell functional activity. The increase of CD16<sup>+</sup>-lymphocytes may be directed to elimination of cells damaged by toxicants from human organism and support of adequate protection under conditions of intensive antigen load.

## PA09/92 ACTIVATION OF NK CELLS VIA STIMULATION OF NKP46 RECEPTORS

A. Karapetyan<sup>1</sup><sup>1</sup>University of Kentucky, Pathology & Laboratory Medicine, Lexington, United States

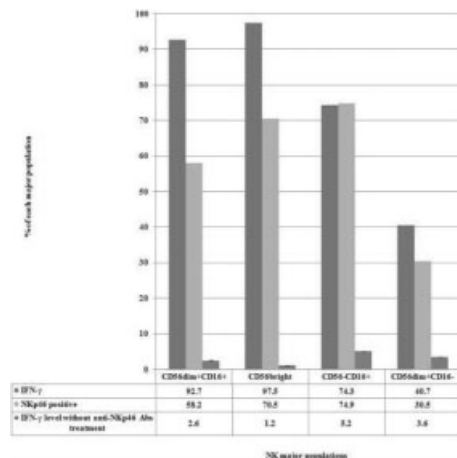
**Main hypothesis:** Anti-NKp46 antibody can activate NKp46 receptor on NK cells and induce IFN- $\gamma$  expression and cytotoxic granule release.

NK (natural killer) cells may release IFN- $\gamma$  or/and cytotoxic granules upon activation by infected or abnormal cells. NKp46 is an activating receptor, expressed on most NK cells. To activate NKp46 we used anti-NKp46 antibody with the cocktail of antibodies to surface receptors CD3, CD56, CD16 (cocktail1) and anti-CD107a to monitor cytotoxic granule release. The expression of IFN- $\gamma$  was determined by intracellular staining. Surface staining was done in whole blood and on peripheral blood mononuclear cells (PBMC) in RPMI + 10% heat inactivated CCS. Blood was donated by healthy young and elderly female volunteers participating in research study. Samples were ran on LSR II. Below are histograms for IFN- $\gamma$  and CD107a of NKp46 activated NK cells. Shown are only NK populations defined as CD3<sup>+</sup>CD16<sup>+</sup> and/or CD56<sup>+</sup> under lymphocyte gate.

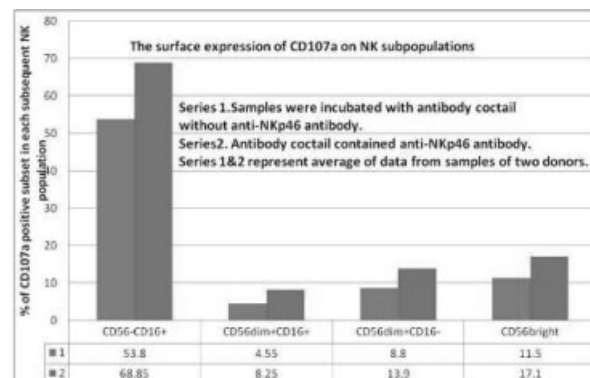


[histograms for IFN-gamma and CD107a]

Experimental data are summarised in graphs 1&2.



[Graph1.]



[Graph2.]



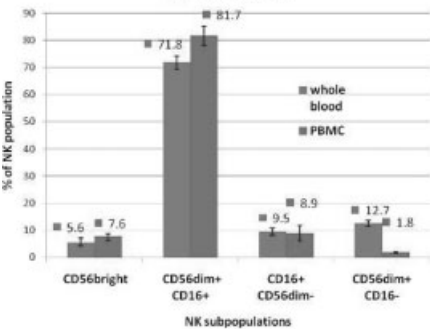
After 4 hours of incubation with surface antibody cocktail1, monensin and anti-NKp46 antibody ~95% of NK population has expressed IFN- $\gamma$ . CD107a surface expression was enhanced after 1 hour incubation and median fluorescence of CD56<sup>dim</sup>CD16<sup>+</sup> population was increased 4 times. In one of the elderly donors NKp46 positive IFN- $\gamma$  positive population (60% of all NK population) was CD107a positive.  
**Conclusion:** Anti-NKp46antibody mimics NKp46 ligand and can induce cytotoxic granule release and IFN- $\gamma$  expression.  
**Acknowledgments:** The author is thankful to Dr.Lutz and Jennifer Strange for support.

PA09/93 THE EXPRESSION OF NK SURFACE MARKERS CD56 AND CD16 IS REGULATED BY IMMUNOLOGIC NETWORK IN WHOLE BLOOD

A. Karapetyan<sup>1</sup>  
<sup>1</sup>University of Kentucky, Pathology & Laboratory Medicine, Lexington, United States

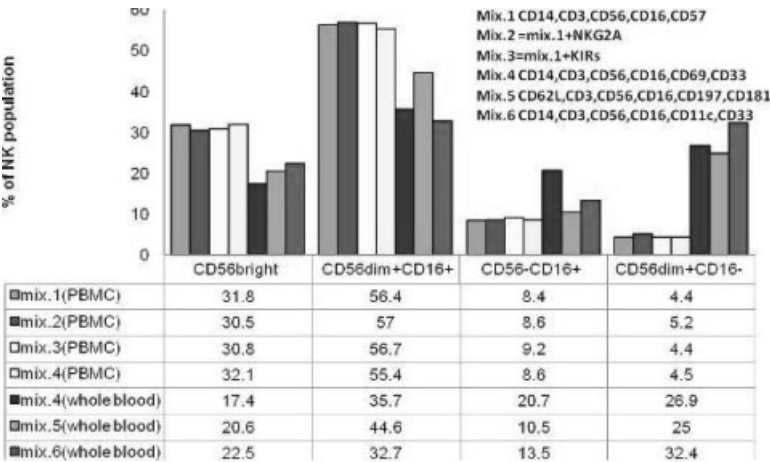
**Hypothesis:** The expression of natural killer cells' (NK) surface markers CD56 and CD16 is regulated by immunologic network in whole blood. NK subpopulations are classified by the expression of surface markers CD56 and CD16 into subpopulations: CD16<sup>+</sup>CD56<sup>+</sup>; CD16<sup>+</sup>CD56<sup>dim</sup>; CD16<sup>+</sup>CD56<sup>bright</sup>. By using multifunctional flow cytometry we examined the expression of surface markers of NK major populations in peripheral whole blood and peripheral blood mononuclear cell pool (PBMC). Samples were ran on LSRII, at least 100,000 events were collected in lymphocyte gate; 6-color antibody panel was used for surface staining; CD14CD3CD56<sup>+/</sup>CD16<sup>+/</sup> population in lymphocyte gate was considered as NK population comprised 4 subpopulations: CD16<sup>+</sup>CD56<sup>+</sup>; CD16<sup>+</sup>CD56<sup>dim</sup>; CD16<sup>+</sup>CD56<sup>bright</sup>; CD56<sup>dim</sup>CD16<sup>-</sup>.

Staining of NK surface receptors in whole blood and in PBMC pool.



[Graph1.]

The samples from three different young female donors were used to graphically display average % values of 4 subpopulations. The fresh peripheral blood from the same healthy donor was divided into two parts. One part of whole blood was stained with the mixtures of antibodies. The other part was used to isolate PBMC by density gradient centrifugation then stained with the same mixute of antibodies: anti-CD3, anti-CD62L, anti-CD56, anti-CD16, anti-CD197, anti-CD181.



[Graph2.]

Mixtures are antibodies to indicated surface receptors. In another study we incubated the whole blood with anti-NKp46 antibody and mixture of anti-CD3, anti-CD56, anti-CD16, during 4 hours. CD56bright population rose up to 50% from 20% with subsequent decline of CD56<sup>dim</sup>CD16<sup>+</sup> population from 60% to 30%. ~98% of CD56<sup>bright</sup> population was IFN- $\gamma$  positive. If staining in PBMC with various mixtures of antibodies does not change the distribution pattern of NK major populations, the expression of CD56 and CD16 during the staining in whole blood greatly depends on antibodies used for staining.

PA09/94 OVEREXPRESSION OF MIF AND BAT3 IN SERA FROM B-CLL AND HL PATIENTS CORRELATES WITH NK CELL SUPPRESSION

E. Pogge von Strandmann<sup>1</sup>, V.R. Simhadri<sup>1</sup>, K. Reiners<sup>1</sup>, H.P. Hansen<sup>1</sup>, M. Hallek<sup>1</sup>, A. Engert<sup>1</sup>  
<sup>1</sup>University Clinic of Cologne, Clinic I for Internal Medicine, Cologne, Germany

Natural Killer (NK)-cells are lymphocytes of the innate immunity involved in tumor cell recognition and surveillance. The major activating receptors directing NK cell function are the NKG2D receptor and the Natural Cytotoxicity Receptors NKp30, 44 and 46. A decrease of the corresponding ligands on tumor cells correlates with impaired NK cell-dependent killing and tumor progression. However, sustained expression and the release of soluble ligands for NK cell receptors may negatively imprint the local and systemic immune response. Here we present evidence that soluble, extracellular MIF (macrophage migration inhibitory factor) and BAT3 (HLA-B associated transcript 3, the cellular ligand for NKp30) impair activation of NK cells in patients with chronic lymphocytic leukaemia (B-CLL) and Hodgkin lymphoma (HL). Serum levels of both factors were significantly elevated in comparison to healthy donors and detectable as soluble factors in the supernatant. FACS analysis using specific antibodies and soluble NKp30-Ig protein revealed that BAT3 was also expressed on the surface of exosomes. Pre-incubation of NK cells derived from healthy donors with the soluble fraction of B-CLL and HL sera dramatically suppressed NK cell cytotoxicity against NK cell-sensitive tumor cell lines. No effect or even activation was observed upon incubation of healthy NK cells with the exosomal serum-fraction. The decrease of NK cell activity correlated with the levels of soluble MIF and BAT3 and was accompanied by decreased surface expression of cytotoxicity receptors on NK cells. Thus MIF and BAT3 appear to be involved in regulatory functions counteracting

the cellular immune response of NK cells in B-CLL and HL patients by contributing to a broad immunosuppression supporting disease progression. Soluble ligands for NKG2D that are already described as NK cell inhibitors such as MICA and MICB were under the detection level in most patients analyzed. The development of immunotherapeutics that modulate the activity of NK cells via targeting MIF and BAT3/NKp30 signalling will be discussed.

#### PA09/95 MECHANISM OF NK CELL ACTIVATION BY RECOMBINANT HSP90 AND HSP90 FROM TUMOR CELLS

H.-R. Kim<sup>1</sup>, J. Kim<sup>1</sup>

<sup>1</sup>Yonsei University College of Medicine, Department of Microbiology and Brain Korea 21 for Medical Sciences, Seoul, Korea, Republic of

**Object:** Almost tumor cells contain heat shock proteins (Hsps) in their cytoplasm and surface and the proteins have multi-activity. Hsps act as chaperone proteins and have influence in cell survival such as preventing apoptosis in cytoplasm. On the other hand, Hsps secreted or on cell surface can stimulate immune system and induce target cell death through activation of macrophage, dendritic cells, T cell, and NK cells. In this study, we investigated the effects of Hsp90 on NK cell activation and their action mechanisms.

**Methods:** NK cells were isolated by negative selection using the RosetteSep™ NK enrichment antibody cocktail. Fresh isolated NK cells were stimulated with Hsp90 and the effects of Hsp90 were estimated by ELISA, RT-PCR, and Flow cytometric analysis. The binding of Hsp90 to NK cell surface was verified by flow cytometric analysis, immunoprecipitation, and immunoblotting.

**Results:** Hsp90 treatment did not alter the expression of mRNAs and proteins of cytotoxic granules such as Perforin and Granzyme B in NK cells. However, endotoxin removed-recombinant Hsp90 induced IFN- $\gamma$  release by NK cells. Hsp90 proteins were found in culture media of some tumor cell lines and IFN- $\gamma$  production was induced by the media from NK cells. We demonstrated that fluorescein labeled Hsp90 protein bound to NK cell surface directly, and we confirmed that Hsp90 interacted with NKG2D receptor through immunoprecipitation. IFN- $\gamma$  and TNF- $\alpha$  production of Hsp90 treated NK cells were blocked by treatment of some inhibitors for PI3 kinase or MEK.

**Conclusion:** Hsp90 didn't influence on the expressions of cytotoxic molecules of NK cells, but could induce IFN- $\gamma$  and TNF- $\alpha$  release by NK cells. Hsp90 appeared to bind to NK cells directly through NKG2D receptor and PI3 kinase and MEK were participated in the activating signals triggered by the receptor.

#### PA09/96 QUANTIFICATION OF IFN- $\gamma$ PRODUCED BY HUMAN PURIFIED NK CELLS FOLLOWING TUMOR CELL STIMULATION: COMPARISON OF THREE IFN- $\gamma$ ASSAYS

E. Lion<sup>1</sup>, E. Smits<sup>1</sup>, Z. Berneman<sup>1,2</sup>, V. Van Tendeloo<sup>1,2</sup>

<sup>1</sup>Vaccine and Infectious Disease Institute (VIDI), University of Antwerp, Laboratory of Experimental Hematology, Antwerp, Belgium, <sup>2</sup>Center for Cell Therapy and Regenerative Medicine, Antwerp University Hospital, Antwerp, Belgium

Interferon (IFN)- $\gamma$  released by natural killer (NK) cells has become a subject of major interest, given its importance in bridging the innate and adaptive immune system. Interestingly, reports concerning tumor cell stimulation of NK cells show divergent data on which stimuli induce IFN- $\gamma$  production. Here, the question remains whether tumor cell recognition is sufficient to trigger IFN- $\gamma$  or whether a second signal is required such as type I IFN. While IFN- $\gamma$  detection methods are abundantly used with peripheral blood mononuclear cells or purified T cell fractions as responder populations, only limited data is available about comparison of these assays with purified NK cells. In this study, we assessed the relationship between stimulation of human purified resting peripheral blood NK cells with one (tumor cell or IFN- $\alpha$ ) and two (tumor cell + IFN- $\alpha$ ) signals by measuring IFN- $\gamma$  using three different assays. We performed the enzyme-linked immunosorbent assay (ELISA), the enzyme-linked immunospot (ELISPOT) assay and intracellular cytokine staining (ICS) assay in parallel per donor and determined whether there was a correlation between these assays.

Our results show that two-signal stimulation of human resting NK cells induces significantly more IFN- $\gamma$  compared to one-signal stimulation, readily picked up by all assays. Moreover, statistical analysis points towards a positive correlation between these assays for IFN- $\gamma$  produced following two-signal stimulation. Importantly, we show that tumor cell stimulation alone is enough to trigger secretion of IFN- $\gamma$ , but this finding was only evidenced by ELISPOT. These results reveal that the choice of IFN- $\gamma$  detection method can markedly influence the outcome regarding induction of NK cell IFN- $\gamma$  by tumor cells.

#### PA09/97 NK CELLS CONSTITUTE A MAJOR RESERVOIR IN VIVO FOR HUMAN T-CELL LYMPHOTROPIC VIRUS TYPE 2 (HTLV-2) INFECTION AND HAVE REDUCED CYTOTOXICITY ACTIVITY IN VITRO

A.L. Oliveira<sup>1</sup>, L. Maron<sup>1</sup>, A. Waters<sup>1</sup>, D. Schor<sup>2</sup>, A.C. Leite<sup>2</sup>, A. Araújo<sup>2</sup>, W. Hall<sup>1</sup>

<sup>1</sup>University College Dublin, Centre for Research in Infectious Diseases, Dublin, Ireland, <sup>2</sup>Oswaldo Cruz Foundation, Evandro Chagas Institute for Clinical Research, Rio de Janeiro, Brazil

**Objectives:** To evaluate the cellular tropism in vivo of HTLV-2 and investigate the cytotoxicity activity in vitro of NK cells from HTLV-2 infected individuals.

**Methods:** Frozen PBMCs from HTLV-2 infected individuals were submitted to immuno-magnetic isolation of CD4+, CD56+, CD8+ and CD19+ lymphocyte subpopulations. Cellular tropism of HTLV-2 was assessed by either measuring the proviral load (PVL) using a multiplex real-time PCR assay or by detecting the production of HTLV-2 Tax protein after short-term culture. Total PBMCs from infected individuals were also stained with antibodies against surface markers for fluorescence-activated cell sorting (FACS). NK-mediated cytotoxicity was assessed using CD107a degranulation assays and flow cytometry based cytotoxicity assays. Statistical analysis was performed using the non-parametric Mann-Whitney U test.

**Results:** Six out of the 12 HTLV-2 infected individuals had undetectable PVL (mean 3.15 Tax copies/100 cells, ranging from 0 to 20.99 Tax copies/100 cells. Among samples with detectable PVL, CD8+ T cells showed the highest PVL levels (mean PVL = 17.84) followed by CD56+ cells (mean PVL = 11.7), CD19+ cells (mean PVL = 7.27) and CD4+ cells (mean PVL = 0.47). Infection of NK cells and NK T cells by HTLV-2 was further confirmed by the detection of proviral sequences in highly purified cellular fractions isolated by FACS. The expression of HTLV-2 Tax protein could be detected in all cellular subsets harboring the provirus after short-term cultures. NK cells from HTLV-2 infected individuals showed a significant reduction in cytotoxicity when compared to NK cells from uninfected individuals when assayed by degranulation and cytotoxicity assays ( $p=0.0433$  and  $p=0.0143$ , respectively).

**Conclusions:** This study provides evidence that HTLV-2 naturally infects NK cells, at high PVL levels. A significant reduction in cytotoxic activity of NK cells was found in HTLV-2 infected individuals when compared to uninfected controls. The impairment of NK mediated cytotoxicity observed in this study may be related HTLV-2 infection of such cells and this may have implications for our understanding of the persistence of HTLV-2 in infected individuals.

### PA11 – DENDRITIC CELL FUNCTIONS AND INTERACTIONS

#### PA11/2 REGULATION OF DENDRITIC CELL MIGRATION TO LYMPH NODES IN RESPONSE TO S1P BY SWAP-70

C. Ocana-Morgner<sup>1</sup>, M. Chopin<sup>1</sup>, S. Braungart<sup>1</sup>, R. Jessberger<sup>1</sup>

<sup>1</sup>Dresden University of Technology, Medical School, MTZ, Institute of Physiological Chemistry, Dresden, Germany

Stimulated dendritic cells (DCs) mature and migrate to lymphoid organs to prime naive T cells. DC maturation augments the migratory capacity of DCs through a series of phenotypic changes, e.g. by increasing the expression of CCR7. Migration of stimulated DCs is also regulated by the chemoattractant sphingosine-1-phosphate (S1P) that binds to G protein-coupled receptors. Signaling of S1P receptors is mediated by the Rho GTPases Rac and Rho. SWAP-70 regulates the expression of peptide-loaded MHCII on bone marrow-derived DCs (BMDCs)<sup>1</sup> and interacts with F-actin, Rac and RhoA-GTP. Unlike wild-type (wt) BMDCs, Swap-70/- BMDCs show constitutively active RhoA, which may affect S1P receptor signaling. Here we report on the migratory capacity and response to S1P of wt and Swap-70/- DCs. Swap-70/- BMDC show reduced migration to lymph nodes when transferred to wt animals. A large fraction of skin DCs from Swap-70/-, but not of wt DCs, remain outside lymphatic vessels in ear explants used to study the entry of skin DCs into lymphatic vessels. This phenotype is also observed in ear explants of wt animals after i.v. injection of the S1P receptor antagonist FTY720, which is known to reduce migration of DCs to lymph nodes<sup>2</sup>. Transwell migration of Swap-70/- BMDC towards S1P is significantly impaired. The response to S1P by Swap-70/- BMDC was restored near wt levels upon SWAP-70 re-expression. Expression of S1P receptors (S1P1-3) by wt and Swap-70/- BMDC is similar suggesting that signaling from these receptors is impaired in Swap-70/- BMDC. Through regulation of S1P signaling, SWAP-70 defines a new pathway to control migration of DCs, a critical yet incompletely understood function in DC-dependent immune responses.

1: Ocana-Morgner et al, Blood. 2009 Feb 12;113(7)

2: Czeloth et al, J Immunol. 2005 Sep 1;175(5)

#### PA11/4 CONDITIONAL ABLATION OF LANGERHANS CELLS LEADS TO ENHANCED PROTECTIVE IMMUNITY AGAINST LEISHMANIA MAJOR

K. Kautz-Neu<sup>1</sup>, M. Noordegraaf<sup>2,3</sup>, S. Dinges<sup>1</sup>, C.L. Bennett<sup>3,4</sup>, B.E. Clausen<sup>2,3</sup>, E. von Stebut<sup>1</sup>

<sup>1</sup>Johannes-Gutenberg University, Dept. of Dermatology, Mainz, Germany, <sup>2</sup>Erasmus University Medical Center, Dept. of Immunology, Rotterdam, Netherlands, <sup>3</sup>Academic Medical Center, University of Amsterdam, Dept. of Cell Biology and Histology, Amsterdam, Netherlands, <sup>4</sup>University College London, Royal Free Hospital, Dept. of Haematology, London, United Kingdom

Because an effective *Leishmania* vaccine does not exist and (skin) dendritic cells (DC) are critical regulators of the Th1/Tc1-dependent protective anti-*Leishmania* immune response, DC are attractive targets for immuno-therapeutic approaches. Thus, it is important to understand the precise contribution of a particular DC subset in the course of leishmaniasis. Recently, knock-in mice expressing a diphtheria toxin (DT) receptor (DTR) cDNA under control of the  *langerin*  promoter were

generated to study the *in vivo* dynamics and function of Langerin<sup>+</sup> DC in general, and Langerhans cells (LC) in particular. Application of DT to Langerin-DTR mice rapidly eliminates all Langerin<sup>+</sup> cells from epidermis, dermis and skin-draining LN. While LC are depleted for at least 2–4 weeks following a single injection of DT, Langerin<sup>+</sup> dermal DC (dDC) recover within 7–14 days from the toxin treatment. In this study we analyzed the role of Langerin<sup>+</sup> skin-derived DC in the physiologically relevant low dose (1x10<sup>3</sup> parasites) *Leishmania* infection model mimicking natural parasite transmission by the bite of a sand fly. DT-treated Langerin-DTR mice developed significantly smaller lesions, increased IFN $\gamma$ /IL-4 ratios and decreased parasite loads compared to control mice. Selective depletion of LC only showed that LC, not Langerin<sup>+</sup> dDC, were responsible for this effect. Interestingly, the number of CD4<sup>+</sup> Foxp3<sup>+</sup> regulatory T cells (Treg) in infected ears was reduced in DT-treated Langerin-DTR mice as compared to control mice. In contrast to prior studies, our data clearly reveal a suppressive role of epidermal LC in the course of *L. major* infection via induction of Treg, as the depletion of these cells leads to a better disease outcome. Finally, results after immunomodulation/therapy in leishmaniasis or other important infectious diseases worldwide might critically depend on the DC subtype targeted.

#### PA11/5 SPHINGOSINE-1-PHOSPHATE – A NOVEL REGULATOR OF DENDRITIC CELL MIGRATION, POSITIONING AND FUNCTION

A. Rathinasamy<sup>1</sup>, G. Bernhardt<sup>1</sup>, R. Förster<sup>1</sup>, O. Pabst<sup>1</sup>

<sup>1</sup>Medizinische Hochschule Hannover (MHH), Institute of Immunology, Hannover, Germany

Sphingosine-1-phosphate (S1P) signals through its receptors (S1P<sub>1</sub>, S1P<sub>2</sub>, S1P<sub>3</sub>, S1P<sub>4</sub>, S1P<sub>5</sub>) thereby resulting in various cellular responses including cell migration, proliferation and apoptosis. Immune cells respond to S1P signals. In particular, S1P<sub>1</sub> has been shown to regulate lymphocyte egress from thymus and lymph nodes. In this study we report a function for S1P<sub>1</sub> and S1P<sub>3</sub> in regulating dendritic cell (DC) migration and positioning *in vivo*.

**Objectives and methods:** S1P<sub>1</sub> and S1P<sub>3</sub> deficient bone marrow cells were differentiated to dendritic cells and tested for their migratory potential *in vitro* using transwell migration assay. *In vivo* DC migration was studied after subcutaneous and intra-tracheal adoptive transfer followed by quantification of DC in the respective draining lymph nodes. Expression of various S1P receptors was studied using quantitative real-time PCR from bone marrow derived mature and immature DC as well as DC isolated from various organs.

**Results:** Murine bone marrow derived DC express S1P receptors among which S1P<sub>1</sub> and S1P<sub>3</sub> are highly upregulated upon DC maturation. Dendritic cells migrate *in vitro* towards S1P as observed in transwell migration assay. This migration is severely impaired in S1P<sub>1</sub> as well as S1P<sub>3</sub> deficient DC. Similarly S1P<sub>1</sub> and S1P<sub>3</sub> deficient DC showed reduced migration to the draining lymph nodes *in vivo*. In particular, migration was severely hampered in S1P<sub>1</sub> deficient DC. Strikingly S1P<sub>1</sub> but not S1P<sub>3</sub> controls the positioning of DC in bridging channels of spleen. Irrespectively, ovalbumin loaded S1P<sub>1</sub> and S1P<sub>3</sub> deficient DC readily induced the proliferation of antigen specific T cells *in vivo*.

**Conclusions:** Our observations reveal a prominent role for S1P<sub>1</sub> and S1P<sub>3</sub> in regulating DC migration and a potential role for S1P<sub>1</sub> in the positioning *in vivo*. Interestingly S1P<sub>1</sub> and S1P<sub>3</sub> showed partially overlapping functions with respect to migratory capacity but not with respect to positioning within lymphoid organs. The dynamic regulation of S1P receptor expression during DC maturation suggests a role for the S1P system in regulating the DC life cycle.

#### PA11/6 T HELPER CELL POLARIZATION TOWARDS APC IS GUIDED BY THE ACTIVATION OF ATYPICAL PKC $\zeta$ AT THE IMMUNOLOGICAL SYNAPSE: DECOY FUNCTION OF TGF $\beta$ 1

F. Bertrand<sup>1</sup>, S. Duchez<sup>1</sup>, M. Esquerre<sup>1</sup>, M. Rodrigues<sup>1</sup>, S. Valitutti<sup>1</sup>

<sup>1</sup>INSERM, Toulouse, France

We investigated the molecular mechanisms and the functional role of T helper (T<sub>H</sub>) cell polarization towards APC. We show that, in T<sub>H</sub> cells interacting with cognate APC, atypical PKCs are rapidly recruited and phosphorylated at the immunological synapse via a PI3K-dependent pathway and that a functional PKC $\zeta$  is required for T<sub>H</sub> cell polarization. Block of PKC $\zeta$  pathway in T<sub>H</sub> cells inhibits IL-12 production by cognate DC (a function dependent on T<sub>H</sub> cell polarization) but does not affect signaling in T cells. Finally, treatment of T cell/APC conjugates with TGF $\beta$ 1 results in atypical PKC phosphorylation outside of immunological synapses and, accordingly, randomizes T<sub>H</sub> cell polarization.

Our results show that localized activation of PKC $\zeta$  at the immunological synapse drives T cell polarization responses, dispensable for sustained signaling in T cells, but required for dedicated help delivery. TGF $\beta$ 1, by hijacking PKC $\zeta$  signaling outside the immunological synapse, spoils T cell polarization responses.

#### PA11/7 CD11c<sup>+</sup>CD8 $\alpha$ <sup>+</sup> DENDRITIC CELLS PROMOTE PROTECTIVE IMMUNITY TO RESPIRATORY INFECTION WITH *BORDETELLA PERTUSSIS*

P.J. Dunne<sup>1</sup>, B. Moran<sup>1</sup>, R.C. Cummins<sup>2</sup>, K.H. Mills<sup>1</sup>

<sup>1</sup>Trinity College Dublin, Biochemistry & Immunology, Dublin, Ireland, <sup>2</sup>RCSI Education & Research Centre, Beaumont Hospital, Pathology Department, Dublin, Ireland

Here we have used a murine model of infection with *Bordetella pertussis* to examine the function of DC subtypes in protective immunity in the lungs. We found a dramatic increase in the numbers of CD11c<sup>+</sup>CD8 $\alpha$ <sup>+</sup> DC in the cervical lymph nodes (CLN) within 4 hrs of challenge with *B. pertussis*. CD11c<sup>+</sup>CD8 $\alpha$ <sup>+</sup> DC also infiltrated the lung with a peak 7 days post *B. pertussis* challenge. The infiltrating CD11c<sup>+</sup>CD8 $\alpha$ <sup>+</sup> DC expressed MHC, co-stimulatory and activation markers, indicative of mature DC. The CD11c<sup>+</sup>CD8 $\alpha$ <sup>+</sup> DC in the CLN expressed IL-4 and IL-10, and lower levels of IFN- $\gamma$ , but in the lungs expressed predominantly IFN- $\gamma$ . Depletion of CD8 $\alpha$ <sup>+</sup> cells early in infection attenuated Th1 responses in the lungs and significantly reduced bacterial clearance. Conversely, transfer of FLT3 ligand (FL)-expanded CD11c<sup>+</sup>CD8 $\alpha$ <sup>+</sup> DC enhanced bacterial clearance, whereas GM-CSF-expanded conventional DC had no effect. The numbers of CD11c<sup>+</sup>CD8 $\alpha$ <sup>+</sup>CD103<sup>+</sup> cells were also increased during the early phase of infection. Blocking CD103 function caused a significant delay in bacterial clearance and a reduction in cellular infiltration into the lungs. These findings demonstrate that CD11c<sup>+</sup>CD8 $\alpha$ <sup>+</sup> and CD11c<sup>+</sup>CD103<sup>+</sup> DC play a protective role in mediating immunity to *B. pertussis* infection in the respiratory tract.

#### PA11/8 THE ADAP/SKAP-HOM COMPLEX IS REQUIRED FOR OPTIMAL DENDRITIC CELL FUNCTION

A. Reinhold<sup>1</sup>, S. Reimann<sup>1</sup>, D. Reinhold<sup>1</sup>, B. Schraven<sup>1</sup>, M. Togni<sup>1</sup>

<sup>1</sup>Otto-von-Guericke-University Magdeburg, Institute of Molecular and Clinical Immunology, Magdeburg, Germany

SKAP-HOM is an ubiquitously expressed cytosolic adaptor protein, whereas its binding partner ADAP is mainly expressed in T cells, myeloid cells and platelets. We have previously shown that SKAP-HOM expression is mandatory for the optimal function of B cells and dendritic cells (DC) while it is dispensable for T cell function. ADAP is known as a positive regulator of T cell adhesion and activation, and its deletion results in the concomitant loss of SKAP-HOM and SKAP55 on protein level. So far, no data are available about the role of ADAP in myeloid cells.

In the present study, we investigated the functional consequences of the simultaneous deletion of both, ADAP and SKAP-HOM, on dendritic cell function. Therefore, we generated the respective ADAP/SKAP-HOM double knock-out mouse (dKO).

Immature dendritic cells lacking SKAP-HOM and ADAP show an enhanced adhesion to fibronectin and ICAM-1 and a reduced spontaneous emigration of DC from ear skin explants. Furthermore, the antigen-dependent conjugate formation between the double deficient DC and wild type T cells is nearly abolished. The antigen-specific T cell stimulatory capacity of dKO DC was also reduced compared to wild type DC. *In vivo*, the migration of *ex vivo*-matured dendritic cells from the skin to the draining lymph nodes is enhanced. Finally, the simultaneous loss of SKAP-HOM and ADAP rendered the mice unsuceptible to MOG-induced experimental autoimmune encephalomyelitis (EAE).

In summary, our data suggest that the expression of the ADAP/SKAP-HOM complex is required for optimal functioning of DC. Further studies are necessary to investigate more dendritic cell functions and to dissect the mechanism underlying the disturbed functions of dendritic cells in the absence of SKAP-HOM and ADAP.

The studies are supported by DFG grant SCHR 533/5-3.

#### PA11/9 HUMAN IMMATURE MYELOID DENDRITIC CELLS TRIGGER A T<sub>H</sub>2-POLARIZING PROGRAM VIA JAGGED-1/NOTCH INTERACTION

F. Frosali<sup>1</sup>, F. Liotta<sup>1</sup>, V. Querci<sup>1</sup>, A. Mantei<sup>2</sup>, L. Filì<sup>1</sup>, L. Maggi<sup>1</sup>, B. Mazzinghi<sup>1</sup>, R. Angeli<sup>1</sup>, E. Ronconi<sup>1</sup>, V. Santarlasci<sup>1</sup>, T. Biagioli<sup>1</sup>, L. Lasagni<sup>1</sup>, C. Ballerini<sup>1</sup>, P. Paronchi<sup>1</sup>, A. Scheffold<sup>2</sup>, L. Cosmi<sup>1</sup>, E. Maggi<sup>1</sup>, S. Romagnani<sup>1</sup>, F. Annunziato<sup>1</sup>

<sup>1</sup>University of Florence, Florence, Italy, <sup>2</sup>Deutsches Rheumaforschungszentrum, Berlin, Germany

**Objectives:** The mechanisms by which human dendritic cells (DCs) activate a T<sub>H</sub>1- or T<sub>H</sub>2-polarizing program are still partially unclear.

The objective of the study was to identify the mechanisms responsible for the T<sub>H</sub>1/T<sub>H</sub>2-polarizing activity by human circulating myeloid DCs before and after ligation of their Toll-like receptors (TLRs).

**Methods:** IL-4 and IFN- $\gamma$  production by CD4<sup>+</sup> T-cells was assessed in co-cultures with myeloid DCs before or after TLR triggering. Expression of Jagged-1 and Delta-4 Notch ligands and of GATA-3 and T-bet transcription factors was evaluated by real-time quantitative PCR. STAT-4 and STAT-6 phosphorylation was assessed by flow cytometry. Knockdown of Jagged-1 or Delta-4 was performed by transfection of DCs with appropriate siRNAs.

**Results:** The results obtained showed that myeloid immature DCs (iDCs) constitutively expressed Jagged-1 which induces in CD4<sup>+</sup> T cells a T<sub>H</sub>2 polarization, as shown by Jagged-1 gene silencing. The T<sub>H</sub>2 polarization was associated with high GATA-3/T-bet ratio, and was at least partially dependent of the early induction of IL-4 by T cells themselves. Maturation of DCs by TLR triggering resulted in the reduction of Jagged-1 and in the up-regulation of Delta-4, which was at least in part responsible for the polarization of CD4<sup>+</sup> T cells to the T<sub>H</sub>1 phenotype.

**Conclusion:** In conclusion CD4<sup>+</sup> T cell responses, induced by myeloid iDC, are usually characterized by a prevalent T<sub>H</sub>2 phenotype unless TLR are triggered on DCs by microbial components. These findings may have important implications for the explanation of the biological bases of the hygiene hypothesis and for the development of both preventive and immunotherapeutic strategies in allergic disorders.



**PA11/10 ACTIVATION OF CDCS INDUCES SPECIFIC PDC COOPERATION TO PROMOTE A COORDINATED IMMUNE RESPONSE**B. Pérez-Cabezas<sup>1,2</sup>, P. Bastos-Amador<sup>1,2</sup>, M. Naranjo-Gomez<sup>1,2</sup>, M. Bofil<sup>2,3</sup>, F. Carmona<sup>4</sup>, R. Pujol-Borrell<sup>2,5</sup>, F. Nuñez<sup>6</sup>, F.E. Borrás<sup>1,2</sup><sup>1</sup>Laboratory of Immunobiology (LIRAD-BST), Germans Trias i Pujol Research Institute, Badalona, Spain, <sup>2</sup>Autonomous University Barcelona (UAB), Dept. Cell Biology, Physiology & Immunology, Badalona, Spain, <sup>3</sup>Irsicaixa Foundation, Badalona, Spain, <sup>4</sup>Statistics Department, University of Barcelona, Barcelona, Spain, <sup>5</sup>Fundació Institut Investigació Germans Trias i Pujol, Immunology, LIRAD-BST, Badalona, Spain, <sup>6</sup>Unit of Scientific-Technical Support (UCTS), Research Institute of Vall d'Hebron, Barcelona, Spain

Two well-characterized blood dendritic cell populations, conventional (cDC) and plasmacytoid (pDC) have been described. Both populations exhibit multiple differences including phenotype, TLR-expression and cytokine and chemokine secretion. cDCs are highly efficient at exogenous antigen presentation and they traffic from tissues to local lymph nodes for antigen presentation via afferent lymphatic vessels. In contrast, pDCs are less competent in antigen uptake, they are present in the thymus and secondary lymph nodes and are rarely found in non-inflamed tissues and afferent lymphatics. These marked differences and some evidences reported by our and other laboratories suggest specialized and may be complementary and coordinated functions between DC subsets to induce a potent immune response.

**Aims:** To verify the coordination between pDC and cDC in the generation of immune responses, elucidating the mechanisms of pDCs-conditioning by activated cDCs and looking for specific differences depending on the cDC activation stimulus.

**Methods:** cDCs and pDCs were sorted from buffy coat preparations obtained from healthy blood donors. Sorted cDCs were stimulated with LPS or R848 during 16h. Meanwhile, pDCs from the same donor were CFSE-labeled and maintained in IL3. Then, CFSE-pDCs and stimulated-cDCs were mixed (2:1) and co-cultured for additional 5h. CFSE-pDCs were sorted again and the RNA was extracted for microarray analyses and real time-PCR. Functional features of the conditioned-pDC included phenotype changes, induction of alloproliferation and production of IFN $\alpha$ .

**Results:** Phenotypically, activated cDC induced the up-regulation of several maturation markers in pDCs, including CD25, CD83 and CD86. Also, conditioning of pDC by activated-cDC promoted the expression of several genes such as chemokines and proinflammatory cytokines such as IL6. Qualitatively, the gene expression profile of pDCs conditioned by LPS-cDCs or by R848-cDCs was very similar and both were able to induce alloresponses. However, as expected, the larger number of changes in gene expression was found when pDCs were conditioned by R848-cDC.

**Conclusions:** Our results demonstrate that different types of cDC activating factors promote specialized gene and functional activation profiles in pDC that may in turn contribute to establish an appropriate immune-regulatory environment.

**PA11/11 ICOS/B7H BIDIRECTIONAL SIGNAL TO INDUCE SECRETION OF IL-1BETA AND IL-17**A. Chiochetti<sup>1</sup>, R. Mesturini<sup>1</sup>, S. Nicola<sup>1</sup>, U. Dianzani<sup>1</sup><sup>1</sup>University of Eastern Piedmont, Dept. Medical Sciences, Novara, Italy

B7h is constitutively expressed by antigen presenting cells (APC) and binds ICOS expressed by activated T cells. ICOS triggering costimulates T helper (TH) cell activation and modulates their cytokine secretion, whereas the effect of B7h triggering in APC is unknown.

**Objective:** This work investigated B7h reverse signalling in dendritic cells (DC).

**Methods:** We induced DC maturation with LPS in the presence or absence of a soluble ICOS-Ig fusion protein; then, both cytokine secretion and allostimulatory activity were evaluated.

**Results:** B7h stimulation substantially modulated LPS-induced DC maturation by influencing cytokine secretion. The most striking effect was 80-fold increase of IL-1 $\beta$  secretion, but other changes were 3-fold increase of IL-10 and 2-fold decrease of TNF- $\alpha$ . In IL-1 $\beta$  secretion, B7h triggering acted as a "second signal" by activating caspase-1, required to cleave pro-IL-1 $\beta$  whose synthesis was induced by LPS. B7h triggering on LPS-activated DC also modulated their capacity to activate allogeneic T cells in mixed lymphocyte cultures (MLC) by decreasing the proliferative response and changing cytokines secretion, including 20-fold decrease of IFN- $\gamma$  and 5-fold increase of IL-17. This effect was partly ascribable to the cytokine milieu secreted by DC and particularly to the high IL-1 $\beta$  levels, known to be involved in differentiation of human Th17 cells. However, ICOS triggering too played a role in IL-17 secretion since blocking of the B7h/ICOS interaction inhibited IL-17 secretion in MLC with DC. Moreover, in naive Th cells activated with anti-CD3 antibodies, ICOS stimulation induced IL-17 secretion in the presence of exogenous IL-1 $\beta$ .

**Conclusions:** In this work we showed that B7h-mediated reverse signaling induced IL-1 $\beta$  by DCs which in turn favors IL-17 secretion by Th cells. On the other side, we demonstrated that ICOS costimulation directly favors Th17 differentiation.

**PA11/12 REGULATORY T CELLS PREVENT THE PRIMING OF CD8<sup>+</sup> T CELLS IN CONTACT HYPERSENSITIVITY REACTIONS BY INTERACTION WITH DENDRITIC CELLS VIA GAP-JUNCTIONS IN VIVO**S. Ring<sup>1</sup>, T. Johnson<sup>1</sup>, S. Karakhanova<sup>1</sup>, A.H. Enk<sup>1</sup>, K. Mahnke<sup>1</sup><sup>1</sup>University of Heidelberg, Heidelberg, Germany

To analyze the influence of regulatory T cells (Treg) on CD8<sup>+</sup> T cell mediated immune responses *in vivo*, we used the model of hapten-induced contact hypersensitivity (CHS) in mice. CHS is induced by sensitization of individuals against haptens. This sensitization occurs in the draining lymph nodes (dLN) where the antigen-specific priming of CD8<sup>+</sup> T cells by dendritic cells (DCs) takes place. Our initial studies showed that the immune reaction, as measured by the ear swelling response induced by challenging the mice 5d after sensitization, can significantly be inhibited by injection of CD62L<sup>+</sup> LN-homing CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells (Treg) before the first hapten application. Thereby, Treg migrated to dLN and established cellular contact to DC, which was demonstrated by fluorescence microscopy of cryosections of dLN stained with fluorescence markers for Treg, DCs or CD8<sup>+</sup> T cells, respectively. Further detailed analysis of this interaction revealed that fluorescently labelled Treg transfer cytoplasmic dyes to DC *in vivo*, and that Connexin 43, a marker for gap junctions, is present at the Treg–DC interfaces. As a consequence DC display reduced expression of CD80, CD86 and are impaired in stimulating T cells. Therefore we conclude that gap junctional intercellular communication led to downregulation of T cell co-stimulatory molecules on the surface of the DC abrogating the priming, the activation and the proliferation of hapten-specific CD8<sup>+</sup> T cells. Thus, Treg do not only modulate ongoing CD4<sup>+</sup> T cell mediated immune reactions at tissue sites but also abrogate the *de novo* induction of CD8<sup>+</sup> T cell driven immune reactions by interfering with T cell stimulatory activity of DC via gap junctional intercellular communication.

**PA11/13 ACTIVIN A INDUCES DENDRITIC CELL MIGRATION THROUGH THE POLARIZED RELEASE OF CXCL12 AND CXCL14**L. Salogni<sup>1</sup>, T. Musso<sup>2</sup>, D. Bosio<sup>1</sup>, M. Mirolo<sup>3</sup>, V.R. Jala<sup>4</sup>, B. Haribabu<sup>4</sup>, M. Locati<sup>3</sup>, S. Sozzani<sup>1</sup><sup>1</sup>University of Brescia, Department of Biomedical Sciences and Biotechnology, Section of General Pathology, Brescia, Italy, <sup>2</sup>University of Torino, 2Department of Public Health and Microbiology, Torino, Italy, <sup>3</sup>University of Milan, IRCCS Istituto Clinico Humanitas, Department of Translational Medicine, Milan, Italy, <sup>4</sup>James Graham Brown Cancer Center, University of Louisville, Louisville, United States

Activin A is a dimeric protein, member of the TGF- $\beta$  family that plays a crucial role in wound repair and in foetal tolerance. Emerging evidence also proposes Activin A as a key mediator in inflammation. This study reports that Activin A induces the directional migration of immature myeloid dendritic cells (DC) through the activation of ALK4 and ActRIIA receptor chains. Conversely, Activin A was not active on plasmacytoid DC or mature myeloid DC. DC migration to Activin A was PI3K $\gamma$ -dependent, *Bordetella pertussis* toxin and cycloheximide sensitive and was inhibited by M3, a viral-encoded chemokine binding protein. In a real time video microscopy based migration assay, Activin A induced polarization of DC but not migration. These characteristics clearly differentiated the chemotactic activities of Activin A from TGF- $\beta$  and classic chemokines. By the use of combined pharmacological and low-density microarray analysis it was possible to define that Activin A-induced migration depends on the selective and polarized release of two chemokines, namely CXCL12 and CXCL14. This study extends the pro-inflammatory role of Activin A to DC recruitment and provides a cautionary message about the reliability of the *in vitro* chemotaxis assays in discriminating direct versus indirect chemotactic agonists.

**PA11/14 UNRAVELING GLOBAL PLASMACYTOID DENDRITIC CELL RESPONSES TO VIRAL INFECTION IN VIVO**N. Zucchini<sup>1</sup>, B. Thomas<sup>1</sup>, R. Scott<sup>1</sup>, G. Bessou<sup>1</sup>, S. Traub<sup>1</sup>, L. Chasson<sup>1</sup>, S. Uematsu<sup>2</sup>, S. Akira<sup>2</sup>, L. Alexopoulou<sup>1</sup>, M. Dalod<sup>1</sup><sup>1</sup>CNRS UMR6102, Centre d'Immunologie de Marseille-Luminy, Marseille Cedex, France, <sup>2</sup>Research Institute for Microbial Diseases, Department of Host Defense, Osaka, Japan

**Objectives:** Plasmacytoid dendritic cells (pDC) are characterized by their ability to rapidly produce high amounts of IFN- $\alpha/\beta$  in response to many viruses, including during murine cytomegalovirus (MCMV) infection *in vivo*. pDC also produce other cytokines/chemokines. However, their quantitative contribution to this function as compared to other cells was not clear. Furthermore, whether multiple cytokines are simultaneously produced within individual cells in response to the same stimuli, or occur in specialized pDC subsets, was unknown. Finally, the global role of pDC in host resistance to viral infection is difficult to study rigorously, partly due to the lack of a method allowing efficient and specific depletion of these cells *in vivo*.

**Methods:** IFN- $\alpha/\beta$ , IL-12 and TNF- $\alpha$  expression were examined by multiparametric flow cytometry to identify the cellular sources and molecular triggers for production of these innate cytokines in various tissues early after MCMV infection. Gene expression profiling of leukocyte subsets isolated from naïve versus MCMV-infected mice was performed, to evaluate the extent of the specific versus common antiviral responses of pDC, and to help identifying genes specific to pDC even under activation conditions.

**Results:** Splenic pDCs were the major source of the cytokines studied early after MCMV infection, with simultaneous production of all three cytokines in individual cells, and overlapping roles of TLR-7 and -9 for this function. However, the kinetics and expression pattern of IL-12 as compared to IFN- $\alpha/\beta$  and TNF- $\alpha$  suggested that different intracellular signalling events were involved in the production of these 2 groups of cytokines. pDC selectively repressed or induced many genes during infection. However, genes were identified which expression stayed pDC-specific and which could thus be used for genetic targeting of this cell type.

**Conclusions:** pDC rapidly respond to a systemic infection with a DNA virus in vivo in tissue-specific manner and with a coordinated expression of multiple cytokines by individual cells in a TLR-9 and -7-regulated manner. pDC undergo a complex and profound genetic reprogramming during viral infection in vivo, suggesting their implication in other functions than cytokine production. To investigate these functions, a mutant mouse strain for pDC depletion in vivo is being engineered.

#### PA11/15 HARNESSING THE POWER OF COMPARATIVE GENOMICS TO EXPLORE INTRA- AND INTER-SPECIES RELATIONSHIPS BETWEEN DENDRITIC CELL SUBSETS

S. H. Robbins<sup>1</sup>, R. Guiton<sup>1</sup>, K. Crozat<sup>1</sup>, T. Walzer<sup>1</sup>, G. Bessou<sup>1</sup>, D. Dembélé<sup>2</sup>, C. Thibault<sup>2</sup>, A. Defays<sup>1</sup>, H. Xu<sup>3</sup>, E. Vivier<sup>1</sup>, M. Sellars<sup>2</sup>, P. Pierre<sup>1</sup>, F. R. Sharp<sup>3</sup>, S. Chan<sup>2</sup>, P. Kastner<sup>2</sup>, M. Y. Dalod<sup>1</sup>

<sup>1</sup>CNRS UMR6102, Centre d'Immunologie de Marseille-Luminy, Marseille Cedex, France, <sup>2</sup>CNRS UMR7104, Institut de Génétique et de Biologie Moléculaire et Cellulaire, Strasbourg, France, <sup>3</sup>University of California at Davis Medical Center, The Medical Investigation of Neurodevelopmental Disorders Institute, Sacramento, United States

**Objectives:** Dendritic cells (DCs) play a key role in the induction of immune responses to foreign antigens and in the regulation of tolerance to self. Mouse DCs have been extensively characterized. Protective immunity against tumors or intracellular pathogens can be induced in mice by targeting antigens specifically to the CD8 $\alpha$  subset of conventional DCs (cDCs), which is selectively endowed with a strong capacity for cross-presentation of exogenous antigens to CD8 T lymphocytes. In sharp contrast, harnessing the DC subsets naturally present in human lymphoid organs for medical purposes is currently hampered by a profound lack of knowledge about these cells. To contribute in overcoming this cognitive gap, we used comparative genomics to explore potential equivalences between human blood and mouse spleen DC subsets.

**Methods:** Using a combination of our own Affymetrix gene chips data and of compatible public data, we established a compendium for gene expression profiling of DC subsets, as compared to lymphocytes and myeloid cells, within and across species. To determine the relationships between leukocyte subsets, and identify subset-specific transcriptional signatures, we used a variety of bioinformatics analyses based on different statistical methods.

**Results:** Mouse and human DC subsets formed a distinct group within the leukocyte family, and displayed a common and evolutionarily conserved transcriptional signature. Mouse and human plasmacytoid DCs shared a large specific gene expression program supporting a strong conservation of the functions of this cell type from mouse to human. Conserved gene expression profiles were also observed between mouse CD8 $\alpha$  and human BDCA3 cDCs, as well as between mouse CD11b and human BDCA1 cDCs.

**Conclusions:** To the best of our knowledge, our study was the first to perform in depth comparative genomics analyses of leukocyte subsets between species (Robbins et al. Genome Biol. 2008). This work builds the foundations for future investigation of the evolutionarily conserved molecular pathways governing DC subset ontogeny and functions. Importantly, it suggests that human BDCA3 cDCs are homolog to mouse CD8 $\alpha$  cDCs and may thus be a promising target for vaccination or immunotherapy against cancer or intracellular pathogens, and for treatment against certain autoimmune diseases.

#### PA11/16 DENDRITIC CELL MIGRATION TO LUNG DRAINING LYMPH NODES IN AN INFLUENZA INFECTION

A. W. Ho<sup>1</sup>, X. Dai<sup>1</sup>, P. A. Macary<sup>1</sup>, D. M. Kemeny<sup>1</sup>

<sup>1</sup>National University of Singapore, Microbiology, Singapore, Singapore

The lung is an organ that is constantly exposed to the environment and thus routinely encounters pathogens. Lung dendritic cells (DCs) play a crucial role as sentinels, surveying the environment through antigen uptake, and migrating to the draining lymph nodes to present the antigen to T and B cells. Using a mice model of influenza, we investigated DC migration to the lung draining lymph nodes shortly after infection. A lipophilic fluorescent dye, DiD, was used to label the lipid coat of influenza virus to allow detection of cells that have been infected by the virus. We then analysed DCs from both lung parenchyma and mediastinal lymph nodes within the thoracic cavity for the presence of DiD label. We report the following observations. Firstly, DCs with DiD label drain only to posterior mediastinal lymph node and not to the anterior mediastinal lymph node. Secondly, by 48 hours post infection, the majority (70%) of DiD positive DCs were the CD11b<sup>neg</sup> subset in the lymph node. This is in contrast to the lung tissue where the CD11b<sup>+</sup> dendritic cell subset formed the majority (87%) of DiD positive DCs. Finally, we observed proliferation of clone 4 T-cells in the posterior mediastinal lymph nodes, but not in the anterior mediastinal lymph nodes when these cells were adoptively transferred into mice infected with influenza. This distinct pattern of DC migration in an influenza infection and provides insight into the anatomical site where early immune response against influenza is mounted and has important implications for understanding lung DC biology in the context of viral antigen uptake and presentation.

#### PA11/17 ENVIRONMENTAL AND DENDRITIC CELL GLYCOSYLATION DICTATES DENDRITIC CELL MIGRATION

S. J. van Vliet<sup>1</sup>, M. Bax<sup>1</sup>, L. C. Paessens<sup>1</sup>, J. J. Garcia-Vallejo<sup>1</sup>, Y. van Kooyk<sup>1</sup>

<sup>1</sup>VU University Medical Center, Molecular Cell Biology & Immunology, Amsterdam, Netherlands

The correct positioning of immune cells is crucial for the orchestration of effective immune responses. However, little is known on the regulatory mechanisms which control dendritic cell (DC) migration to draining lymph nodes. Here, we provide evidence for two separate mechanisms involving glycosylation and cognate lectins that influence the migratory capacity of dendritic cells. By using mouse Fc-chimera of the human C-type lectin MGL as a probe, MGL ligands could be detected on the endothelial structures in lymph node and skin. MGL binding correlated with the expression of  $\alpha$ -GalNAc, the preferred MGL ligand. Strikingly, addition of blocking MGL antibodies increased DC migration towards RANTES in a transwell system and enhanced DC motility on coated GalNAc structures as visualized by time-lapse video microscopy. Furthermore, 3-fold more DCs emigrated from human skin explants in the presence of blocking MGL antibodies (van Vliet et al. 2008). Upon DC maturation profound changes in DC expression patterns of lectins and glycans occur. Mature DCs have lost all MGL expression, thereby releasing them from their GalNAc constraints. Maturation is also accompanied with profound changes in sialylation, showing a marked increase in polysialic acid, a homopolymer of multiple  $\alpha$ 2-8-linked sialic acids (Bax et al. 2007). Expression of polysialic acid was only observed on highly migratory DCs cultured under strong polarizing conditions, whereas DC cultured under tolerogenic regimens failed to upregulate polysialic acid and exhibited poor migration. Polysialic acid served as a capture molecule for the chemokine CCL21, whereby CCL21 directly bound the  $\alpha$ 2-8 linked sialic acids. Indeed, removal of polysialic acid led to a loss of DC migration towards CCL21. Together our results show that the glycosylation of the DC and of its local environment, combined with the expression of lectin receptors on the DC determine the ability of the DC to respond to chemotactic stimuli such as the chemokine CCL21. Our results have strong implications for DC-based therapies, where DC migration to lymph node is required to induce full immune protection. Employing DCs with a MGL negative and polysialic acid positive phenotype could enhance DC migration and thus the outcome of the vaccination therapy.

#### PA11/18 GM-CSF DIFFERENTLY STIMULATES ACCESSORY FUNCTION OF THYMIC DENDRITIC CELL SUBSETS BUT NOT THEIR CAPACITY TO INDUCE FOXP3 EXPRESSION

S. Vasiljic<sup>1</sup>, D. Vucevic<sup>1</sup>, I. Majstorovic<sup>1</sup>, B. Bozic<sup>2</sup>, P. Milosavljevic<sup>1</sup>, M. Colic<sup>1</sup>

<sup>1</sup>Military Medical Academy, Institute for Medical Research, Belgrade, Serbia, <sup>2</sup>University of Belgrade, Faculty of Biology, Belgrade, Serbia

**Objectives:** GM-CSF is well known cytokine with important role for development of conventional DC, but its influence on functional capabilities of CD11b<sup>+</sup> and CD11b<sup>-</sup> subsets of thymic dendritic cells (TDC) is unresolved. The aim of this work was to study the effect of GM-CSF on endocytic activity of TDC subsets, their ability to induce proliferation and apoptosis of thymocytes, as well as thymocyte Foxp3 expression.

**Methods:** TDC subsets of AO rats were separated according to the expression of CD11b and cultivated overnight in the presence of GM-CSF. The endocytic and phagocytic activity were studied by uptake of FITC-dextran and CMFDA labeled apoptotic thymocytes, respectively. Accessory function of TDC was assessed in thymocyte proliferation and apoptosis assays. Adoptive transfer assay was performed by TDC subsets intravenously transferred in allogeneic rats. After 3 days, recipients were sacrificed and apoptosis of thymocytes as well as hyporesponsiveness of lymphocytes were tested. The expression of Foxp3 was analyzed by flow cytometry after 4 days coculture of TDC and thymocytes. Statistical analysis was performed using Student's t-test.

**Results:** We found that CD11b<sup>+</sup> and CD11b<sup>-</sup> subsets have a similar phenotypical characteristics, but after cultivation, they show different functional properties. CD11b<sup>-</sup> TDC have a higher endocytic activity and strongly stimulate thymocyte proliferation, whereas CD11b<sup>+</sup> TDC are more potent in induction of thymocyte apoptosis. Both subsets to the same extent induce the expression of Foxp3 molecules on thymocytes. However, TDC cultured with GM-CSF, showed a higher accessory activity but not ability to induce Foxp3 expression. By the influence of GM-CSF, both subsets acquired a significantly higher ability to induce proliferation and apoptosis of thymocytes, but only CD11b<sup>+</sup> subset showed a significantly higher endocytic and phagocytic activity. A higher stimulatory effect of GM-CSF on CD11b<sup>+</sup> TDC was confirmed in vivo. It was found that only GM-CSF treated CD11b<sup>+</sup> TDC induced a significant hyporesponsiveness of lymphocytes, which was in correlation with increased apoptosis of alloreactive thymocytes.

**Conclusion:** These results suggest that GM-CSF could play modulatory role in central tolerance mediated by CD11b<sup>+</sup> TDC and its effect can be based more on depletion of self-reactive thymocytes than induction of regulatory T cells.

**PA11/19 B7-H1 EXPRESSION ON DENDRITIC CELLS NEGATIVELY REGULATES MHC II- AND CD1D-RESTRICTED CD4+ T CELLS BUT NOT INVARIANT NKT CELLS**C. Brandl<sup>1</sup>, S. Ortler<sup>2</sup>, H. Wiendl<sup>2</sup>, M. Lutz<sup>1</sup><sup>1</sup>University of Würzburg, Virology and Immunology, Würzburg, Germany, <sup>2</sup>University Hospital Würzburg, Neurology, Würzburg, Germany

Dendritic cells (DC) can act tolerogenic at immature and semi-mature stages. Previously, we showed that bone marrow (BM)-derived and with TNF-matured DC protected mice in a peptide-specific manner from Experimental Autoimmune Encephalomyelitis (EAE). Repetitive injections of these tolerogenic TNF-DC induced protective CD4+ T cell and NKT cell responses, which were mediated by their release of IL-10, IL-4 and IL-13. Here we examined the role of the co-inhibitory molecule B7-H1 on DC in this tolerance model. TNF-DC were generated from B7-H1<sup>-/-</sup> mice and applied to mice before EAE induction. B7-H1-deficient TNF-DC showed a stronger protective effect on EAE as compared to wild type (WT)-DC. Injections of B7-H1<sup>-/-</sup> TNF-DC induced higher levels of peptide-specific IL-10 and IL-13 after restimulation *in vitro* together with the elevated serum cytokines IL-4 and IL-13, while IL-17 and IFN- $\gamma$  were reduced in the CNS. EAE protection experiments in CD1d<sup>-/-</sup> and *Ja281*<sup>-/-</sup> mice revealed that CD1d-restricted CD4+ T cells (type II NKT cells), but not invariant type I NKT cells, were responsible for the increased serum cytokine production in the absence of B7-H1. Together, our data indicate that B7-H1 expressed on DC specifically interferes with conventional CD4+ T cells and type II NKT cells *in vivo* to release tolerogenic cytokines as demonstrated by the EAE tolerance model.

**PA11/20 EFFICIENT LISTERIA CLEARING NEEDS COLLABORATION BETWEEN TLR AND INTEGRIN ON ANTIGEN PRESENTING CELLS**A. Reinhold<sup>1</sup>, D. Schlüter<sup>2</sup>, D. Reinhold<sup>1</sup>, B. Schraven<sup>1</sup>, M. Togni<sup>1</sup><sup>1</sup>Otto von Guericke Universität Magdeburg, Institut für Molekulare und Klinische Immunologie, Magdeburg, Germany, <sup>2</sup>Institut für Medizinische Microbiologie, Magdeburg, Germany

*Listeria monocytogenes* is a facultative intracellular bacterium which may cause severe infections in immunocompromised patients, very young and old persons, and pregnant women. It is generally acquired by the ingestion of contaminated food. Although the way of infection is clear, the exact mechanisms leading to the efficient elimination of the pathogen are only partially understood. Here, we report that mice lacking the cytosolic adapter molecule Skap-hom are more sensitive to listeriosis. In particular, Skap-hom-deficient mice develop a reduced number of *Listeria*-specific CD8 T cells and cannot completely clear the pathogen. This seems to be related to the fact that skap-hom-deficient dendritic cells, the most potent antigen presenting cells (APC), show an altered proximal signalling following the encounter of the pathogen, at least *in vitro*. In fact, TLR signalling seems to be unaltered since stimulation of DC results in I $\kappa$ B degradation in both wild type and Skap-hom deficient DCs. However, downstream of integrins, Pyk2 and Fak are not activated in the absence of Skap-hom. These data suggest that both, TLR and integrin signalling on the APC, are needed for an efficient activation of the adaptive immune system and for an efficient clearing of the pathogen.

**PA11/21 MECHANISMS OF PERIPHERAL CD8+ T CELL TOLERANCE INDUCTION BY DENDRITIC CELLS**A. Schildknecht<sup>1</sup>, S. Brauer<sup>2</sup>, C. Brenner<sup>1</sup>, K. Lahl<sup>3</sup>, T. Sparwasser<sup>3,4</sup>, M. Van den Broek<sup>1</sup>, H.C. Probst<sup>2</sup><sup>1</sup>Institute of Experimental Immunology, University Hospital Zurich, Zurich, Switzerland, <sup>2</sup>Institute for Immunology, Universitätsmedizin Mainz, Mainz, Germany, <sup>3</sup>Institut für Medizinische Mikrobiologie, Immunologie und Hygiene, Technische Universität München, Munich, Germany, <sup>4</sup>Centre of Experimental and Clinical Infection Research, Institute for Infection Immunology, Twincore, Hanover, Germany

Peripheral T cell tolerance is thought to significantly contribute to the prevention of autoimmunity and it was shown that antigen-presenting steady state dendritic cells efficiently induce peripheral tolerance. Using a transgenic mouse model that allows inducible expression of viral CTL epitopes selectively on Dendritic cells we show that dendritic cell-induced tolerance is a T cell-intrinsic process and depends on the co-inhibitory molecules CTLA-4 and PD-1. We found that the induction of peripheral tolerance by steady state DC was severely impaired in the absence of regulatory T cells. Rather than inducing tolerance, antigen presentation by steady state DCs resulted in priming of a functional CTL response if FoxP3+ T cells were missing.

**PA11/22 MAST CELLS PROMOTE T<sub>H1</sub> IMMUNE RESPONSES BY MODULATION OF DENDRITIC CELL FUNCTION**A. Dudeck<sup>1,2</sup>, C. Suender<sup>1</sup>, E. von Stebut-Borschitz<sup>3</sup>, S. Heydrich<sup>1</sup>, M. Maurer<sup>1</sup><sup>1</sup>Charité-Universitätsmedizin Berlin, Department of Dermatology and Allergy, Allergie-Centrum-Charité, Berlin, Germany, <sup>2</sup>Technical University Dresden, Medical Faculty Carl-Gustav Carus, Institute of Immunology, Dresden, Germany, <sup>3</sup>University Hospital Mainz, Department of Dermatology, Mainz, Germany

**Objectives:** Mast cells (MCs) contribute critically to the T cell-dependent host defense in *Leishmania* major infections. However, the underlying mechanisms of this novel MC function remain to be elucidated in detail. Here, we asked whether MCs can influence the maturation and function of dendritic cells (DCs) required for the generation of effective T cell-mediated immune responses.

**Methods:** Murine peritoneal cultured MCs (PCMCs) undergo a direct cell-to-cell contact to immature murine bone marrow derived DCs (imDCs). Therefore, we assessed the DCs primed by crosstalk with MCs for their surface expression of costimulators, their capacity to induce CD4+ T cell proliferation and the DC activated CD4+ T cells for their cytokine release.

**Results:** We found that DC crosstalk with PCMCs induced the expression of the costimulatory signals CD80, CD86, and CD40 on the cell surface suggesting the induction of DC maturation. Furthermore, MC/DC interaction induced the release of IFN- $\gamma$  and increased the IL12p70 secretion of LPS-matured DCs. MC-primed DCs subsequently induced a strong proliferation of naïve CD4+ T cells. Surprisingly, we observed, that MC-primed DCs stimulated CD4+ T cells to release high IFN- $\gamma$  levels, but no IL-4 and IL-5, demonstrating a polarization to T<sub>H1</sub> response. In contrast, soluble MC mediators alone failed to induce DC maturation or modulate DC-mediated T-cell responses.

**Conclusion:** Our data demonstrate that the cellular crosstalk of immature DCs and mature MCs initiates the maturation of DCs and modulates their capacity to induce naïve CD4+ T cell proliferation and polarization towards T<sub>H1</sub>. Thus, MCs may contribute to adaptive immune responses by inducing the maturation of DCs cells and regulating their function.

**PA11/23 EVALUATION OF MYCOBACTERIUM LEPRAE HEAT-SHOCK PROTEIN 65 AS AN IMMUNE-MODULATING MOLECULE FOR DENDRITIC CELLS**P.R.M. Souza<sup>1</sup>, C.R. Zárate-Bladés<sup>1</sup>, R.R.S. Júnior<sup>1</sup>, W.M. Rios<sup>1</sup>, L.M. Massis<sup>1</sup>, I.T. Brandão<sup>1</sup>, A.P. Masson<sup>1</sup>, A.F. Gembre<sup>1</sup>, V.L.D. Bonato<sup>1</sup>, C.L. Silva<sup>1</sup><sup>1</sup>The Centre for Tuberculosis Research, School of Medicine of Ribeirão Preto – University of São Paulo, Department of Biochemistry and Immunology, Ribeirão Preto, Brazil

**Objective:** To establish if mycobacterial heat-shock protein 65 functions as an immune-modulating molecule for dendritic cells (DCs).

**Methods:** DCs were differentiated from bone marrow cells of C57BL/6 mice. On day 0, cells (1x10<sup>6</sup>/m) were plated in RPMI-complete medium containing 20 ng/ml murine GM-CSF and 10ng/ml murine IL-4. On day 3, an equal volume of RPMI-complete medium was added. On day 6, one-half of the volume of RPMI medium was replaced. The DCs were stimulated with recombinant Hsp65 (rHsp65) on day 8 in presence of polymixin-B. LPS was used as positive control. Cytokine production (IL-6, IL-10 and IL-12) was measured by ELISA. The expression of co-stimulatory molecules and the ability of DCs to stimulate splenocytes were also evaluated. The data were analyzed by ANOVA and P < 0.05 values were considered significant.

**Results:** DCs stimulated with 20 mg of rHsp65 produced pro-inflammatory cytokines IL-12 and IL-6, exhibit up-regulation of co-stimulatory molecules CD40 and CD86 and were able to stimulate the secretion of IFN- $\gamma$  by splenocytes. In contrast, DCs that were not stimulated with rHsp65 or that were incubated with LPS, displayed polarized results from no-cytokine production to maximal secretion of cytokines (IL-6 and IL-12), respectively. These same patterns were observed for co-stimulatory molecules CD40 and CD86. Finally, IL-10 production was detected only in LPS stimulated DCs.

**Conclusion:** These results show the ability of mycobacterial Hsp65 to directly stimulate DCs and the capacity of these cells to activate splenocytes to produce IFN- $\gamma$ . Considering that several reports showed the ability of *M. leprae* Hsp65 in the form of a genetic vaccine to prevent and treat infections as tuberculosis and paracoccidioidomycosis, and also to decrease immunopathologic lesions observed in these pathologies, the results presented here provide new evidences for the explanation of those immunotherapeutic effects.

**Financial support:** FAPESP, CNPq, CAPES.

**PA11/24 ROLE OF P50 NF-KB IN DENDRITIC CELLS FUNCTIONS**P. Larghi<sup>1</sup>, C. Porta<sup>2</sup>, A. Mancino<sup>3</sup>, A. Mantovani<sup>3,4</sup>, A. Sica<sup>2</sup><sup>1</sup>Fondazione Humanitas per la Ricerca, Immunology and Inflammation, Rozzano, Italy, <sup>2</sup>DISCAFF, Università del Piemonte Orientale 'A. Avogadro', Novara, Italy, <sup>3</sup>Istituto Clinico Humanitas, Rozzano, Italy, <sup>4</sup>University of Milan, Milano, Italy

Tumor growth is supported by tumor stroma, which is made by matrix and infiltrating cells, such as tumor associated macrophages (TAM) and tumor associated dendritic cells (TADC). We have recently reported that TAM display massive nuclear localization of the p50 NF- $\kappa$ B inhibitory homodimer, which correlates with impaired inflammatory functions. The functional significance of this observation was demonstrated in p50 NF- $\kappa$ B deficient mice, which displayed tumor growth inhibition. More recently, in order to evaluate whether this tolerogenic mechanisms may target other compartments of the immune system, we characterized the role of p50 NF- $\kappa$ B in dendritic cells (DC) functions, including their differentiation and maturation.

Our data clearly show that p50 NF- $\kappa$ B plays a non redundant role in DC survival and APC functions. p50 NF- $\kappa$ B has pro-apoptotic functions in bone marrow derived DC, as its absence leads to a reduced rate of apoptosis/necrosis in DC activated for 48h with LPS. Moreover, after 48h of LPS stimulation, p50<sup>-/-</sup> DC that are still alive (80% vs 40% of WT DC) display higher expression of MHC molecules, as well as higher secretion of pro-inflammatory cytokines such as IL-1b, TNF- $\alpha$  and IL-18. This correlates with the enhanced capability of p50<sup>-/-</sup> DC to activate T cell responses, *in vitro* and *in vivo*.

Therefore, our data suggest that targeting p50 NF- $\kappa$ B activity may represent a strategy to enhance selective functions of DC, with potential application in anti-tumor vaccination strategies.



**PA11/25 SPECIFIC ENDOCYTOSIS MECHANISMS OF HUMAN DENDRITIC CELLS ARE DIFFERENTLY AFFECTED BY SIALIC ACID REMOVAL**M. G. Cabral<sup>1</sup>, D. Ligeiro<sup>2</sup>, A. R. Piteira<sup>1</sup>, P. A. Videira<sup>1</sup><sup>1</sup>Faculdade de Ciências Médicas from Universidade Nova de Lisboa, Lisbon, Portugal, <sup>2</sup>Centro de Histocompatibilidade do Sul, Lisbon, Portugal

**Objectives:** Sialic acids feature prominently at terminal positions of many cell surface glycoconjugates. We demonstrated that human monocyte derived-Dendritic Cells (mo-DCs) are highly sialylated (1) and became particularly interested in studying the contribution of sialic acid for their endocytosis processes. Previously, we observed that this sugar removal from DCs surface decreases their ability to uptake antigens mainly through macropinocytosis (1). This specific work aimed to compare the effect of sialic acid lack in macropinocytosis and phagocytosis.

**Methods:** The influence of sialic acid in DC endocytosis was evaluated in mo-DCs (1) neuraminidase (enzyme that cleaves sialic acid) treated or untreated by flow cytometry and confocal microscopy, using FICT-conjugated ovalbumin (for macropinocytosis) and *E.coli* (for phagocytosis). The uptake of these tracers were also analysed after DCs maturation with LPS or TNF $\alpha$ .

**Results:** Concordantly with our previous results, in DCs lacking sialic acid, Ovalbumin internalization is 40% reduced (1). In contrast, *E.coli* internalization increases 15% with neuraminidase treatment. We have shown earlier that the exclusion of this sugar induces DCs maturation (2) and we associated this feature with the lesser capacity of DCs to uptake certain antigens. However, this justification does not explain the results herein obtained for phagocytosis. To examine the influence of other maturation stimulus in these mechanisms we conduct endocytosis assays after DC activation with LPS or TNF  $\alpha$  and we observed that neuraminidase treatment synergistically decreases the uptake of ovalbumin but surprisingly improves the uptake of *E.coli*.

**Conclusions:** Sialic acid cleavage influences negatively macropinocytosis but not phagocytosis. The retention of high phagocytic capacity by DCs neuraminidase treated, especially when pre-activated, suggests that sialic acid has different roles in particular endocytosis mechanisms. Removal of sialic acid differently affects macropinocytosis and phagocytosis signalling in DCs. Thus the activity of Rho GTPases, which are involved in the cytoskeletal reorganization required for these processes, is being investigated. These results will provide new insight into DC biology contributing to novel approaches designed to deliver antigens to DCs, regarding the development of DC based therapies.

(1) Videira et al, 2008, Glycoconj J 25:259-268

(2) Crespo et al, 2009, Immunol, in press IMM3047

**PA11/26 INCREASED ANTIGEN CROSS-PRESENTATION BUT IMPAIRED CROSS-PRIMING AFTER ACTIVATION OF PPARG IS MEDIATED BY UP-REGULATION OF B7H1**S. Hucke<sup>1</sup>, L. Klotz<sup>1</sup>, D. Thimm<sup>1</sup>, J. Schultze<sup>2</sup>, F. Edenhofer<sup>3</sup>, C. Kurts<sup>1</sup>, T. Klockgether<sup>4</sup>, A. Limmer<sup>1</sup>, P. Knolle<sup>1</sup>, S. Burgdorf<sup>1</sup><sup>1</sup>University of Bonn, Institute of Molecular Medicine and Experimental Immunology, Bonn, Germany, <sup>2</sup>University of Bonn, Institute for Life and Medical Sciences, Bonn, Germany, <sup>3</sup>University of Bonn, Life and Brain Center, Bonn, Germany, <sup>4</sup>University of Bonn, Department of Neurology, Bonn, Germany

Dendritic cells (DCs) are able to take up exogenous antigens and present antigen-derived peptides on MHC class I molecules, a process termed cross-presentation. The mannose receptor (MR), an endocytic receptor expressed on a variety of antigen-presenting cells, has been demonstrated to target soluble antigens exclusively towards cross-presentation. In this study, we investigated the role of the murine nuclear receptor peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ), a ligand-activated transcription factor with immunomodulatory properties, in MR-mediated endocytosis and cross-presentation of the model antigen ovalbumin (OVA). We could demonstrate both *in vitro* and *ex vivo* that activation of PPAR $\gamma$  resulted in increased MR expression, which in consequence led to enhanced MR-mediated endocytosis of soluble OVA and elevated cross-presentation of OVA-derived peptides. Concomitantly, activation of PPAR $\gamma$  in DCs induced up-regulation of the co-inhibitory molecule B7H1, which, despite enhanced cross-presentation, caused an impaired activation of OVA-specific CD8<sup>+</sup> T cells and the induction of T cell tolerance characterized by strongly reduced cytokine production as well as impaired cytotoxicity. These data provide a mechanistic basis for the immunomodulatory action of PPAR $\gamma$  which might open new possibilities in development of therapeutical approaches aimed at the control of excessive immune responses, e.g. in T cell-mediated autoimmunity.

**PA11/27 A NOVEL, COMPLEMENT-MEDIATED WAY TO ENHANCE THE INTERPLAY BETWEEN MACROPHAGES, DENDRITIC CELLS AND T LYMPHOCYTES**N. Sándor<sup>1</sup>, D. Pap<sup>1</sup>, J. Prechl<sup>1,2</sup>, A. Erdei<sup>1,2</sup>, Z. Bajtay<sup>1,2</sup><sup>1</sup>Eötvös Loránd University, Department of Immunology, Budapest, Hungary, <sup>2</sup>Research Group of the Hungarian Academy of Sciences, Budapest, Hungary

Complement is known to regulate adaptive responses in several ways. Here we investigated how component C3 influences the phenotype and functional activity of human dendritic cells. We found that monocyte-derived dendritic cells (MDCs) when incubated with native, hemolytically active C3, are able to bind the activation fragments of C3 two ways; i.e. in addition to the well-known interaction of iC3b through complement receptor type 3 (CR3) and 4 (CR4) the cells are also able to bind freshly generated, nascent C3b covalently via their cell surface acceptor sites. We demonstrate that covalent fixation of C3b directs MDCs to increase surface expression of MHCII and the costimulatory molecules CD83 and CD86, moreover it results in significantly enhanced secretion of TNF- $\alpha$ , IL-6 and IL-8. We also show that the important functional consequence of C3b-fixation is the much elevated capacity of DCs to stimulate allogeneic T cells. The distinct role of covalently fixed C3-fragments is strongly supported by our results obtained with MDCs where CD11b expression was downregulated by siRNA. We found that native C3 treated MDCs are as potent stimulators of T cell proliferation as LPS-matured MDCs even in the absence of CR3 expression. To reveal the possible *in vivo* significance of our findings, we modelled a phenomenon occurring during inflammation in various tissues, where C3 is produced locally by activated macrophages. In these cocultures MDCs were found to fix substantial amounts of C3-fragments on their cell membrane. Our data provide compelling evidence that antigen presenting cells arising in complement-sufficient environment mature to competent stimulators of T cells after fixing C3b covalently. This work was supported by OTKA Fund, Grant T63038 and T047151.

**PA11/28 SELECTIVE EXPRESSION OF THE CHEMOKINE RECEPTOR XCR1 ON CD8<sup>+</sup> DENDRITIC CELLS DETERMINES COOPERATION WITH CD8<sup>+</sup> T CELLS**X. Zhou<sup>1</sup>, A. Mora<sup>1</sup>, S. Güttler<sup>1</sup>, H.-W. Mages<sup>1</sup>, V. Henn<sup>1</sup>, R.A. Kroczeck<sup>1</sup><sup>1</sup>Robert Koch-Institute, P21 – Molecular Immunology, Berlin, Germany

ATAC (XCL1/lymphotactin/SCM-1) has previously been identified as a chemokine-like protein abundantly secreted by activated CD8<sup>+</sup> T cells and NK cells, but its function remained as elusive as the expression pattern of its receptor XCR1. With a new, highly specific PCR approach we now can clearly demonstrate that XCR1 mRNA is exclusively expressed in CD8<sup>+</sup> murine dendritic cells (DC), but not in populations previously reported as XCR1-positive (including B-, T- and NK cells). Our *in vitro* migration tests revealed a potent chemotactic capacity of ATAC for CD8<sup>+</sup> DC, but not for other DC subsets or lymphoid populations, and provided for the first time a definitive proof for the function of ATAC as a chemokine. To elucidate the *in vivo* function of ATAC and XCR1, we targeted antigen to CD8<sup>+</sup> DC by injection of an ovalbumin-coupled antibody against DEC-205 ( $\alpha$ DEC205:OVA) and analyzed the activation of adoptively transferred OVA-specific TCR-transgenic CD8<sup>+</sup> T cells (OT-I). OT-I cells abundantly secreted ATAC 8–36 hours after antigen recognition, which coincides with a period of intense T-DC interactions. During the first 3 days, OT-I wild type T cells responded to antigen in a similar fashion as ATAC-deficient OT-I T cells (OT-I ATAC-KO), although the kinetics of activation differed. At later time points, however, the size of the OT-I T cell population decreased substantially in the absence of ATAC and this was accompanied by a markedly diminished cytotoxicity *in vivo*. At the same time, the differentiation of OT-I T cells to IFN- $\gamma$  secreting effectors was diminished in the absence of ATAC. Taken together with the demonstrated chemotactic activity of ATAC these data indicate that ATAC very specifically enhances the physical contact between CD8<sup>+</sup> T cells and CD8<sup>+</sup> DC upon recognition of antigen. This enhanced interaction leads to an optimized co-stimulation of CD8<sup>+</sup> T cells and thus facilitates their expansion/ survival and differentiation to effector cells upon recognition of antigen.

**PA11/29 IMMUNE MODULATION BY SILENCING CD40 EXPRESSION IN DENDRITIC CELLS USING SMALL INTERFERENCE RNA**M. H. Karimi<sup>1</sup>, A. A. Pourfathollah<sup>2</sup>, A. S. G. Lotfi<sup>3</sup>, S. M. Moazzeni<sup>2</sup>, P. Ebadi<sup>4</sup><sup>1</sup>Shiraz University of Medical Sciences, Transplant Research Center, Shiraz, Iran, Islamic Republic of, <sup>2</sup>Tarbiat Modares University, Faculty of Medical Sciences, Immunology, Tehran, Iran, Islamic Republic of, <sup>3</sup>Tarbiat Modares University, Faculty of Medical Sciences, Biochemistry, Tehran, Iran, Islamic Republic of, <sup>4</sup>Faculty Member of Islamic Azad University, Kazeroun Branch, Biology, Kazeroun, Iran, Islamic Republic of

RNA interference is a mechanism of posttranscriptional gene silencing that functions in most eukaryotic cells, including human and mouse. Specific gene silencing is mediated by short strands of duplex RNA of ~21 nt in length (termed small interfering RNA or siRNA) that target the cognate mRNA sequence for degradation. We demonstrate here that RNAi can be used for immune modulation by targeting dendritic cell (DC) gene expression. Transfection of DC with siRNA specific for the CD40 gene resulted in potent suppression of gene expression and blockade of CD40 surface expression without affecting unrelated genes or cellular viability. Inhibition of CD40 expression was associated with increased IL-4 and decrease of IL-12 production, which endowed the DC with the ability to stimulate production of Th2 cytokines from allogeneic T cells *in vitro*.

**PA11/30 CAN DUSP9 EXPRESSION EXPLAIN DIFFERENCES IN CYTOKINE PRODUCTION BETWEEN PDC AND MDC?**M. Niedzińska<sup>1</sup>, K. Lahl<sup>2</sup>, H. Dietrich<sup>2</sup>, J. Mages<sup>2</sup>, M. Schiemann<sup>2</sup>, T. Sparwasser<sup>3</sup>, R. Lang<sup>1</sup><sup>1</sup>University Hospital Erlangen, Institute of Clinical Microbiology, Immunology and Hygiene, Erlangen, Germany, <sup>2</sup>Technical University Munich, Institute of Medical Microbiology, Immunology and Hygiene, Munich, Germany, <sup>3</sup>TWINCORE – Centre for Experimental and Clinical Infection Research, Hannover, Germany

Plasmacytoid dendritic cells are known to efficiently produce large amounts of type I interferon upon viral exposure. On the other hand, classical DCs effectively trigger immune responses due to processing and presentation of antigens as well as secretion of cytokines such as IL12. It is not fully understood why these two dendritic cell subsets respond so differently to stimulation through TLR9 by CpG ODN.

In search for a molecular basis underlying the distinct phenotype, transcriptome analysis of FACS-sorted splenic pDCs and mDC was performed. Among the 'pDC specific' genes we observed the MAPK phosphatase DUSP9 that targets ERK1/2 activation and is required for placenta development. To date no immunological function of DUSP9 has been described. We confirmed the constitutive, strong expression of DUSP9 in pDC generated *in vitro* by culture with Flt3L at the mRNA and protein level. This data focused our work on two aspects: why DUSP9 is exclusively and constitutively present in pDCs and what the function is in context of TLR9 signaling.

To investigate which factors may drive pDC-specific DUSP9 expression, *in silico* analysis of DUSP9 promoter with Genomatix software was performed and identified a large number of transcription factor binding sites. By 'intersecting' this data with information about transcription factor expression from the microarray data we found a limited number of candidate regulators of pDC-specific Dusp9 expression (e.g. IRF3, IRF7 and HoxA9). In terms of DUSP9 function, analysis of MAPK activation in sorted pDC and mDC revealed impaired phosphorylation of ERK1/2 in pDC upon CpG stimulation. It remains to be tested whether the observed inverse correlation between DUSP9 and ERK1/2 activation in different DC subsets is causally involved in the specification of cytokine and interferon responses to TLR9 stimulation.

**PA11/31 INHIBITION OF  $\alpha$ -1,2-MANNOSIDASE IMPAIRS DENDRITIC CELL MATURATION/MIGRATION AND PROLONGS CORNEAL ALLOGRAFT SURVIVAL**S. Schlickeiser<sup>1</sup>, S. Stanojlovic<sup>2</sup>, C. Appelt<sup>1</sup>, T. Ritter<sup>3</sup>, H.-D. Volk<sup>1</sup>, U. Pleyer<sup>4</sup>, B. Sawitzki<sup>1</sup><sup>1</sup>Charité – University Medicine Berlin, Institute of Medical Immunology, Berlin, Germany, <sup>2</sup>University Clinical Center, Belgrade, Institute of Ophthalmology, Belgrade, Serbia, <sup>3</sup>National University of Ireland, Galway, Regenerative Medicine Institute, Galway, Ireland, <sup>4</sup>Charité – University Medicine Berlin, Department of Ophthalmology, Berlin, Germany

**Objectives:** N-glycosylation is tightly controlled during both the differentiation and activation of leukocytes. It determines their ability to respond to extracellular stimuli and mediates cell-cell interactions. Indeed, we could also recently demonstrate, that the transcription of an enzyme, catalyzing essential steps during N-glycan modification, is regulated during rejection of allogeneic transplants. Here we investigated the role of  $\alpha$ -1,2-mannosidase for DC maturation, migration and *in vivo* function.

**Methods:** Glycophenotype of bone marrow-derived DCs was analyzed by lectin-staining using flow cytometry. Migration of specific  $\alpha$ -1,2-mannosidase inhibitor Kifunensine-treated TNF- $\alpha$ -matured DCs through an endothelial monolayer, as well as homing of CFSE labeled BALB/c DCs to lymph nodes of C57BL/6 mice was quantified. *In vivo* C57BL/6 mice received 2.0mm BALB/c corneal allografts. Either 4mg/(kg body weight) Kifunensine or PBS was administered i.p. at days -1, 2, 5, 8 and 11. Grafts were daily graded based on corneal clarity. Day 10 intra-graft mRNA expression of DC markers was analyzed by RT-PCR, as well as splenic allo-specific memory T-cell responses by IFN- $\gamma$  ELISPOT.

**Results:** DCs differentially regulated expression of  $\alpha$ -1,2-mannosidase mRNA and subsequently showed altered expression of PHA-reactive complex N-glycans upon maturation. Inhibition of complex N-glycosylation diminished maturation of DCs (MHC class II, CD86, CCR7 expression), thereby reducing transendothelial migration of DCs towards CCL19 *in vitro* and absolute number of lymph node homing of allogeneic DCs by 80%. *In vivo* Kifunensine-treatment significantly delayed corneal rejection (MST: PBS=11 $\pm$ 2d, n=9 vs. Kifunensine=18 $\pm$ 3d, n=7). Corneal CD11c and DC-SIGN expression was significantly increased in Kifunensine-treated mice compared to vehicle group, indicating a reduced migration of graft residing DCs. Consistently, numbers of IFN- $\gamma$ -producing allospecific memory T-cells were diminished.

**Conclusions:** Targeting modification of N-glycans demonstrates high immunoregulatory potential and might provide novel therapeutic options.

**PA11/32 ROLE OF HUMAN CD103<sup>+</sup> DCS IN THE GENERATION OF INTESTINAL IMMUNE RESPONSES**H.A. Uronen-Hansson<sup>1</sup>, E. Jaansson<sup>1</sup>, P.-L. Berg<sup>2</sup>, T. Davidsson<sup>3</sup>, B. Johansson-Lindbom<sup>1</sup>, W. Agace<sup>1</sup><sup>1</sup>Lund University, Medical and experimental sciences, Lund, Sweden, <sup>2</sup>University Hospital in Lund, Surgery, Lund, Sweden, <sup>3</sup>University Hospital in Lund, Urology, Lund, Sweden

The intestinal lamina propria (LP) is packed with a subepithelial network of DC, which drain via lymphatics to the mesenteric lymph nodes (MLN) where they present luminal antigen to naïve T cells. We have recently identified the integrin CD103 as a marker for LP DCs that have entered the MLN. Importantly, only CD103<sup>+</sup> DCs are capable of generating gut homing CCR9<sup>+</sup>a $\alpha$ b $\gamma$ <sup>+</sup> T cells. Here we have examined the localization and phenotype of CD103<sup>+</sup> DC in mice as well as in MLN, small intestine and colon in control subjects and in patients with inflammatory bowel diseases (Crohn's and colitis). The role of CD103<sup>+</sup> DCs in gut immune responses was also studied after LPS induced inflammation in mice.

Under steady state, murine CD103<sup>+</sup> DCs were located within the paracortex of the MLN close to the B cell follicles, while CD103<sup>+</sup> DCs were distributed throughout the T cell zone. Human MLN DCs expressed CD103 similarly to mice and also clustered near B cell follicles. CD103<sup>+</sup> and CD103<sup>+</sup> DC both in humans and mice expressed partially overlapping, and some distinct chemokine receptors and integrins as well as classical DC activation markers. During LPS induced inflammation the numbers of CD103<sup>+</sup> and CD103<sup>+</sup> DCs in the MLN increased 4 and 3 fold *in vivo*, respectively, and both subsets localized throughout the T cell zone. LPS induced maturation of both DC subsets *in vivo*, but only CD103<sup>+</sup> DC were capable of inducing CCR9 and a $\alpha$ b $\gamma$  on responding T cells.

A further understanding of the mechanisms underlying DC induced generation of tissue tropic T cells and the role of the tissue microenvironment in this process will provide important insights into the pathogenesis of T cell mediated diseases and to identify novel targets and strategies for the treatment of chronic inflammation in these specific sites.

**PA11/33 IDENTIFICATION OF A CHEMOTACTIC PATHWAY IN THE MOUSE CD8 $\alpha$ <sup>+</sup> DENDRITIC CELL SUBSET THAT IS PUTATIVELY CONSERVED IN HUMAN BDCA3 DENDRITIC CELLS**K. Crozat<sup>1,2,3</sup>, R. Guiton<sup>1,2,3</sup>, G. Bessou<sup>1,2,3</sup>, S.H. Robbins<sup>1,2,3</sup>, M. Dalod<sup>1,2,3</sup><sup>1</sup>Centre d'Immunologie Marseille-Luminy, Marseille, France, <sup>2</sup>INSERM – U631, Marseille, France, <sup>3</sup>CNRS UMR 6102, Marseille, France

Dendritic cells (DCs) comprise distinct subsets according to surface marker expression and functions. Plasmacytoid DCs are professional type I IFN producers upon viral infection, whereas conventional DCs comprising CD8 $\alpha$ <sup>+</sup> and CD11b<sup>+</sup> subsets, are mostly involved in regulating T cell responses. In mice, the control of mouse cytomegalovirus (MCMV) infection depends on the promotion of natural killer (NK) cell functions by DCs. Reciprocally, NK cell antiviral activity preserves the integrity of CD8 $\alpha$ <sup>+</sup> DCs, and allows a rapid mounting of CD8 T cell responses. The mechanisms for this cross-talk are still undetermined. To unravel the molecular bases for DC subset-specific functions, we performed gene expression profiling of mouse splenic and human blood DC subsets under steady-state condition (Robbins SH et al. 2008). We have extended this analysis to DC subsets isolated from MCMV-infected mice. We show here some results focused on the analysis of the transcriptome of CD8 $\alpha$ <sup>+</sup> DCs. We include functional studies for a candidate chemokine receptor potentially involved in the cross-talk with NK cells and in the promotion of the adaptive immune responses.

We performed 1) gene chips analysis on DC subsets sorted from MCMV-infected mice as compared to naïve controls, 2) real-time PCR to confirm the expression profile of the selected genes; 3) a comparison with the expression of their orthologs on human blood DC subsets, and 4) transwell assays to determine the migrating capacity of DC subsets in response to chemokines.

We have identified new genes selectively expressed in CD8 $\alpha$ <sup>+</sup> DCs under steady state conditions and/or after MCMV infection. The human orthologs of some of these genes were specifically expressed in BDCA3 DCs, which are most likely the human counterparts of mouse CD8 $\alpha$ <sup>+</sup> DCs. One of these genes encodes a chemokine receptor, which specific expression and activity on mouse CD8 $\alpha$ <sup>+</sup> DCs were confirmed by chemotactic assays *in vitro*.

We have identified a chemokine $\rightarrow$ receptor signaling axis in mouse CD8 $\alpha$ <sup>+</sup> DCs most likely conserved in human BDCA3 DCs. This pathway may contribute to the recruitment of these DC subsets to specific sites for promoting adaptive immune responses to intracellular pathogens or cancer.

**PA11/34 A NOVEL AP4A SECOND MESSENGER SIGNALING IN DENDRITIC CELL MATURATION, DIFFERENTIATION, AND FUNCTION**C.M. Yang<sup>1</sup>, E. Razin<sup>2</sup>, D.M. Kemeny<sup>1</sup><sup>1</sup>National University of Singapore, Immunology Programme, Singapore, Singapore, <sup>2</sup>Hebrew University-Hadassah Medical School, Department of Biochemistry, Jerusalem, Israel

It has recently been established that oligomerization of the IgE Fc gamma receptors on mast cells triggers a cascade signaling reaction in which the phosphorylation of Lysl tRNA synthetase induces its dissociation from the MSC (multi-synthetase complex) and subsequently produces Ap4A (Diadenosine tetraphosphate) that acts as a second messenger to effect the activation of the MITF family of transcription factors. Ap4A elicits a conformational change on Hint-1, a binding partner and repressor of MITF, thereby releasing MITF from an inactive state. MITF is thereafter able to influence gene expression as a DNA binding transcription factor. This is a novel signaling pathway first described in mast cells.

In this study we sought to investigate the presence and significance of the Ap4A second messenger signaling pathway in dendritic cells (DC). We studied a DC cell line (DC2.4) that expressed MITF and Ap4A hydrolase (an enzymic regulator of Ap4A which degrades Ap4A). DC maturation and activation were found to induce

Ap4A hydrolase mRNA 1.5-fold. A potential candidate for MITF gene regulation is TRACP5, which is predominantly expressed in osteoclasts and dendritic cells. Mouse bone marrow dendritic cells and the DC2.4 cell line were activated with a panel of Toll-like receptors ligands and specific cytokines IL-1 $\alpha$ , TNF- $\alpha$ , IFN- $\gamma$ . Stimulation with flagellin and IFN- $\gamma$  increased expression of Ap4A hydrolase increased two-fold. Nucleofection with Ap4A hydrolase specific siRNA effectively reduced the corresponding mRNA levels by 70%, with or without DC activation. Furthermore, a two-fold increase of TRACP5 mRNA levels was detected in activated Ap4A hydrolase specific siRNA treated DC2.4.

These data suggest that Ap4A may be an important second messenger signaling pathway in DC and that TRACP5 is a candidate for functional MITF gene regulation.

#### PA11/35 THE ROLE OF THE ARYL HYDROCARBON RECEPTOR IN SUPPRESSION OF CONTACT ALLERGY

S. Kadow<sup>1</sup>, B. Jux<sup>1</sup>, C. Esser<sup>1</sup>

<sup>1</sup>Institut für umweltmedizinische Forschung (IUF), Molecular Immunology, Düsseldorf, Germany

The aryl hydrocarbon receptor (AhR) is a transcription factor which responds to small chemicals (SC), and controls xenobiotic metabolizing enzymes. Hyperactivation by substance like 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), an environmental pollutant, results in strong systemic immunosuppression in rodents. The underlying mechanisms are not clear; presumably, several immune cell types and their functions are involved. We asked whether contact allergy – a relevant skin immune response – is AhR-dependent.

AhR<sup>d</sup> (responsive) C57BL/6 mice or AhR-deficient (AhR<sup>tm-bra</sup>) were injected with an immunosuppressive dose of 10 $\mu$ g TCDD/ kg body weight, and CHS response to the fluorescent contact sensitizer FITC was assessed in comparison to untreated/wild type mice. Ear thickness was measured 24 hours after challenge.

TCDD exposure resulted in significant suppression of ear thickness (50% reduction), compared to solvent injected control. Surprisingly, AhR<sup>-/-</sup> mice similarly displayed suppressed CHS as well. AhR<sup>-/-</sup> are impaired in LC maturation, and we asked whether inefficient CHS is due to a failure of Langerhans cells (LC) in the epidermis and dermal dendritic cells (dDC) in the dermis to carry antigen to the skin draining lymph nodes (sdLN). CHS was induced with FITC, and the FITC<sup>+</sup> cells were detected in sdLN 24h and 96h after induction of CHS.

In TCDD-treated mice, fewer LC and significantly fewer dDC reached the sdLN, which conceivably could result in reduced T cell activation. However, there was no difference between the numbers of FITC<sup>+</sup> LC and dDC in the sdLN of AhR<sup>-/-</sup> and wild-type mice. The number of CD4<sup>+</sup>CD69<sup>+</sup> and CD8<sup>+</sup>CD69<sup>+</sup> cells in sdLN increased after CHS, but did not differ between AhR<sup>-/-</sup> and wild-type mice, indicating that CHS immunosuppression was not a failure of basic T-cell activation. In conclusion, albeit AhR hyper-activation and AhR deficiency both result in diminished CHS, the underlying mechanisms are different, and warrant further investigation.

#### PA11/36 TRANSCRIPTOMIC PROFILE OF ESDCS DURING SALMONELLA TYPHIMURIUM INFECTION

R. Rossi<sup>1,2</sup>, C. Hale<sup>2</sup>, R. Andrew<sup>2</sup>, P. Fairchild<sup>3</sup>, D. Goulding<sup>2</sup>, G. Dougan<sup>2</sup>

<sup>1</sup>Pasteur Institute, Paris, France, <sup>2</sup>Sanger Institute, Hinxton, United Kingdom, <sup>3</sup>Dunn School of Pathology, Oxford, United Kingdom

Our interest in host-pathogen interactions led us to explore new avenues of modern research and their application to infectious diseases. In this abstract I present the results of a study looking at the interactions between embryonic stem (ES) derived dendritic cells (DCs) (esDCs) and Salmonella Typhimurium. Our previous work demonstrated that, during infection, ES cells cannot up- or down-regulate a number of genes consistently and therefore cannot build a reliable immune reaction to bacterial invasion (Yu et al., 2009). In contrast, once ES cells are differentiated into DCs the cell response to bacterial invasion is strong and fast. DCs are the most efficient antigen presenting cell (APC) present in the body involved in the fight against infections. Salmonella is a common bacterial cause of food-borne diseases like typhoid fever and gastroenteritis that can lead to death. The interaction between Salmonella and DCs is of key interest in the understanding of how the bacteria evade the immune response and how the immune system fails to defeat this pathogen. This work compared esDCs characteristics with those of bone marrow derived DCs and employed this new source of somatic cells to applied research by investigating esDCs-bacteria interactions utilizing confocal and electron microscopy and microarray expression profile.

This study revealed how ES cells grown in GM-CSF and IL-3 rich media developed strong antigen presenting cell-like qualities including the ability to process and present antigen to T cells, cytokine production and increased expression of myeloid surface markers. The mRNA expression profile microarray data, during Salmonella Typhimurium invasion, revealed interesting cellular pathways up- or down-regulated highlighting an enhanced immunogenic response and deregulation of basic metabolic processes together with other cellular events.

Yu, J., Rossi, R., Hale, C., Goulding, D. & Dougan, G. (2009). Interaction of enteric bacterial pathogens with murine embryonic stem cells. Infection and immunity 77, 585-597.

#### PA11/37 DISTINCT FUNCTIONAL RESPONSES OF RAT THYMIC AND SPLENIC DENDRITIC CELLS TO TLR-7 AND TLR-3 AGONISTS

J. Djokic<sup>1</sup>, S. Tomic<sup>1</sup>, B. Bozic<sup>1</sup>, S. Vasiljic<sup>1</sup>, M. Colic<sup>1</sup>

<sup>1</sup>Institute of Medical Research, Military Medical Academy, Belgrade, Serbia

**Objectives:** Different functional capacities of DC subtypes in initiation and regulation of the immune response are still being investigated and TLR agonists are recognized as powerful modulators of DCs, thus diverse strategies for their modulation are being developed. Considering differences of the thymus and spleen functions, we postulated that the resident DC subtypes in these organs have specialized functional capacities for responding to TLR ligation.

**Methods:** Rat splenic dendritic cells (SDCs) and thymic dendritic cells (TDCs) were isolated from AO strain and cultivated in the presence of agonists (25 $\mu$ M TLR-3 agonist poly(I:C), 500 $\mu$ M TLR-7 agonist loxoribine) for 3 days. The effects of TLR agonists on the DC phenotype were determined by flow cytometry and cytokine production was measured by ELISA. Allostimulatory and Th polarization capabilities were tested by using allogenic mixed leukocyte reaction (MLR) and by ELISA, respectively.

**Results:** Poly(I:C) up-regulated the expression of CD80, CD86 and ICAM-1 by TDCs. Loxoribine exerted a similar effect on SDCs. In contrast, poly(I:C) up-regulated CD86, but down-regulated CD80 and ICAM-1 by SDCs. Loxoribine showed similar influence on the CD80/CD86 expression by SDCs. Both TLR agonists up-regulated SIRP- $\alpha$  expression by TDCs, but did not change the CD11b<sup>+</sup>/CD11b<sup>-</sup> TDC ratio. Poly(I:C) and loxoribine had similar enhancing effect on the production of TNF- $\alpha$  and IL-12p70 by both DC types. In addition, loxoribine up-regulated IL-10 and down-regulated TGF- $\beta$  production by TDCs. Both TLR agonists inhibited the allostimulatory capability of both DC types and decreased their Th1 polarization activity, as judged by lower production of IFN- $\gamma$  by allogenic CD4<sup>+</sup> T cells. While loxoribine-treated SDCs augmented the production of IL-4, opposite results were obtained with poly(I:C)-treated SDCs.

**Conclusion:** Our results show that the modulatory effects of TLR-7 and TLR-3 agonists on phenotypic properties, cytokine production and Th1/Th2 polarizing capability of TDCs and SDCs were different. These results suggest that the lymphoid tissue-specific microenvironment could be an important factor determining the functional responses of DCs to TLR stimuli.

#### PA11/38 CYTOKINE PRODUCTION AND HELICASE EXPRESSION OF LEUKEMIC PLASMACYTOID DENDRITIC CELLS

Z. Magyarics<sup>1,2</sup>, A. Szabó<sup>1</sup>, K. Pázmándi<sup>1</sup>, L. Gopcsa<sup>1</sup>, A. Bácsi<sup>1</sup>, É. Rajnavölgyi<sup>1</sup>

<sup>1</sup>University of Debrecen, Medical and Health Science Centre, Institute of Immunology, Debrecen, Hungary, <sup>2</sup>Intercell AG, Serology & Immune Assays, Vienna, Austria, <sup>3</sup>Szent Istvan & Szent Laszlo Hospital of Budapest, Department of Haematology & Stem Cell Transplantation, Budapest, Hungary

**Objectives:** Plasmacytoid dendritic cell (pDC) leukemia is a rarely diagnosed malignancy with the enrichment of leukemic cells in peripheral blood and bone marrow. Based on the scarcity of circulating pDC of healthy subjects, leukemic pDC may represent a unique opportunity for studying the phenotypic and functional properties of this cell type. The aim of this study was to measure 1) the expression of intracellular RNA helicases with nucleic acid sensing capacity in leukemic pDC, 2) the response of these cells to different TLR ligands, and 3) the induced cytokine production of leukemic pDC.

**Methods:** pDC leukemia cells were isolated by cell sorting from cryopreserved peripheral blood and bone marrow samples of a male patient (Gopcsa et al. 2005). The levels of secreted IFN- $\alpha$ , TNF- $\alpha$  and IL-6 in culture supernatants were determined by ELISA at 24 hours after treatment with TLR-9 ligands type A (CpG2216) and type B (CpG2006), or the TLR-7 ligand imiquimod. The expression of IFNA1, IFNA2, IRF-7, RIG-I and MDA5 mRNA was measured by real time Q-PCR, and compared to that of normal pDC isolated from buffy coat of healthy volunteers by MACS.

**Results:** Treatment of leukemic cells with type B CpG oligonucleotide or imiquimod induced the production of TNF- $\alpha$  and IL-6. Type A CpG oligonucleotide stimulation resulted in increased IFNA1 expression as measured by Q-PCR, but the production of IFN- $\alpha$  was not detectable in the supernatant of leukemic cells, most likely because of the effect of cryopreservation. The amount of cytokines secreted by CpG-activated leukemic cells was lower than that of normal pDC. Interestingly, we observed RIG-I and MDA-5 induction after type A CpG-treatment of leukemic cells, whereas normal pDC responded to other TLR ligands such as imiquimod and both types of CpG.

**Conclusion:** Our results show that leukemic pDC share functional features with their normal counterpart. The induction of helicase expression after TLR9 ligation in leukemic cells raises the possibility that these cells acquire a sensitized state for intracellular nucleic acid detection through TLR-ligand activation. As pDC are non-responsive to helicase-ligands such as poly-I:C, further studies are needed to investigate the functional consequences of helicase induction.

#### PA11/39 HUMAN MYELIN MODULATES THE IMMUNE-FUNCTION OF DENDRITIC CELLS AND INFLUENCES T CELL PROLIFERATION

V. Gredler<sup>1</sup>, S. Ebner<sup>2</sup>, M. Forstner<sup>2</sup>, K. Schanda<sup>1</sup>, N. Romani<sup>2</sup>, M. Reindl<sup>1</sup>

<sup>1</sup>Innsbruck Medical University, Dept. of Neurology, Innsbruck, Austria, <sup>2</sup>Innsbruck Medical University, Dept. of Dermatology and Venereology, Innsbruck, Austria

**Objectives:** Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS). Dendritic cells (DC) are the most potent antigen presenting cells. In the CNS they reside in the choroid plexus, meninges and in cerebrospinal fluid. Recently it was shown that DC are recruited to MS



lesions were they mature and might contribute to the local activation and expansion of T cells. It has already been shown that uptake of myelin has an immunomodulatory effect on macrophage and microglial function, however, the effect of myelin on the activation and maturation of human DC has not been studied so far. Here we analyzed whether human myelin affects the maturation and activation of human monocyte-derived DC and describe their immunostimulatory capacity.

**Methods:** Cells were grown on myelin coated or control cell culture plates and stimulated with LPS or a defined maturation cocktail. Cell activation and differentiation was analyzed by the expression of cell surface markers (CCR7, CD1a, CD11b, CD14, CD83, CD86, HLA-DR and TLR4), myelin phagocytosis and the concentrations of cytokines and chemokines (IL-6, IL-10, IL-12p70, IL-23, MCP-1 and TGF- $\beta$ 1) in cell culture supernatants. Parts of the supernatants were used to stimulate purified T cells in the presence of an activating anti-CD3 antibody. The immunostimulatory capacity of DC was assessed by allogeneic mixed leukocyte reaction (MLR).

**Results:** Phagocytosis of human myelin resulted in a significantly diminished upregulation of CCR7 in mature DC. T cells incubated with supernatants of DC did not show any myelin-specific changes. In allogeneic MLR we could show that myelin-phagocytosis lead to a decreased proliferation of T cells.

**Conclusion:** Our results indicate an immunomodulatory effect of myelin on DC like a diminished upregulation of CCR7 while maturation or a reduced immunostimulatory capacity in allogeneic MLR.

#### PA11/40 LIMITED INNATE IMMUNE RESPONSE TO LCMV INFECTION IS ASSOCIATED WITH WEAK CD8 AND CD4 T CELL EXPANSION IN INFANT MICE

E. Belnoue<sup>1</sup>, P. Fontannaz-Bozzotti<sup>1</sup>, G. Sealy<sup>1</sup>, A.-F. Rochat<sup>1</sup>, P.-H. Lambert<sup>1</sup>, D. D. Pinschewer<sup>1</sup>, C.-A. Siegrist<sup>1</sup>

<sup>1</sup>University of Geneva, Pathology-Immunology, Geneva, Switzerland

Early life is characterized by an increased vulnerability to infectious agents, and in particular to viral pathogens. Cytopathic viruses such as influenza virus that induce acute infections in immunologically mature hosts, follow a protracted course in early life, characterized by higher viral loads and several weeks of viral replication. To identify the mechanisms responsible for this protracted pattern of infection, we developed an infant infection model in 2-week-old BALB/c mice using the well-characterized LCMV-WE strain. We previously showed that LCMV-specific CD8+ T cells were elicited in infant mice but failed to expand and rapidly control infection. Here, we show that LCMV infection induces a lower number of multifunctional CD4+ T cells in infant compared to adult mice. Adoptive transfer of naïve adult CD4+ T cells did neither restore LCMV-specific CD8+ T cell responses nor the course of infection in infant mice. This suggested that the early life innate responses were not sufficient for optimal activation/induction of CD4+/CD8+ T cells during LCMV infection. Indeed, LCMV infection of infant mice fails to elicit an adult-like increase/activation of plasmacytoid dendritic cells, resulting into lower production of type I interferon. Strategies to increase type I IFN production and restore effective CD8/CD4 T cell responses in infant mice are being assessed.

#### PA11/41 LUNG TISSUE STROMAL CELLS AFFECT CCL18 PRODUCTION BY DENDRITIC CELLS IN STEADY STATE AND RESPONSE TO MYCOBACTERIUM TUBERCULOSIS

A. T. Nguyen Hoang<sup>1</sup>, J. Juárez<sup>1</sup>, J. Fink<sup>1</sup>, S. Rahman<sup>1</sup>, S. A. Betemariam<sup>1</sup>, J. Andersson<sup>1</sup>, S. Brighenti<sup>1</sup>, M. Svensson<sup>1</sup>

<sup>1</sup>Karolinska Institutet, Medicine, Stockholm, Sweden

**Objectives:** Chemokines are small, secreted proteins that are key conductors of immune cell trafficking. The chemokine CCL18 is predominantly expressed in skin and lung tissue, mainly by immune cells including monocytes, macrophages and dendritic cells (DC). CCL18 is believed to mediate recruitment of naïve T cells and Th2 cells. Tuberculosis (TB) is a chronic pulmonary infectious disease and the protective immunity against TB is mediated by IFN- $\gamma$  producing Th1 cells. Although, several immunological processes have been identified in mycobacterial infection, the production of chemokines and regulation of immune cell trafficking in TB is poorly defined. To approach this, we performed studies of *Mycobacterium tuberculosis* (Mtb)-induced chemokine production by DC cultured with 3-dimensional (3D) lung tissue equivalents in combination with studies of chemokine responses and clinical outcome in patients.

**Methods:** The 3D-lung tissue model contained a stratified mucus-producing epithelial layer rested on a basement membrane with fibroblasts underneath. Stimulation of DC with irradiated Mtb was performed in co-cultures with 3D lung tissue equivalents. Real time QRT-PCR and ELISA was used to monitor CCL18 expression in DC. Real time QRT-PCR was also used to analyse mRNA expression of CCL18 and the cytokines IFN- $\gamma$  and IL-13 in lung tissue of Mtb infected patients (n=19) and control lung (n=5) from non-Mtb donors.

**Results:** We have found that lung stromal cells in the 3D-tissue model induce CCL18 expression in DC. In addition, stimulation of lung tissue equivalents and DC with Mtb induced an additional increase of CCL18 expression in DC. Interestingly, we observed that CCL18 expression is also increased in lung tissue of TB patients. Studying cytokine expression in lung tissue from TB patients we have found increased levels of IL-13 and unaltered levels of IFN- $\gamma$  compared to control samples.

**Conclusions:** The increased production of CCL18 by DC in response to *Mycobacterium tuberculosis* may contribute to exaggerated recruitment of IL-13 producing Th2 cells that undermine the efficacy of immunity and contribute to immunopathology in tuberculosis. Our 3D-lung tissue model will allow studies on human immune cell responses in living tissue and can be used to dissect the mechanism regulating CCL18 production in DC.

#### PA11/42 HISTONE DEACETYLASES INHIBITORS AFFECT THE PRO-ANGIOGENIC POTENTIAL OF ALTERNATIVELY ACTIVATED DENDRITIC CELLS

V. Salvi<sup>1</sup>, L. Salogni<sup>1</sup>, X. Vaira<sup>1</sup>, S. Sozzani<sup>1</sup>, D. Bosio<sup>1</sup>

<sup>1</sup>Università degli Studi di Brescia, Dept Biomedical Sciences and Biotechnologies, Brescia, Italy

The balance of histone acetylation, which is maintained by the opposite effects of histone acetyl transferases (HATs) and deacetylases (HDACs), has a crucial role in the regulation of DNA replication, repair, and transcription and is often altered in tumors. Histone deacetylases inhibitors (HDACi) are being successfully tested as antitumor agents in clinical trials, but the molecular mechanism of action remains elusive. Angiogenesis, the growth of new blood vessels, is a relevant process associated to many physiologic as well as pathologic conditions, including tumors. We have shown that alternatively activated dendritic cells (A-DC) display potent angiogenic activity *in vivo* and suggested that the pro-angiogenic action of tumor-infiltrating A-DC may favour tumor growth and dissemination.

Aim of this project is understanding whether HDACi alter the pro-angiogenic potential of A-DC. Low density array analysis shows that HDACi selectively reduce the mRNA of direct and indirect angiogenic factors such as VEGF-A, TNF- $\alpha$  and IL-6. Proteome profiler arrays as well as ELISA will be used to validate these results at the protein level. The physiological relevance of these findings is being evaluated *in vivo* by chicken chorioallantoic membrane (CAM) assay. In addition, we show that DC do migrate in response to VEGF-A. This migration is pertussis toxin and protein synthesis inhibitors sensitive and is blocked by HDACi. The involvement of VEGF-A receptors or second messenger down-modulation by HDACi will be addressed. Altogether, our data suggest that HDACi may decrease the pro-angiogenic potential of A-DC either directly through the reduction of secreted angiogenic molecules and indirectly by altering DC migratory properties.

#### PA11/43 SIGNS OF PDCS ACTIVATION DURING EARLY PRIMARY SIV INFECTION CORRELATE WITH THE VIRAL LOAD PEAK

T. Démoulin<sup>1</sup>, F. Martinon<sup>1</sup>, N. Bosquet<sup>1</sup>, I. Stanescu<sup>2</sup>, M. Ustav<sup>2</sup>, R. Le Grand<sup>1</sup>, B. Vaslin<sup>1</sup>

<sup>1</sup>DSV/IMETI/SIV, Fontenay-aux-Roses, France, <sup>2</sup>FIT Biotech Plc, Tampere, Finland

**Rational:** Plasmacytoid DCs are important for the initiation of both innate and adaptive immune responses to pathogens; they are major type I interferon producers in response to HIV and SIV, which may contribute to both the control of viral replication and regulation of T-lymphocyte clonal expansion.

**Objectives:** Although pDC numbers are increased in lymph nodes during primary infection with SIV, their activation levels and involvement in IFN-I production *in vivo* are not known. We investigated the dynamics of pDCs in relation with their level of activation and the subsequent implication on the initiation of immune response.

**Methods:** Macaques (12) were infected with SIVmac251. Six had been vaccinated previously against several SIV antigens. pDCs were counted longitudinally in the blood and their level of activation measured by flow cytometry (HLA-DR, CD80, CD86 and CCR7 expression). Their ability to produce type I IFNs *in vivo* was analyzed by intra-cellular staining and flow cytometry. Simultaneously, plasma viremia, CD4<sup>+</sup> T-cell counts and antigen specific responses were studied.

**Results:** Peripheral pDCs numbers rapidly increased (day 7) followed by a drastic collapse when viremia rose in plasma (nadir day 11), both of which were attenuated in vaccinated macaques showing one log reduction of peak plasma viral loads. In both groups, this dynamic was associated with significant increase of CD86 and CCR7 during acute viremia. In contrast, HLA-DR was down regulated. In parallel we evidenced a decrease of CD123 expression, also observed *in vitro* after TLR7-8 stimulation of these cells. These data suggest early activation of pDCs during primary infection reminiscent of their increase lymphoid tissues. All studied activation markers exhibited a return to normal levels by day 21.

**Conclusion:** These data demonstrate early activation of pDCs during primary infection with SIV in macaques for the first time and suggest that the initial changes in peripheral pDCs correlate with the intensity of viremia during primary infection but not with anamnestic response to vaccine. This activation is reminiscent of the homing of these cells in lymph nodes observed in a previous study, suggesting a central role for pDCs in the initiation and regulation of SIV specific immune responses.

**PA11/44 THE ROLE OF I $\kappa$ B KINASE (IKK)  $\alpha$  IN THE CROSS-TALK BETWEEN INNATE AND ADAPTIVE IMMUNITY**A. Mancino<sup>1</sup>, E. Johnson<sup>2,3</sup>, M.P. Seed<sup>2</sup>, T. Lawrence<sup>1</sup><sup>1</sup>Institute of Cancer, Bart's and The London School of Medicine and Dentistry, Centre for Cancer & Inflammation, London, United Kingdom, <sup>2</sup>William Harvey Research Institute, Bart's and The London School of Medicine and Dentistry, London, United Kingdom, <sup>3</sup>Imperial College London, South Kensington Campus, London, United Kingdom

The nuclear factor- $\kappa$ B (NF- $\kappa$ B) pathway plays a pivotal role in inflammation and immunity. Two separate pathways leading to NF- $\kappa$ B activation has been described: the canonical pathway is regulated by I $\kappa$ B kinase (IKK)  $\beta$  and leads to activation of RelA/p50 through phosphorylation and degradation of I $\kappa$ B $\alpha$ ; the 'alternative' pathway for NF- $\kappa$ B activation is mediated by IKK $\alpha$  and results in activation of RelB/p52 through the phosphorylation and proteolysis of p100 (NF- $\kappa$ B2). The canonical NF- $\kappa$ B pathway is essential for activation of innate immunity and for maintaining survival of immune cells, whereas the alternative pathway is required for lymphoid organogenesis and B cell function, suggesting an important role in adaptive immunity. We have used transgenic mice that express a mutant form of IKK $\alpha$  (IKK $\alpha^{\Delta 4}$ ), which cannot be activated by the upstream kinase NIK, to study the role of IKK $\alpha$  in cell-mediated immunity. Here we show that IKK $\alpha$  activity is required for priming antigen-specific inflammation *in vivo* and antigen-specific IFN $\gamma$  production by T cells *ex vivo*. However IKK $\alpha$  is not required for T cell proliferation in response to TCR stimulation but is required for TLR-mediated activation of dendritic cells, in particular for IL-12 production. Therefore our data suggest IKK $\alpha$  activity may exert an important role in cell-mediated immunity especially in priming IFN $\gamma$  dependent Th1 immune responses.

**PA11/45 LANGERHANS-LIKE DCS LOADED WITH COMPLEMENT-OPSONISED HIV EFFICIENTLY INDUCE EXPANSION OF NAIVE CD8<sup>+</sup> T CELLS**W. Posch<sup>1</sup>, M.P. Dierich<sup>1</sup>, D. Wilflingseder<sup>1</sup><sup>1</sup>Innsbruck Medical University, Department of Hygiene, Microbiology and Social Medicine, Innsbruck, Austria

In our study, we compared the commonly used IL-4 dendritic cells (DCs) with the recently described Langerhans-like IL-15 DCs exposed to differentially opsonised HIV. *In vitro* experiments in our laboratory revealed differences with respect to productive infection of immature IL-4-generated dendritic cells (iDCs) with differentially opsonised HIV (Wilflingseder et al, 2007). Furthermore, the antigen-presenting capacity of these cells differed dependent on the opsonisation pattern of the virus (Wilflingseder et al, submitted). Since IL-15 DCs were recently demonstrated to efficiently prime naive CD8<sup>+</sup> T cells to differentiate into melanoma-specific CTLs (Dubsky et al, 2007), we tested if IL-15 DCs loaded with differentially opsonised HIV are more potent in stimulating CD8<sup>+</sup> T cell expansion.

In preliminary experiments, we characterised IL-15 and IL-4 DCs after exposure to LPS or differentially opsonised HIV, for DC markers (e.g. C-type lectins, CD83, HLA-ABC, HLA-DR), complement receptors (CR3, CR4) and Fc $\gamma$ Rs (CD16, CD32, CD64) by FACS analyses or RT-PCR. Subsequently, IL-4 and IL-15 DCs were infected with differentially opsonised R5-, R5X4- and X4-tropic HIV preparations and virus production was monitored over several days after infection by p24-ELISA. Finally, we investigated whether HIV-loaded Langerhans-like DCs are more potent in initiating expansion of autologous naive CD8<sup>+</sup> T cells compared to HIV-IL-4 DCs.

We found that similar to IL-4 DCs, all HIV preparations activated IL-15 DCs with regard to characteristic maturation markers. As observed with IL-4 DCs, infection of the Langerhans-like cells was enhanced when HIV was coated with complement fragments compared to non-opsonised virus (HIV) or HIV opsonised with specific IgGs (HIV-Ig). However, IL15-DCs exposed to HIV-C were more efficient in inducing proliferation of naive CD8<sup>+</sup> T cells than IL4-DCs loaded with HIV-C. Whilst functionality (IFN- $\gamma$  secretion, expression of perforin and Granzyme B, killing of target cells) remains to be determined, our data indicate that HIV-loaded IL15 DCs are more potent in priming CD8<sup>+</sup> T cells than IL4 DCs.

**PA11/46 SIGNALING THROUGH TLR-7 STIMULATES TH-17 POLARIZATION CAPABILITY OF HUMAN MONOCYTE-DERIVED DENDRITIC CELLS**T. Dzopalic<sup>1</sup>, A. Dragicevic<sup>1</sup>, S. Vasiljic<sup>1</sup>, I. Majstorovic<sup>1</sup>, G. Vukovic<sup>2</sup>, D. Vucevic<sup>1</sup>, P. Uskokovic<sup>2</sup>, M. Colic<sup>1</sup><sup>1</sup>Military Medical Academy, Institute for Medical Research, Belgrade, Serbia, <sup>2</sup>University of Belgrade, Faculty of Technology and Metallurgy, Belgrade, Serbia

**Objectives:** It is believed that intracellular Toll-like receptor 7 (TLR-7) is not expressed by human monocyte-derived dendritic cells (Mo-DCs). However, recent data showed that its expression could be induced by activation stimuli, such as IFN- $\beta$ . Based on our previous preliminary results, we postulated that high concentrations of TLR-7 specific agonists could activate human Mo-DCs and trigger their maturation.

**Methods:** Mo-DCs were obtained by cultivation of human Mo for 6 days with GM-CSF and IL-4. Such prepared Mo-DCs were treated for additional 2 days with loxoribine or immunosine, two specific TLR-7 agonists, both at concentrations of 250  $\mu$ M. Mo-DCs were also treated with multi-wall carbon nanotubes (CNTs), to which immunosine was covalently bound. Control Mo-DCs were treated with control oxidized multi-wall CNTs. Phenotype of the cells was determined by flow cytometry, cytokine production was assayed by ELISA, whereas the allostimulatory capability was tested using an allogeneic MLR assay.

**Results:** We showed that both TLR-7 agonists stimulate differentiation and maturation of Mo-DCs as judged by up-regulation of CD40, CD54, CD80, CD83 and CCR7, and higher proliferation of allogeneic CD4<sup>+</sup>T cells in MLR. Mo-DCs stimulated with TLR-7 agonists produced only basal levels of IL-12 and moderate levels of IL-23, IL-27 and IL-10. Allogeneic CD4<sup>+</sup>T cells in coculture with Mo-DC activated with TLR-7 agonists secreted significantly higher levels of IL-17 compared to control Mo-DC/CD4 cultures, whereas no difference in Th1/Th2 cytokine production was observed. Similar phenotypical and functional capability of Mo-DCs occurred in the presence of 10 times lower concentrations of immunosine bound to CNT particles which were efficiently endocytosed by Mo-DCs.

**Conclusion:** Our results suggest that TLR-7 signaling pathway favours Th17 immune response and that carbon nanoparticles could be efficiently functionalized for intracellular delivery of TLR-7 agonists.

**PA11/47 YEAST BETA-GLUCAN MEDIATED MODULATION OF TLR-INDUCED ACTIVATION OF DENDRITIC CELLS: A FUNGAL ESCAPE MECHANISM?**S. Brix<sup>1</sup>, H. Frøkiær<sup>2</sup><sup>1</sup>Technical University of Denmark, Department of Systems Biology, Kgs. Lyngby, Denmark, <sup>2</sup>University of Copenhagen, Faculty of Life Sciences, Frederiksberg, Denmark

Dendritic cells (DC) provide an immediate response to microbial-derived structures by binding through specific pattern recognition receptors such as the toll-like receptors (TLRs). TLR-mediated recognition by DC is a key element for appropriate activation of adaptive immune responses, and modification of TLR-induced responses may result in reduced ability to clear of pathogenic microorganisms. The impact on DC of concurrent presence of TLR ligands and response modifiers in pathogenic microbes is yet incompletely defined. The present objective was to study the specific immune regulation in DC by yeast beta-glucan when present alone or in conjunction with TLR-ligands.

While blocking specific surface receptors (dectin-1, CD11b, DCAL-2) or kinase activities, we tested the effect of beta-glucan on DC's ability to produce cytokines, to up-regulate surface marker expression, and to activate naive CD4<sup>+</sup> T cell subsets (Th1, Th17, Th2, and Treg).

Yeast beta-glucan was found to suppress TLR-induced IL-12p70 and IL-23, while synergistically enhancing IL-10 production from DC. Beta-glucan *per se* induced up-regulation of CD40, CD80, CD86 and MHC class II on DC, but in presence of TLR-ligands, the display of CD40 was down-regulated, whereas CD86 was increased. Activation of naive CD4<sup>+</sup> T cell subsets was significantly reduced by yeast beta-glucan in presence of TLR-ligands. While the dectin-1 receptor was found to be partly involved in synergistical induction of IL-10 and IL-2 in DC by yeast beta-glucan in presence of TLR-ligands, the TLR-modulating effect of yeast beta-glucan was independent of CD11b and DCAL-2 receptors. The regulatory activity was also independent of MAPK (ERK1/2, JNK, p38), Akt, Raf-1 as well as Syk activation.

Collectively, we here demonstrate that yeast beta-glucan modulates the TLR-induced immune response in DC by yet unrecognised receptors and signal modifiers leading to weak IL-12, IL-23, and CD40 expressing cells with reduced capacity to activate naive CD4<sup>+</sup> T cells. These data indicate that fungal-derived sugar moieties, such as yeast beta-glucans, induce a non-effective phenotype in DC that may reduce the potency of host immune responses.

**PA11/48 A CONVENTIONAL SKIN LYMPH OVINE DC SUBSET SHARES FUNCTIONAL AND TRANSCRIPTOMIC PROPERTIES WITH THE CROSS-PRIMING CD8 $\alpha$  MURINE DC**V. Contreras<sup>1</sup>, C. Urien<sup>1</sup>, M. Bonneau<sup>1</sup>, L. Jouneau<sup>1</sup>, N. Bertho<sup>1</sup>, A. Savina<sup>2</sup>, S. Amigorena<sup>2</sup>, M. Dalod<sup>3</sup>, I. Schwartz-Cornil<sup>1</sup><sup>1</sup>INRA, Jouy en Josas, France, <sup>2</sup>Institut Curie-INSERM, Paris, France, <sup>3</sup>CIML, Cnrs UMR6102, Inserm U631, Univ UMR 6546, Marseille, France

Dendritic cell subsets differentially direct specific types of immune responses. In the mouse, the conventional CD8 $\alpha$  DC subset, that resides in lymphoid organs, presents the exquisite capacity to stimulate a Th1 response and to induce antigen specific CD8 T cells after capture of exogenous antigens and/or apoptotic bodies, a property called "cross-priming". Several innovative and highly successful vaccine strategies that specifically target the CD8 $\alpha$  murine DC have been developed to elicit anti-viral and anti-tumoral CD8 T cells. In other species, the CD8 $\alpha$  DC equivalent is a matter of intense research. Interestingly, in sheep, cattle, pig and rat afferent lymph, a CD172- DC subset that migrates as a minor subset in lymph at steady state has been found to transport apoptotic bodies. Based on this observation, we used the sheep model, in which large amount of afferent lymph can be collected for months, to evaluate whether the CD172- DC subset could be a functional equivalent of the murine CD8 $\alpha$  DC. We found that sheep lymph CD172- DC are more prone than the CD172+ DC to capture exogenous apoptotic bodies after *in vivo* injection. The CD172- DC subset had a higher propensity to activate a Th1 response in CD4 allogeneic naive T cells as compared to the CD172+ DC subset that rather oriented a Th2 response. After ovalbumin (OVA) endocytosis, the CD172- DC subset was more capable to cross-prime OVA-specific CD8 T cell than the CD172+ DC. This enhanced cross-priming property was associated with a lower capacity for rapid endosomal acidification. Several key proteins in the CD8 $\alpha$  DC biology were found over-expressed by the CD172- DC, such as surface DEC-205 and secreted IL-12p40. Finally, gene expression profiling using the ovine Agilent

microarrays, demonstrated that, as compared to their CD172+ counterparts, ovine CD172- DC expressed to higher levels genes that are either more strongly expressed in, or known to play a major role in the biology of, mouse CD8 $\alpha$  DC, as compared to their CD11b counterparts, including CADM1, IRF8, BatF3 and Id2. Several of these genes encode for cell surface molecules that could be targeted by vaccination.

**PA11/49 SACCHAROMYCES BOULARDII INHIBITS LIPOPOLYSACCHARIDE INDUCED ACTIVATION OF PRIMARY HUMAN DENDRITIC CELLS AND T-CELL PROLIFERATION**

S. Thomas<sup>1,2</sup>, I. Przesdzin<sup>1</sup>, D. Metzke<sup>1</sup>, J. Schmitz<sup>2</sup>, A. Radbruch<sup>2</sup>, D.C. Baumgart<sup>1</sup>

<sup>1</sup>Charité Campus Virchow Klinikum, Medizinische Klinik mit Schwerpunkt Hepatologie und Gastroenterologie, Berlin, Germany, <sup>2</sup>Deutsches Rheumaforschungszentrum, Berlin, Germany

*Saccharomyces boulardii* (Sb) is a probiotic yeast preparation that has demonstrated efficacy in inflammatory and infectious disorders of the gastrointestinal tract in controlled clinical trials. Although patients obviously benefit from treatment with Sb, little is known on how Sb unfolds its anti-inflammatory properties in humans. Dendritic cells (DC) balance tolerance and immunity and are critically involved in the control of T-cell activation. Thus, they are believed to have a pivotal role in the initiation and perpetuation of chronic inflammatory disorders not only in the gut. We therefore decided to investigate if Sb modulates dendritic cell function. Culture of primary (native, non-monocyte derived) human myeloid CD1c+CD11c+CD123- DC (mDC) in the presence of Sb culture supernatant (active component molecular weight < 3kDa as assessed by membrane partition chromatography) significantly reduced expression of the co-stimulatory molecules CD40 and CD80 ( $p < 0.01$ ) and the DC mobilization marker CC-chemokine receptor (CCR7) [CD197] ( $p < 0.001$ ) induced by the prototypical microbial antigen lipopolysaccharide (LPS). Moreover, secretion key pro-inflammatory cytokines like TNF- $\alpha$  and IL-6 were notably reduced, while the secretion of anti-inflammatory IL-10 did increase. Finally Sb supernatant inhibited the proliferation of naïve T-cells in a mixed lymphocyte reaction (MLR) with mDC. In summary, our data suggests that Sb may exhibit part of its anti-inflammatory potential through modulation of dendritic cell phenotype, function and migration by inhibition of their immune response to bacterial microbial surrogate antigens such as LPS.

**PA11/50 NATURALLY OCCURRING SHORT SPLICE VARIANT OF CYLD POSITIVELY REGULATES DENDRITIC CELL FUNCTION**

J. Masri<sup>1</sup>, C.C. Srokowski<sup>2</sup>, N. Hövelmeyer<sup>1</sup>, A.K. Krembel<sup>2</sup>, C. Tertilt<sup>2</sup>, D. Strand<sup>1</sup>, K. Mahnke<sup>3</sup>, R. Massoumi<sup>4</sup>, H. Schild<sup>2</sup>, A. Waisman<sup>1</sup>

<sup>1</sup>Universitätsmedizin der Johannes Gutenberg-Universität, I. Med. Klinik und Poliklinik, Mainz, Germany, <sup>2</sup>Universitätsmedizin der Johannes Gutenberg-Universität, Institut für Immunologie, Mainz, Germany, <sup>3</sup>Universität Heidelberg, Universitäts-Hautklinik Heidelberg, Heidelberg, Germany, <sup>4</sup>Lund University, Department of Laboratory Medicine, Clinical Research Center, Malmö, Sweden

**Objectives:** CYLD is a tumor suppressor gene and codes for a deubiquitinase which removes lysine-63 polyubiquitin chains from distinct members of the NF- $\kappa$ B pathway. Mutations in CYLD lead to dysregulation of NF- $\kappa$ B signaling. We have recently identified a short splice variant of this protein and investigated the impact of its sole expression in the absence of the full length protein on dendritic cell (DC) function.

**Methods:** Using bone marrow derived dendritic cells from CYLD<sup>ex7/8</sup> mice, which lack the full-length CYLD (fCYLD) transcript and overexpress the short splice variant (sCYLD) and CYLD complete knock-out mice, we analysed DC function by performing FACS analysis and immune tolerance experiments. Moreover, we assessed the consequences of sCYLD overexpression on NF- $\kappa$ B signaling by western blotting, electromobility shift assay, real-time PCR and NF- $\kappa$ B luciferase assay.

**Results:** Bone marrow derived DCs from CYLD<sup>ex7/8</sup> mice display a hyperactive phenotype *in vitro* and *in vivo* and have a defect in establishing tolerance using DEC-205-mediated antigen targeting to resting DCs. The combination of sCYLD overexpression and lack of fCYLD in CYLD<sup>ex7/8</sup> DCs leads to enhanced NF- $\kappa$ B activity accompanied by an increased nuclear translocation of the I $\kappa$ B molecule Bcl-3, along with nuclear p50 and p65.

**Conclusion:** We propose a model where the exclusive overexpression of sCYLD with high nuclear levels of Bcl-3 in BMDCs is accompanied by an increased NF- $\kappa$ B activation resulting in a hyperactive phenotype. It seems that in contrast to fCYLD, sCYLD is a positive regulator of NF- $\kappa$ B activity and its overexpression induces a hyperactive phenotype in DCs.

**PA11/51 SUPPRESSION OF PHOSPHOINOSITIDE 3-KINASE SIGNALING BY CIGARETTE SMOKE ATTENUATES RELEASE OF IFN- $\alpha$  BY HUMAN PLASMACYTOID DENDRITIC CELLS**

E. Mortaz<sup>1</sup>, M. Ezzati Givi<sup>1</sup>, F.P. Nijkamp<sup>1</sup>, G. Folkerts<sup>2</sup>

<sup>1</sup>Section Pharmacology and Pathophysiology, Department of Pharmaceutical Sciences, Utrecht, Netherlands, <sup>2</sup>Section Pharmacology and Pathophysiology, Department of Pharmaceutical Sciences, Faculty of Sciences, Utrecht University, Utrecht, Netherlands

Myeloid and plasmacytoid dendritic cells (mDCs, pDC) are crucial immune cells detecting microorganisms and linking innate and adaptive immunity. mDC are antigen presenting cells and pDC are intermediate cells. They produce large amounts of IFN- $\alpha$  after stimulation with CpG motifs and are also antigen presenting cells. The antiviral effect exerted by IFN- $\alpha$  is due to the induction of IFN response genes. Chronic airway inflammation is a cardinal feature of chronic obstructive pulmonary disease (COPD), a destructive cigarette smoke-induced lung disease. COPD patients are more susceptible to viral infections. Previously, we have demonstrated that exposure of mDC to cigarette smoke extract (CSE) lead to release of chemokines from these cells. Not much is known about the number and role of pDC in smokers with COPD. In this study, we addressed several key questions with respect to the mechanism of action of CSE on human pDC in an *in vitro* model. Human pDCs were isolated from normal healthy volunteers and subjected to fresh CSE. The release of protein and expression of mRNA of IFN- $\alpha$  were studied by ELISA and real time PCR methods, respectively. We observed that CSE suppressed release of IFN- $\alpha$ . Moreover, CSE suppressed PI3K/Akt signalling in pDC. Thus, our data indicate that cigarette smoke by suppression of releases of IFN- $\alpha$  production may play role in diminishing anti-viral immunity.

**PA11/52 DISTINCT EFFECT OF CD40 AND TNF-SIGNALING ON THE CHEMOKINE/ CHEMOKINE RECEPTOR EXPRESSION AND FUNCTION OF THE HUMAN MONOCYTE-DERIVED DENDRITIC CELLS**

J. Dai<sup>1</sup>

<sup>1</sup>Suzhou University, Medical Biotechnology Institute, Suzhou, China

A key and limiting step in the process of human monocyte-derived dendritic cells (mDCs) for clinical use is their *in vitro* maturation and *in vivo* migration. We previously observed that CD40 signal facilitated human mDC growth and maturation. To further explore this process, mDCs generated with GM-CSF and IL-4 were co-cultured with apoptotic tumor cells for 24 hours, followed by incubating with anti-CD40 monoclonal antibody or TNF- $\alpha$  for 48 hours to generate mature DCs. The chemokine/chemokine receptor expression and functions of mature DCs upon various stimuli were determined. The expression of costimulatory molecules on apoptotic tumor cell-loaded mature DCs co-cultured with either anti-CD40 antibody (anti-CD40-DCs) or TNF- $\alpha$  (TNF-DCs) were up-regulated compared to immature DCs, consistent with the abilities of these cytokine to drive DC maturation *in vitro*. The mRNA levels of chemokines such as stromal cell-derived factor-1 $\alpha$  (SDF-1 $\alpha$ ), EBV-induced molecule 1 ligand chemokine (ELC), and IFN inducible protein-10 (IP-10) in anti-CD40 activated DCs were increased and the dendritic cell-specific chemokine 1 (DC-CK1) was moderately up-regulated as compared with other mature DCs.

The corresponding chemokine receptors CXCR4 and CCR7 of anti-CD40-DCs were significantly expressed. The CXCR3 expression on activated T cells stimulated by anti-CD40-DCs was also increased. Moreover, the anti-CD40-DCs had a stronger ability to stimulate T cell proliferation than any other DCs. The NF- $\kappa$ B activity was much higher in anti-CD40-DCs than that of TNF-DCs. These results offer further evidence of the importance of the CD40 signal in developing efficient human DC vaccines for cancer immune therapy.

Cellular & Molecular Immunology. 2008;5(2):121-131.

**PA11/53 COORDINATED REGULATION OF PLASMACYTOID DENDRITIC CELL SURFACE RECEPTORS UPON STIMULATION WITH HERPES SIMPLEX VIRUS TYPE 1**

P. Schuster<sup>1</sup>, N. Donhauser<sup>1</sup>, S. Haupt<sup>1</sup>, N.A. Kittan<sup>1,2</sup>, K. Korn<sup>1</sup>, B. Schmidt<sup>1</sup>

<sup>1</sup>Institute of Clinical and Molecular Virology, University of Erlangen-Nürnberg, German National Reference Centre for Retroviruses, Erlangen, Germany, <sup>2</sup>University Hospital Regensburg, Internal Medicine, Regensburg, Germany

**Objectives:** Human plasmacytoid dendritic cells (PDC) are crucial for innate and adaptive immune responses against viral infections, mainly through production of type I interferons. Recently, evidence is accumulating that direct cell to cell contact plays an important role in this process. However, the effects of single PDC surface receptors in these interactions are poorly investigated. Therefore the aim of our study was to characterize the PDC phenotype in more detail, in order to provide the basis for further functional studies.

**Methods:** A chip-based expression analysis of surface receptors was combined with respective flow cytometry data obtained from fresh PDC, PDC cultivated in the presence of interleukin (IL)-3 or additionally exposed to UV-inactivated and infectious herpes simplex virus type 1 (HSV-1). Statistics was performed using paired or unpaired t-tests on logarithmically transformed mean fluorescence values corrected for multiple comparisons. The viral replication capacity on PDC was evaluated by HSV-1 real-time PCR and experiments involving autofluorescing HSV-1 particles.

**Results:** We were able to detect the expression of 52 receptors. Several receptors including CD81, CD229, CD305, and CD319 were newly identified on the surface of PDC. A total of 16 and 22 receptors were found to be significantly regulated upon IL-3 or HSV-1 exposure, respectively. These were receptors involved in chemotaxis, antigen uptake, activation and maturation, migration, apoptosis, cytotoxicity, and costimulation. UV-inactivated and infectious HSV-1 did not differentially affect surface receptor regulation, consistent with the lack of productive virus infection in PDC.

**Conclusion:** Our data provide evidence that the recognition of HSV-1 results in a coordinated regulation of PDC surface markers, which play a specific role in different aspects of PDC function such as attraction to inflamed tissue, antigen recognition, and subsequent migration to secondary lymphatic tissue. This knowledge will further be used to characterize PDC surface receptor functions in interactions with other cells of the innate and adaptive immune system, in particular natural killer cells and cytotoxic T-lymphocytes.



**PA11/54 REGULATION OF INTERLEUKIN-12 AND INTERLEUKIN-23 PRODUCTION IN MOUSE SPLENIC AND BONE-MARROW DERIVED DENDRITIC CELLS**P.Y. Low<sup>1</sup>, C. Yang<sup>1</sup>, K.L. Wong<sup>1</sup>, M. Tang<sup>1</sup>, D.M. Kemeny<sup>1</sup><sup>1</sup>National University of Singapore, Immunology Programme and Department of Microbiology, Yong Loo Lin School of Medicine, Singapore, Singapore

Interleukin-12 (IL-12) and interleukin-23 (IL-23) are structurally related heterodimeric cytokines sharing a common IL-12p40 subunit. Bioactive IL-12p70 is composed of p40 and p35 subunits while IL-23 is composed of p40 and p19 subunits. Although IL-12 and IL-23 exhibit overlapping pro-inflammatory and immunoregulatory responses, they have divergent roles in both the innate and adaptive immunity. However, what remains unclear is whether dendritic cells (DCs) produce IL-12 and IL-23 in a mutually exclusive fashion. Here we have compared the production of IL-12 and IL-23 in mouse splenic DCs and GM-CSF-derived bone marrow dendritic cells (BMDC) induced by an array of TLR agonists (PAM3Csk4, PolyI:C, LPS, Flagellin, Gardiquimod and CpG). While IL-12p70 production requires a combination of signals consisting of toll-like receptor (TLR) agonists and cytokines, IL-23 production can be induced by TLR-agonists alone. Although both splenic DCs and BMDCs produce IL-12p70, the ability to produce IL-23 appears to be restricted to BMDCs. Among the TLR agonists tested, CpG is the most potent inducer of IL-12p70 production while LPS induces the highest production of IL-23 followed by CpG. Real-time PCR analysis on LPS and LPS/IFN- $\gamma$  treated DCs showed more rapid kinetics of p40 and p35 mRNA induction in BMDCs compared to splenic DCs. As for p19 mRNA, both splenic and BMDCs exhibited induction at 2 hours followed by a rapid decline to basal levels by 4 hours. However, the induction of p19 mRNA is over 100 fold higher in BMDCs. Addition of IFN- $\gamma$  inhibited LPS induced p19 mRNA while enhancing the induction of p35 and p40 mRNA in both the splenic and BMDCs. These data shows that although IL-12 and IL-23 can be produced simultaneously, they appear to be differentially regulated under certain conditions. Furthermore, the difference in ability of splenic DCs and BMDCs to produce IL-23 implies a preferential production of IL-23 under conditions of inflammation.

**PA11/55 HUMAN IL-10 MODULATED DENDRITIC CELLS MIGRATE TO SECONDARY LYMPHOID TISSUES BUT DO NOT AFFECT LETHAL GVHD IN A HUMANIZED MOUSE MODEL**H.S. Adler<sup>1</sup>, F. Hermann<sup>1</sup>, H. Martin<sup>2</sup>, S. Sudowe<sup>1</sup>, C. Taube<sup>2</sup>, K. Steinbrink<sup>1</sup><sup>1</sup>Johannes Gutenberg University, Dermatology, Mainz, Germany, <sup>2</sup>Johannes Gutenberg University, III. Med. Klinik, Mainz, Germany

IL-10 modulated human dendritic cells (IL10DC) display a tolerogenic phenotype, associated with low expression of MHC II and costimulatory molecules of the B7 and TNFR families and impaired production of proinflammatory cytokines. *In vitro*, IL-10DC induce an anergic phenotype in CD4<sup>+</sup> and CD8<sup>+</sup> T cells, characterized by a CDK inhibitor p27<sup>Kip1</sup>-mediated cell cycle arrest. The induction of anergy is dependent on increased activity of MAP kinase p38 during primary activation and can be prevented by inhibition of p38 or high concentrations of IL-2. IL-10DC-generated anergic T cells exhibit suppressor effects on the function of effector T cells *in vitro* and are, thus, characterized as induced regulatory T cells (iTreg). Induction of iTregs by IL-10DC might be exploited therapeutically for suppression of cellular immune responses in severe allergic or autoimmune diseases or transplantation rejections (e.g. graft versus host disease, GVHD). As a preclinical screening system for the *in vivo* efficacy of tolerogenic IL10DC, we choose a model of GVHD in humanized NOD-Scid mice. Transfer of human PBMC into newborn NOD-Scid mice resulted in lethal GVHD within 2-3 months, characterized by growth retardation, weight loss, inflammatory reaction of the skin and multiple organs and death of affected animals. Treatment of GVHD with a single (at time point of PBMC transfer) or multiple doses (3x in weekly intervals) of IL-10DC, which were not additionally loaded with murine antigens, did neither prevent growth retardation, nor improve symptom-free survival and overall survival of the animals to a significant extent. Human IL-10DC, which express CXCR4 but reduced levels of CCR7, did reach secondary lymphoid tissue as demonstrated by recovery from spleen and lymph nodes after 48 h. Organ infiltration (lung, skin) by human cells and the percentage of human CD4<sup>+</sup> and CD8<sup>+</sup> T cells in spleen, lymph nodes and peritoneum were comparable in animals treated with IL-10DC and control mice. In summary, *in vitro* generated human IL-10DC migrate to secondary lymphoid tissues in NOD-Scid mice but do not exert any beneficial effect on experimental GVHD in humanized mice.

**PA11/56 ROLE OF COMPLEMENT IN VIRUS-SPECIFIC CTL RESPONSE INDUCED BY BONE-MARROW DERIVED DCs**Z. Banki<sup>1</sup>, C.G. Ammann<sup>2</sup>, A. Ejaz<sup>1</sup>, U. Dittmer<sup>3</sup>, K. Hasenkrug<sup>2</sup>, M.P. Dierich<sup>1</sup>, H. Stoiber<sup>1</sup>, D. Wilflingseder<sup>1</sup><sup>1</sup>Innsbruck Medical University, Department of Hygiene, Microbiology and Social Medicine, Innsbruck, Austria, <sup>2</sup>Rocky Mountain Laboratories, Laboratory of Persistent Viral Diseases, Hamilton, United States, <sup>3</sup>University of Duisburg-Essen, Institute of Virology, Essen, Germany

The generation of a robust cytotoxic T-cell (CTL) response is crucial to control retroviral infections. For the induction of both primary and secondary immune responses, dendritic cells (DCs) are thought to be the most potent antigen presenting cells. To investigate the role of the complement system in the induction of specific CTL responses by DCs upon infections with retroviruses, the Friend virus (FV)-complex has been chosen. *In vitro* infection assays with mouse bone-marrow derived DCs (bmDCs) demonstrated that both non-opsonised and complement (C)-opsonised FV can productively infect bmDCs. However, C-opsonisation significantly enhanced the infection of bmDCs. To study the impact of C-opsonisation on the antigen presentation, bmDCs were loaded with non-opsonised and C-opsonised FV and were then co-cultured with FV-specific TCR transgenic CD8<sup>+</sup> T-cells. Compared to non-opsonised virus, co-culture of bmDCs loaded with C-opsonised FV resulted in a significant higher frequency of activated FV-specific CD8<sup>+</sup> T-cells, as demonstrated by the expression of activation markers CD69, CD25. Furthermore, bmDCs loaded with C-opsonised FV induced a more pronounced proliferation and IFN- $\gamma$  secretion of FV-specific CD8<sup>+</sup> T-cells. In summary, our data emphasize a role of complement in the induction of FV-specific CTL response by DCs.

**PA11/57 INTERLEUKIN-10 ANTAGONIZES DENDRITIC CELL MATURATION BY INTERLEUKIN-24 VIA NF-KB PATHWAY**Y. Jiang<sup>1</sup>, G. Chen<sup>1</sup>, C. Wu<sup>1</sup>, Y. Long<sup>1</sup>, X. Cao<sup>1</sup><sup>1</sup>Second Military Medical University, Institute of Immunology, Shanghai, China

**Objectives:** Interleukin 24 (IL-24) has a unique tumor suppressor property and Dendritic cells (DCs) are important initiator for tumor immune responses. Whether IL-24 can affect the function of DCs was investigated in the present study.

**Methods:** Monocyte-derived DCs (MoDCs) were generated from human peripheral blood CD14<sup>+</sup> monocytes and treated with or without recombinant human IL-24 (1-50 ng/ml) for 24h. Recombinant human IL-10 with the same amount was co-administrated with IL-24 in the inhibition assay. These IL-24-treated MoDCs with or without IL-10 were then collected for the phenotypic and functional analysis and the related signal transduction investigation.

**Results:** IL-24 could markedly increase expression of MHC class II and costimulatory molecule CD40, CD80, CD86 on MoDCs. Meanwhile, IL-24 induced secretion of high levels of IL-12 and pro-inflammatory cytokines IL-1, IL-6, and TNF- $\alpha$  from MoDCs in dose- and time-dependent manner. A dose-dependent increase in IP-10 and MIP-1 $\alpha$  secretion and increased expression of chemokine receptors CCR7 and CXCR4 but decreased CCR5 on MoDCs by IL-24 were also detected. Moreover, IL-24 induced very low level of IL-10 production from MoDCs. The T cell-stimulating capacity of MoDCs was also enhanced by IL-24. The above results firmly support that IL-24 can enhance DCs function. However, the up-regulation of co-stimulatory molecule expression, secretion of pro-inflammatory cytokines and capacity to stimulate T cells proliferation of MoDCs by IL-24 were effectively suppressed by IL-10 via NF- $\kappa$ B pathways.

**Conclusion:** IL-24 could enhance the function of MoDCs by increased expression of co-stimulating molecules, cytokines and chemokines, which could be effectively inhibited by IL-10 via NF- $\kappa$ B pathways. Therefore, the anti-tumor effects of IL-24 might partially ascribe to activation of DCs by IL-24 *in vivo*. The inhibition of IL-24-mediated enhancement of MoDCs function by IL-10 suggests that these two related protein family members may have antagonistic functions, directly affecting the immunotherapeutic outcome of tumor by IL-24 in future clinical utilization.

**PA11/58 LEISHMANIA INFANTUM EXCRETED/SECRETED MOLECULES MODIFY DENDRITIC CELLS ACTIVATION STATUS**M. Resende<sup>1</sup>, R. Silvestre<sup>1</sup>, N. Santarém<sup>1</sup>, S.G. Pereira<sup>1</sup>, A. Cordeiro-da-Silva<sup>2,3</sup>, Parasite Disease Group<sup>1</sup>IBMC, Porto, Portugal, <sup>2</sup>IBMC, University of Porto, Porto, Portugal, <sup>3</sup>Faculdade de Farmácia da Universidade do Porto, Porto, Portugal

Several evidences suggested that *Leishmania* excreted/secreted (E/S) molecules can play a role in the initial steps of infection, contributing to a successful parasite establishment inside the host. Dendritic cells (DC), known as "professional" antigen presenting cells, act as sentinels for intruding pathogens. Since DCs are essential in orchestrating an effective immune response during infection, we explored the modulation of their functions by *Leishmania* E/S molecules in the presence or absence of infection.

Balb/c mice bone marrow derived DCs (BM-DC) were incubated with *L. infantum* promastigotes expressing the luciferase (LUC) gene and/or with E/S molecules. These E/S molecules were obtained after promastigotes subculture in a serum free medium concentrated by membrane filtration. After 24 hours of incubation, BM-DC culture supernatants were recovered for cytokines quantification by ELISA (IL10, TGF- $\beta$ , IL12p40, IL12p70, TNF- $\alpha$ ). In parallel, BM-DCs were harvested and the levels of CD40, CD80, CD86 or MHCII-FITC molecules was analysed by flow cytometry. The parasite load was evaluated by measuring the luciferase activity of the LUC-recombinant parasites from BM-DCs infected cells. BM-DCs from MyD88KO and TLR2KO mice were also used in order to ascertain the activation pathway. *L. infantum* E/S products induced BM-DCs activation as observed by the dose-dependent up-regulation of DC activation markers. This effect was abolished in the absence of MyD88 suggesting a role for toll-like receptors (TLR) in the signal transduction events. TLR2, which was shown to recognize surface *Leishmania* lipophosphoglycan, is not involved in the recognition of E/S molecules. The cytokine profile differs among the E/S stimuli concentration: while high dose of E/S leads to a predominant IL-10 release, low E/S dose mainly induces TNF- $\alpha$  secretion.

Our results showed that infection with *L. infantum* promastigotes do not induce an up-regulation of BM-DCs co-stimulatory molecules neither a significant cytokine secretion. However, E/S molecules (co-administered with the infection) induced an up-regulation of the co-stimulatory CD40, CD80 and CD86 molecules, with the production of either anti (IL-10) and pro (IL-12, TNF- $\alpha$ ) inflammatory cytokines.

Overall, this data demonstrates that E/S molecules induce BM-DC activation through TLR pathway suggesting a potential role of these molecules in the modulation of DC functions during infection.

**PA11/59 POSSIBLE MECHANISMS OF IMMUNE DOWNMODULATION OF VIRAL T-CELL RESPONSES USING DENDRITIC CELLS**A.H. Nuyts<sup>1</sup>, N. Cools<sup>1</sup>, P. Ponsaerts<sup>1</sup>, G. Nijls<sup>1</sup>, Z.N. Berneman<sup>1</sup>, V.F. Van Tendeloo<sup>1</sup><sup>1</sup>Antwerp University, Laboratory of Experimental Hematology, Vaccine & Infectious Disease Institute (VIDI), Edegem, Belgium

**Objectives:** Dendritic cells (DC) are currently being used to modulate immune responses in a variety of disorders: cancer, infectious diseases and auto-immune diseases. In this study, we aim to investigate the cellular mechanisms of immune suppression induced by human dendritic cells.

**Methods:** The *in vitro* T-cell stimulatory capacity of immature (i)DC, cytokine cocktail-matured DC (CC-mDC) or lipopolysaccharide-matured DC (LPS-mDC) was analyzed after coculture with peripheral blood lymphocytes (PBL) stimulated with a CEF peptide pool.

**Results:** Antigen-specific interferon-gamma (IFN- $\gamma$ ) secretion of PBL is significantly reduced after culture with iDC or CC-mDC as compared to LPS-mDC. Importantly, these T-cells could not be reactivated after addition of stimulating substances, such as IL-2 or LPS-mDC, suggesting that T-cell suppression is not (entirely) mediated by induction of T-cell anergy. In addition, annexin-V and propidium iodide staining reveals a significant higher amount of apoptotic and dead cells, respectively in cocultures with iDC in comparison with mDC. This contributes to the hypothesis that iDC and CC-mDC operate in down modulation of the immune response through a (partially) different suppression mechanism. Currently, this is further investigated by analyzing the effect of DC in cell cycle analysis of T-cells in DC/PBL cocultures. Finally, there is a significantly higher number of CD4+CD25+FOXP3+ cells as well as an increased amount of CD4+IL10+ T-cells in cocultures of PBL with iDC and with CC-mDC, but not with LPS-mDC, as analyzed by multiparametric flow cytometry.

**Conclusion:** Our observations show that the decreased T-cell stimulatory capacity of iDC and CC-mDC, is caused by simultaneous induction of apoptosis and of regulatory T-cells. However, apoptosis is more pronounced in cocultures with iDC. To further unravel the mechanisms of suppression, we are currently investigating the role of DC in polarizing the immune response in a Th1, Th2 or Treg direction.

**PA11/60 IMMUNOMODULATION OF DENDRITIC CELLS BY VIRULENT AND ATTENUATED LEISHMANIA INFANTUM STRAINS**B. Neves<sup>1</sup>, R. Silvestre<sup>2</sup>, J. Cunha<sup>2</sup>, J. Tavares<sup>2,3</sup>, M. Resende<sup>2</sup>, A. Ouassii<sup>2,4</sup>, M.C. Lopes<sup>1</sup>, M.T. Cruz<sup>1</sup>, A. Cordeiro-da-Silva<sup>2,3</sup>

<sup>1</sup>Faculdade de Farmácia e Centro de Neurociências e Biologia Celular de Coimbra, Universidade de Coimbra, Coimbra, Portugal, <sup>2</sup>Parasite Disease Group, Instituto de Biologia Molecular e Celular da Universidade do Porto, Porto, Portugal, <sup>3</sup>Faculdade de Farmácia da Universidade do Porto, Porto, Portugal, <sup>4</sup>INSERM; UMR CNRS 5235, Université Montpellier II, Montpellier, France

*Leishmania* needs to subvert the host innate and adaptive immune responses shortly after inoculation into the host. In the natural course of infection, these events occur in the dermis where dendritic cells (DC), as major antigen-presenting cells connecting innate and adaptive immunity, may act as host targets for *Leishmania*. Therefore, the understanding of the complex interactions between *L. infantum* and DCs are central to the outcome of this infection.

**Methods:** The levels of phosphorylated signaling proteins were evaluated by western blot and the expression of surface molecules was quantified by cytometry. Also, the transcription levels and secretion of pro- and anti-inflammatory cytokines was accessed by quantitative real-time RT-PCR (qPCR) and ELISA, respectively. In the present study, we showed that the initial contact of virulent *L. infantum* parasites with a skin derived DC cell line or with bone-marrow derived DC (BM-DC) in different stages of differentiation induced a rapid yet transient activation of the intracellular signaling pathways AKT and ERK1/2. The effect seems to be specific to these pathways, since we did not observed any relevant activation of JNK1/2 and p38MAPK. Interestingly, attenuated *Leishmania* parasites (*SIR2KO*) were capable to activate the same signaling pathways although at higher extent, since increased levels of phosphorylated AKT and ERK1/2 were detected. Nevertheless, both virulent and *SIR2KO L. infantum* infections were unable to fully activate BM-DCs, as observed by the low expression of cell surface co-stimulatory markers CD80, CD86 and class I and II MHC molecules. Despite the apparent absence of BM-DCs maturation after virulent and *SIR2KO* infections, BM-DCs were shown to up-regulated the transcription of both pro- (IL-12p40) and anti-inflammatory (IL-10) cytokines as determined by qPCR and the IL-12p40 secretion as evaluated by ELISA. Remarkably, only after *SIR2KO* infection, an increased transcription of TNF- $\alpha$  and IL-6 was observed, which might suggest a critical role for these endogenous pro-inflammatory cytokines in the *Leishmania*/DC interaction.

These results indicate that a comparative study between virulent and attenuated *Leishmania* strains will definitely contribute to the understanding of the pathogenic intracellular mechanisms controlling host innate cell machinery.

**PA11/61 FULLY AUTOMATED ISOLATION OF HIGHLY PURIFIED HUMAN PLASMACYTOID DENDRITIC CELLS IN A SINGLE-STEP BY NEGATIVE SELECTION**S. Holland<sup>1</sup>, M. Fairhurst<sup>1</sup>, A. Kokaji<sup>1</sup>, T. Thomas<sup>1</sup>, B. Guibault<sup>1</sup><sup>1</sup>STEMCELL Technologies Inc., Vancouver, Canada

**Objective:** The isolation of plasmacytoid dendritic cells (pDC) from peripheral blood samples is difficult and time consuming as these cells are extremely rare, comprising around 0.5% of total mononuclear cells. In order to obtain highly purified cells, current methods of isolation consist of multiple steps that include either flow cytometry-based sorting or positive immunomagnetic cell selection, which can have detrimental effects on cell structure and function. Our objective was therefore to develop a rapid single-step method for the isolation of plasmacytoid dendritic cells from peripheral blood mononuclear cell (PBMC) suspensions with minimal impact on cell function.

**Methods:** A cocktail of monoclonal antibodies incorporated into tetrameric antibody complexes was used to crosslink unwanted cells in the sample to EasySep<sup>®</sup> magnetic particles. Following incubations times of 30 minutes for the antibody cocktail and 10 minutes for the magnetic particles, the sample was placed in an EasySep<sup>®</sup> magnet for 5 minutes. Desired cells were recovered by simply pouring off the cell suspension while unwanted cells were held to the walls of the tube where they remained. An additional particle incubation and magnetic separation enabled the generation of highly purified pDC fractions. The resulting single-step method was quick, requiring approximately one hour from start to finish.

**Results:** Starting with samples of peripheral blood mononuclear cells, purities of 93.8%  $\pm$  3.8% pDC (defined as Lin<sup>-</sup>, HLA-DR<sup>+</sup>, BDCA-4<sup>+</sup>) were obtained with cell recoveries of 65.6%  $\pm$  16.2% (n=9). This method was also fully automated using a RoboSep<sup>®</sup> cell separator with true walk-away capability. Using the RoboSep<sup>®</sup> cell separator enabled the processing of PBMC samples up to one billion cells in size in just one hour, with total sample handling time of about five minutes. Preliminary culture experiments suggest that purified pDC isolated using RoboSep<sup>®</sup> can provide robust alpha interferon production responses to treatment with live viruses (influenza, rhinovirus) or a TLR-7 agonist (Gardiquimod<sup>TM</sup>).

**Conclusion:** We report here the successful development of a rapid and efficient method for the isolation of pure pDC in a single-step by negative immunomagnetic cell selection.

**PA11/62 SKIN DENDRITIC CELLS SUPPRESS ADAPTIVE IMMUNITY AFTER BURN INJURY**L.M. van den Berg<sup>1</sup>, M.A.W.P. de Jong<sup>1</sup>, T.B.H. Geijtenbeek<sup>1</sup><sup>1</sup>VU University Medical Centre, Molecular Cell Biology and Immunology, Amsterdam, Netherlands

**Introduction:** Burn injury is associated with excessive local innate inflammation on the one hand and suppressed systemic adaptive immunity on the other hand. Dendritic cells (DCs) are professional antigen presenting cells present in peripheral tissues such as skin and are key players in inducing appropriate adaptive immune responses. Here we investigate the role of DCs in the induction of an effective immune response after burn injury.

**Methods:** The Human Ex vivo Adjustable Temperature regulating – Machine (HEAT-M) was used to burn ex vivo human skin pieces at different temperatures (60°C–100°C). The HEAT-M is able to burn surfaces of 2 mm by 10 mm at temperatures ranging from 22°C to 160°C. Sheets of skin were floated onto medium and migrating antigen presenting cells (APCs) were analysed for their functionality.

**Results:** The data demonstrate that the number of migrating APCs from burned and unburned skin remained unaltered. Furthermore, migrating APCs from burned skin showed no substantial differences in maturation status compared to unburned skin. However, APCs from burned skin strongly suppressed T cell responses compared to those from unburned skin as shown in a mixed leukocyte reaction (MLR).

**Conclusion:** APCs from burned skin strongly suppress T cell responses, even though they have a similar maturation phenotype and migratory properties as those from healthy skin. This indicates the important role of skin APCs in directing an effective immune response after burn injury. Our data suggest that skin APCs are key players in the suppressed systemic adaptive immunity observed in burn patients.

This work was supported by a grant from the Dutch Burns Foundation.

**PA11/63 INFLAMMATORY MEDIATORS ARE INSUFFICIENT FOR FULL DENDRITIC CELL ACTIVATION**W. Kratky<sup>1</sup>, R. Spörri<sup>1</sup><sup>1</sup>ETH Zurich, Institute for Microbiology, Zurich, Switzerland

Activation of dendritic cells (DCs) is a prerequisite for the priming of naive T cells and their differentiation to fully functional effectors. DCs can be activated either directly by the engagement of pattern recognition receptors or, indirectly, by inflammatory signals produced by pathogen-exposed immune and non-immune cells. There is accumulating evidence that the two pathways result in qualitatively different DC activation states. We have shown previously that indirectly-activated DCs support the expansion of CD4+ T cells but fail to instruct T helper cell differentiation. However, it remains unclear whether activation by inflammatory mediators generates DCs with the capacity to promote the differentiation of naive CD8+ T cells to CTLs. We report here that indirectly-activated DCs primed CD8+ T cells that lack cytolytic activity and fail to produce effector cytokines. Although indirectly-activated DCs increased the expression of MHC and costimulatory molecules they were unable to provide the newly-activated T cells with differentiation factors such as IL-12. In contrast, DCs activated by the triggering of pattern recognition receptors not only upregulated MHC and costimulatory molecules but in addition also produced IL-12 and other proinflammatory cytokines, enabling them to prime fully functional CD8+ T cells. These results indicate that inflammation cannot substitute for contact with pathogen components in DC activation and demonstrate the importance of a third signal for the generation of effector T cell responses.

## PA11/64 DENDRITIC CELLS SHOW EVIDENCE OF A CELL SURFACE SIALYTRANSFERASE ACTIVITY

A.R. Piteira<sup>1</sup>, M.G. Cabral<sup>1</sup>, D. Ligeiro<sup>2</sup>, R. Brossmer<sup>3</sup>, P.A. Videira<sup>1</sup><sup>1</sup>Faculdade de Ciências Médicas from Universidade Nova de Lisboa, Lisbon, Portugal, <sup>2</sup>Centro de Histocompatibilidade do Sul, Lisbon, Portugal, <sup>3</sup>University of Heidelberg, Heidelberg, Germany

**Objectives:** Sialic acids are typical terminal sugars of cell surface glycoconjugates and are functional important for many biological interactions, including some immune responses. We have observed that, during human monocyte derived-DC (mo-DCs) differentiation, the expression of sialylated structures augments, due to specific sialyltransferases (ST). This sialylation has a significant role in DC maturation, immunogenicity and endocytosis capacity (1, 2). Although, STs are preferentially localized to the Golgi apparatus, cell surface ST are reported for B cells and neutrophils, to permit a rapid modulation of surface sialylation and alter specific immune functions. In this work we aimed to investigate the existence of this particular ST activity in DCs.

**Methods:** mo-DCs, treated or not with neuraminidase (enzyme that cleaves sialic acid), were incubated, in serum free medium, with a fluorescent ST substrate, CMP-FITC-Neu, and the incorporation of sialic acid was determined by Flow Cytometry and Confocal Microscopy.

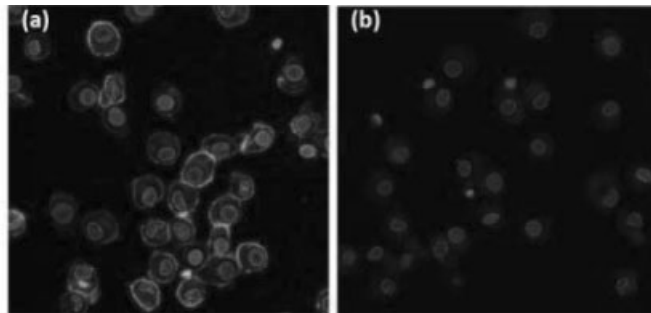
**Results:** Neuraminidase treated mo-DCs significantly incorporated sialic acid into cell surface and this incorporation increased in time and was inhibited by a ST inhibitor, the CTP (Fig 1). This activity was not affected by maturation, since we found no significant differences in LPS stimulated mo-DCs. After proving the existence of a ST activity which rapidly re-sialylated mo-DC surface, we are presently investigating whether it modulates the mo-DC endocytosis capacity, in opposition to sialic acid shortage, which we have previously found to decrease macropinocytosis and increase phagocytosis (1). Based on the literature, we are also analysing the contribution of this ST activity to the DC adhesion capacity to other cells, including cancer cells.

**Conclusion:** Although, physiologically, the presence of CMP-Neu in the outer milieu was never demonstrated, it is important to reveal the existence of a ST activity, which rapidly restores or increases surface sialylation without de novo synthesis of sialylated structures. Given the role of sialylation, this activity is likely to modulate DCs function and it may serve as a simple way to alter in vitro mo-DC immunogenicity, with particular relevance to DC-based therapies.

**References:**

(1) Videira et al, 2008, Glycoconj J 25:259-268

(2) Crespo et al, 2009, Immunology, IMM3047



**Figure 1: Incorporation of fluorescent Sialic acid in Dendritic cells treated with Neuraminidase, assessed by Confocal Laser-scanning microscope. (a) in presence of fluorescent CMP-FITC-Neu; (b) Presence of fluorescent CMP-FITC-Neu and the inhibitor of ST activity, CTP. Dye TOPO3 was used to stain the nucleus.**

[Figure 1]

PA11/65 MYCOPHENOLIC ACID DISABLED HUMAN DENDRITIC CELLS TO INDUCE ALLOGENEIC CYTOTOXIC CD8<sup>+</sup> T CELLS THROUGH INHIBITION OF INTERFERON GAMMA SYNTHESIS IN DENDRITIC CELLSR. Lemoine<sup>1</sup>, F. Velge-Roussel<sup>1</sup>, H. Nivet<sup>1,2</sup>, Y. Lebranchu<sup>1,2</sup>, C. Baron<sup>1,2</sup><sup>1</sup>EA 4245 'Cellules Dendritiques, Immunomodulation et Greffes', UFR de Médecine, Université François Rabelais, Tours, France, <sup>2</sup>Service de Néphrologie et d'Immunologie Clinique, CHRU, Tours, France

**Purpose:** Several studies have highlighted that human dendritic cells (DC) can activate CD8<sup>+</sup> T cell cytotoxic activity independently of CD4<sup>+</sup> helper T cells and have reported that CD8-dependent rejection might be resistant to immunotherapeutic agents, especially for memory CD8<sup>+</sup> T cells. Modulating CD4 independent-CD8<sup>+</sup> T cell cytotoxic alloresponse might be important for inducing organ acceptance. We have previously shown that DC pretreated with mycophenolic acid (MPA) could regulate CD4<sup>+</sup> T cell alloresponse. In this study, we found a great interest to know how MPA could affect the ability of DC to induce the differentiation of allogeneic CD8<sup>+</sup> T cells into effector cytotoxic cells independently of CD4<sup>+</sup> T cells.

**Materials and methods:** The maturation of human monocyte-derived DC was induced by LPS (50ng/ml) in the presence or not of 100μM MPA. Allogeneic CD8<sup>+</sup> T cells, positively selected by magnetic beads were cultured for 6 days with either LPS-DC or MPA-DC. T cell proliferation was analyzed by [<sup>3</sup>H]-thymidine incorporation and cytokine productions were assessed by ELISA. The cytotoxic function was also assessed by analyzing granzymes A and B, perforin and CD107 expression (markers associated with cytotoxic granules) and killing of targets was measured by CFSE/7-AAD double staining.

**Results:** Firstly, we found that LPS-DC support the proliferation of allogeneic CD8<sup>+</sup> T cells independently of CD4<sup>+</sup> T cells whereas DC modified by MPA did not (22150 cpm ± 1240 versus 5900 cpm ± 480; p < 0.001; n = 10). Secondly, MPA-DC induced antigen-specific CD8<sup>+</sup> T cell anergy in both naive and memory CD8<sup>+</sup> T cells that secrete high levels of IL-4, IL-5, IL-10 and TGF-β. Importantly, MPA strongly reduced the capacity of DC to induce cytotoxic activity in allogeneic CD8<sup>+</sup> T cells. Finally, we found that exogenous IFN-γ completely restores IL-12 and TNF-α synthesis in MPA-DC and reconstitute their ability to activate CD8<sup>+</sup> T cells.

**Conclusion:** These results may provide a new approach to modulate CD8 cytotoxic alloresponse to promote allograft tolerance.

## PA11/66 DIRECT CONTACT OF PLATELETS AND THEIR RELEASED PRODUCTS EXERT DIFFERENT EFFECTS ON HUMAN DENDRITIC CELL MATURATION

H. Hamzeh-Cognasse<sup>1</sup>, F. Cognasse<sup>1,2</sup>, S. Palle<sup>3</sup>, P. Chavarin<sup>2</sup>, T. Olivier<sup>3</sup>, O. Deléay<sup>1</sup>, B. Pozzetto<sup>1</sup>, O. Garraud<sup>1,2</sup><sup>1</sup>Université Jean Monnet, GIMAP- EA 3064, Saint-Etienne Cedex, France, <sup>2</sup>EFS Auvergne-Loire, Saint-Etienne, France, <sup>3</sup>Université Jean Monnet, Centre de Microscopie Confocale Multiphotonique 4D, Saint-Etienne Cedex, France

**Background:** Dendritic cells (DCs) are antigen presenting cells capable of inducing innate and adaptive immune responses. According to the stimulus and their maturation state, DCs induce immunogenic or tolerogenic responses. Platelets (PLTs), which are involved in haemostasis and inflammation, can also interact with DCs. In this study, we examined the effect of PLTs on DC maturation *in vitro*. Human monocyte-derived DCs were co-cultured for 2 days with homologous PLTs either in the same well or in 0.4 μm-pore size filter-separated compartments.

**Results:** Confocal microscopy showed the attachment of PLTs to DC membranes. The DC receptor involved in this interactions was found to be CD162. In addition, we observed that DCs co-cultured with PLTs in filter-separated compartments acquired a mature phenotype (high CD80, CD86, and intermediate CD83 expression; IL-12(p70) production; efficient stimulation of autologous CD4<sup>+</sup> T cell proliferation), while DCs co-cultured with PLTs in the same compartment did not undergo phenotypic maturation, did not secrete IL-12(p70) or IL-1β, but instead induced moderate Th2-polarized T cell proliferation.

**Conclusions:** These data indicate that

(i) PLTs secrete a soluble DC-activating factor that was demonstrated not to be soluble CD40-Ligand (CD154; as could have been expected from *in vivo* and previous *in vitro* work) but to be nucleotide, and

(ii) that cell-to-cell contact did not induce DC maturation, possibly because nucleotide release by PLTs was prevented by direct contact with DCs.

This work demonstrates that PLTs are active elements of the immune system that might play a role in balancing the ability of DCs to polarize T cell responses, therefore making them critical factors in transfusion processes.



**PA11/67 HUMAN DERMAL FIBROBLASTS SUPPORT THE DIFFERENTIATION OF IL-17 PRODUCING T-CELLS VIA UP-REGULATION OF IL-23 PRODUCTION BY DENDRITIC CELLS**C. Schirmer<sup>1,2</sup>, C. Klein<sup>1</sup>, M. von Bergen<sup>2</sup>, J.C. Simon<sup>1</sup>, A. Saalbach<sup>1</sup><sup>1</sup>Universität Leipzig, Klinik für Dermatologie, Venerologie und Allergologie, Leipzig, Germany, <sup>2</sup>Helmholtz Centre for Environmental Research, Proteomics, Leipzig, Germany

To trigger an effective T cell-mediated immune response in the skin, upon antigen contact dendritic cells (DC) migrate into locally-draining lymph nodes where they present antigen to naïve T cells and induce activation and differentiation of T cells (TC). During their migration to secondary lymphoid organs, DC travel through the stromal microenvironment comprised of the extracellular matrix and stromal cells such as fibroblasts, macrophages and endothelial cells. Little is known about the interaction of DC with the stromal microenvironment. Recently, we have shown that DC interact with dermal fibroblasts in vivo and in vitro in inflamed skin.

To study the effects of this interaction, monocyte-derived DC were partially matured by adding Lipopolysaccharide (DC-LPS) to imitate antigen contact in inflamed skin. Following, they were cocultured with dermal fibroblasts for 24h. We could demonstrate that LPS-stimulated DC activated fibroblasts via tumour necrosis factor (TNF)  $\alpha$  and interleukin (IL)-1 $\beta$ . Consequently, activated fibroblasts produced prostaglandin (PG) E<sub>2</sub> which in turn increased IL-23 production by DC compared to LPS-stimulated DC. Since IL-23 is an important factor in the differentiation of TH17 cells, we stimulated CD3<sup>+</sup> T cells or memory T cells with either supernatants of DC-LPS-fibroblast-coculture or DC-LPS in the presence of anti-CD3/CD28-beads. Indeed, supernatants of DC-LPS-fibroblast-coculture significantly increased IL-17 production of CD3<sup>+</sup> T cells or memory T cells compared to T cells stimulated with DC-LPS supernatant alone.

In brief, we were able to demonstrate that dermal fibroblasts regulate cytokine production of DC and thus are involved in the control of TC differentiation.

**PA11/68 FACTORS REGULATING DENDRITIC CELL ACCUMULATION IN MESENTERIC LYMPH NODES, AND THEIR IMPACT ON T CELL RESPONSES**K. Fredriksson<sup>1</sup>, W.W. Agace<sup>1</sup>, B. Johansson-Lindbom<sup>1</sup><sup>1</sup>Lund University, Department of Experimental Medical Science, Lund, Sweden

The intestinal immune system faces the challenging task of maintaining tolerance to ingested antigen and commensal gut bacteria while still being capable of mounting protective responses against pathogens. Dendritic cells (DCs) play an important role in the activation and differentiation of naïve T cells. In response to inflammatory stimuli, signaling through TLR adaptor proteins MyD88 and TRIF and/or receptors for inflammatory cytokines, such as type I IFN and TNF- $\alpha$ , influence the properties of the resulting immune response. It is currently not clear how these molecular pathways regulate intestinal DC migration and function in the steady state. Using BrdU pulse chase experiments we have determined the kinetics by which newly formed BrdU-labeled DCs appear in gut-draining mesenteric lymph nodes (MLN) during steady state conditions. We see no significant differences in DC migration from the intestinal mucosa to draining MLN in TRIF, type-I IFN receptor or TNFR1 and 2 double knockout mice. In MyD88 knockout mice preliminary results indicate decreased numbers and impaired accumulation of both tissue-derived and lymph node resident DCs in the MLN. We are currently investigating DC responses and the generation of different T cell subsets in these mice in response to different types of adjuvants.

**PA11/69 INDUCTION OF TH2- AND REGULATORY-TYPE IMMUNE RESPONSE BY TRICHINELLA SPIRALIS ANTIGEN – PRIMED DENDRITIC CELLS IN DARK AGOUTI RATS**A. Gruden-Movsesijan<sup>1</sup>, N. Ilic<sup>1</sup>, S. Vasilev<sup>1</sup>, L. Sofronic-Milosavljevic<sup>1</sup><sup>1</sup>Institute for the Application of Nuclear Energy-INEP, University of Belgrade, Belgrade, Serbia

Dendritic cells (DCs) are important members of the first line of defense and have a key role in initiation and regulation of immune responses to invading pathogens. Upon contact with various pathogen-derived stimuli, DCs become activated and undergo process of maturation that could strongly influence the development of appropriate immune response, both innate and acquired. Rats infected with parasite nematode *Trichinella spiralis* (*T. spiralis*) mount Th2 and regulatory type of responses, essential for the survival of both parasite and the host. We have shown that excretory-secretory antigens of *T. spiralis* muscle larvae (ES L1) provoke incomplete maturation of rat DCs *in vitro*. These cells displayed moderate up-regulation of surface activation markers CD86 and ICAM1, but the expression of MHC II was not significantly altered. ES L1 primed DCs secreted much higher levels of IL-10, but lower level of IL-12 compared to control – unstimulated DCs. Analysis of the effect of ES L1-primed DCs on the immune response polarization showed that these cells were capable to promote mixed Th1/Th2 cytokine response when injected intravenously into rats. Spleen cells of the recipient animals exhibited elevated production of IL-10 and IL-4, with no change in IFN- $\gamma$  production, compared to untreated controls. Spleen cells from rats injected with ES L1 – primed DCs exhibited expansion of CD4+CD25+Foxp3+ cells. Obtained results indicated that DCs possess the capacity to present *T. spiralis* antigens and subsequently induce *in vivo* polarization of immune response toward Th2 and regulatory type. (Ministry of Science and Technological Development, Republic of Serbia, Grant No: 143047).

**PA11/70 INTERACTION OF DENDRITIC CELLS WITH CHITOSAN VIA TLR4 RECEPTORS INDUCES A MAJOR ALTERATION IN THE ACTIVATION OF THE CELLS WITH A POTENTIAL IMPACT ON THE TH1/TH2 BALANCE**C.L. Villiers<sup>1</sup>, M. Chevallier<sup>2</sup>, H. Diemer<sup>3</sup>, R. Couderc<sup>1</sup>, H. Freitas<sup>1</sup>, A. Van Dorsselaer<sup>3</sup>, P.N. Marche<sup>1</sup>, T. Rabilloud<sup>2</sup><sup>1</sup>INSERM 823, Université Joseph Fourier Grenoble, Grenoble, France, <sup>2</sup>CEA, CNRS UMR 5092, iRTSV/LBBSI, Grenoble, France, <sup>3</sup>CNRS UMR7178, Strasbourg, France

Dendritic cells are known to be activated by a wide range of microbial products, leading to cytokine production, and increased levels of membrane markers such as MHC Class II molecules. Such activated dendritic cells possess the capacity to activate naïve T cells. We demonstrate here that immature dendritic cells secrete both the YM1 lectin and lipocalin2. By testing the ligands of these two proteins, respectively chitosan and siderophores, we also demonstrate that chitosan, a degradation product of various fungal and protozoal cell walls, induce an activation of dendritic cells at the membrane level, as shown by the upregulation of membrane proteins such as class II molecules, CD80 and CD86, via a TLR4-dependent mechanism, but is not able to induce cytokine production. This leads to the production of activated dendritic cells unable to stimulate T cells. However, costimulation with other microbial products overcomes this partial activation and restore the capacity of these activated dendritic cells to stimulate T cells. In addition, successive stimulation with chitosan and then by LPS induced a switch in dendritic cells from a Th1 response (IL12-producing) to a Th2 response (IL10-producing).

**PA11/71 RESIDENT KIDNEY DENDRITIC CELLS PREVENT UNCONTROLLED COLLATERAL DAMAGE BY RECRUITED PHAGOCYTES IN URINARY TRACT INFECTION**A. Tittel<sup>1</sup>, D. Engel<sup>1</sup>, J. Maurer<sup>1</sup>, C. Kurts<sup>1</sup><sup>1</sup>University of Bonn, Institute of Molecular Medicine and Experimental Immunology, Bonn, Germany

Urinary tract infections, such as cystitis and pyelonephritis (PN), affect a large proportion of the population worldwide and are mostly caused by the uropathogenic gram-negative bacteria *Escherichia coli* (UPEC). It has been shown previously that the kidney contains a contiguous network of CX3CR1<sup>+</sup> dendritic cells (DCs), but the function of these cells in the immune response against UPECs is unresolved. To study the role of DCs in PN, we established a murine PN model, which is induced by inoculation of the UPEC strain 536 derived from a cystitis patient into the bladder. The numbers of inflammatory DCs and neutrophilic granulocytes (PMN), but not of resident DCs were increased 24 hours after infection. To investigate the role of resident and inflammatory DCs, we depleted these DCs by clodronate liposomes and we found increased numbers of UPECs in the kidney and elevated lethality demonstrating that DCs contribute to the defense against UPECs. To selectively analyze the role of resident DCs, we infected mice at later time points (day 4) after depletion by clodronate liposomes, where resident DC, but not inflammatory DCs were absent. The number of UPECs and the kidney damage by inflammatory phagocytes was increased in these mice indicating a protective role of resident DCs.

**PA11/72 DENDRITIC CELLS FROM PATIENTS WITH SJÖGREN'S SYNDROME HAVE SLIGHTLY ENHANCED ENDOCYTIC CAPACITY**G.S. Lier<sup>1</sup>, P. Vogelsang<sup>1</sup>, J.G. Brun<sup>2,3</sup>, R. Jonsson<sup>1,2</sup>, S. Appel<sup>1</sup><sup>1</sup>University of Bergen, Broegelmann Research Laboratory, The Gade Institute, Bergen, Norway, <sup>2</sup>Haukeland University Hospital, Department of Rheumatology, Bergen, Norway, <sup>3</sup>University of Bergen, Institute of Medicine, Section for Rheumatology, Bergen, Norway

**Aim:** Sjögren's syndrome (SS) is an autoimmune disease of unknown etiology. It is characterized by chronic inflammation of the exocrine glands which leads to dryness of the eyes and the mouth. Dendritic cells (DC) are the most potent antigen-presenting cells of the immune system. They acquire antigens in the periphery and migrate to the lymph nodes where antigen specific T lymphocytes recognize the presented peptide antigens and mount an immune response.

So far, not much is known about the possible role of DC in Sjögren's syndrome. In this study, we analyzed the endocytic capacity of monocyte derived DC from patients with primary SS (pSS) compared to healthy controls.

**Methods:** Peripheral blood mononuclear cells were isolated from freshly heparinized blood samples from pSS patients fulfilling the American European Consensus group criteria (AECC) and gender- and age-matched healthy controls. Monocytes were isolated by plastic adherence, and immature DC were generated by culturing the monocytes with IL-4 and GM-CSF for 5-6 days. A fraction of the cells was stimulated for 48 hours with TLR7/8 ligand CL-097. For the analysis of endocytic activity, the cells were incubated for one hour with FITC-dextran (40 000 MW) at 37°C. Cells incubated at 4°C served as a negative control. After several washing steps, the cells were immediately analyzed by flow cytometry. Endocytic activity was calculated by subtracting median fluorescence intensity of cells incubated at 4°C from cells incubated at 37 °C.

**Results:** Immature DC from SS patients had a slightly enhanced endocytic capacity compared to DC from healthy controls. No obvious difference was observed comparing mature DC from SS patients and healthy controls, both took up reduced amounts of dextran compared to immature DC.  
**Conclusions:** The enhanced endocytic ability of monocyte derived DC from patients with primary Sjögren's syndrome might reflect that the DC in those patients are more active taking up antigens in an inflammatory environment. A higher proportion of autoantigens could therefore be presented on these cells. Eventually, this might induce an immune response instead of tolerance resulting in the autoimmune reactions seen in SS patients.

PA11/73 CD14<sup>+</sup> CELLS TREATED WITH TGF-β1 AND CD4<sup>+</sup> CD25<sup>+</sup> FOXP3<sup>+</sup> T-CELLS

S. Abediankenari<sup>1</sup>, M.R. Parsaie<sup>1</sup>  
<sup>1</sup>Faculty of Medicine, Microbiology and Immunology, Sari, Iran, Islamic Republic of

During antigen capture and processing, mature DCs provide variety of positive and negative regulatory signals. In this research, we studied the capacity of dendritic cells to expand antigen-specific T regulatory cells. We also investigated the role of TGF-beta in induction inhibitory functions of dendritic cells in mixed leukocyte reactions. Dendritic cells were generated from blood CD14<sup>+</sup> monocytes with granulocyte-Monocyte colony stimulating factor and interleukin-4 with or without TGF-beta (TGF-β-GM-DC or GM-DC). CD4<sup>+</sup> T cell were isolated to detect lymphocyte proliferation by thymidin incorporation assay and the ratio of CD4<sup>+</sup>FOXP3<sup>+</sup>CD25<sup>+</sup> T cells were determined by fluorescence-activated-cell-sorter. T cell proliferation responses in GM-DC showed a significance antigen-specific proliferative response when compared with TGFβ-GM -DC, that T Cell proliferation was inhibited in co-culture system containing DC-treated TGF-β. In conclusion, we can suggest that the expansion of T regulatory via TGF-β-GM-DC will provide a means for antigen specific control of unwanted immune reactions.

PA11/74 REGULATORY T CELL INDUCTION BY MONOCYTE-DERIVED DENDRITIC CELLS MODULATED BY 1α,25-DIHYDROXYVITAMIND<sub>3</sub> OR DEXAMETHASONE: DIFFERENTIAL ROLE FOR PD-L1

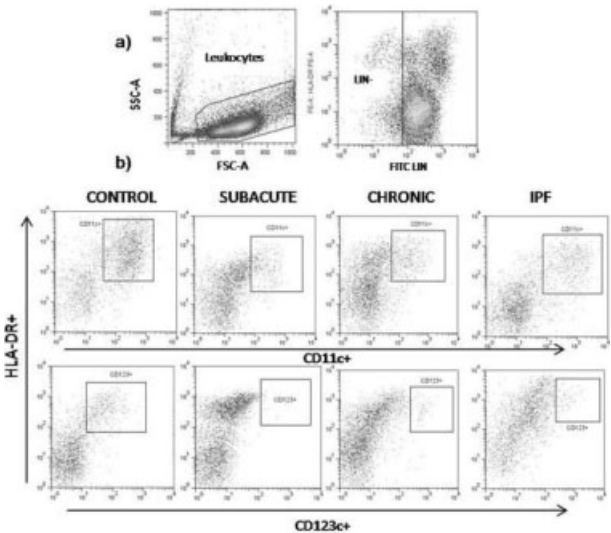
W.W. Unger<sup>1,2</sup>, S. Laban<sup>2</sup>, A.R. van der Slik<sup>2</sup>, B.O. Roep<sup>2</sup>  
<sup>1</sup>VUMC, Molecular Cell Biology and Immunology, Amsterdam, Netherlands, <sup>2</sup>LUMC, Immunohematology and Blood Transfusion, Leiden, Netherlands

Specific therapy with modulated dendritic cells (DC) may restore immunological tolerance, thereby obviating the need for chronic immunosuppression in transplantation or autoimmunity. In this study we compared the tolerizing capacity of dexamethasone (Dex) and 1α,25-dihydroxyvitamin D<sub>3</sub> (VD3) modulated DC. Treatment of monocytes with either VD3 or Dex resulted in DC with stable, semi-mature phenotypes compared to standard DC, with intermediate levels of co-stimulatory and MHC class II molecules, which remained unaltered after subsequent pro-inflammatory stimulation. IL-12p70 secretion was lost by VD3- and Dex-DC, whereas IL-10 secretion was unaffected. VD3-DC distinctly produced large amounts of TNFα. Both VD3- and Dex-DC possessed the capacity to convert CD4 T-cells into IL-10-secreting Treg potently suppressing the proliferation of responder T-cells. Yet, only Treg induced by VD3-DC exhibited antigen-specificity. VD3-, but not Dex-DC expressed significant high levels of PD-L1 upon activation. Blockade of PD-L1 during priming redirected T-cells to produce IFN-γ instead of IL-10 and abolished acquisition of regulatory capacity. Our findings demonstrate that both VD3- and Dex-DC possess durable but differential tolerogenic features, acting via different mechanisms. Both are potentially useful to specifically down-regulate unwanted immune responses and induce immune tolerance. These modulated DC appear suitable as adjuvant in antigen-specific clinical vaccination intervention strategies.

PA11/75 ALTERED BALANCE BETWEEN MYELOID AND PLASMACYTOID DENDRITIC CELLS AND IDENTIFICATION OF A NEW MYELOID-DERIVED SUPPRESSOR CELLS SUBPOPULATION IN BRONCHOALVEOLAR LAVAGE OF PATIENTS WITH HYPERSENSITIVITY PNEUMONITIS

L.M. Barrera<sup>1</sup>, F. Mendoza<sup>1</sup>, I. Sada-Ovalle<sup>1</sup>, M. Selman<sup>1</sup>  
<sup>1</sup>Instituto Nacional de Enfermedades Respiratorias 'Ismael Cosío Villegas', Immunochemistry, Mexico City, Mexico

**Objectives:** In order to gain a better understanding on the T cell regulation in hypersensitivity pneumonitis (HP), we evaluated: 1)the dendritic cells (DCs) profile; 2)a potential myeloid-derived suppressor cells (MDSCs) subpopulation and 3)apoptotic state of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in HP patients.  
**Methods:** We included 5 patients with subacute and 5 chronic HP, 5 healthy subjects and 5 with IPF. Bronchoalveolar lavage (BAL) dendritic cells were flow cytometrically identified as lineage (CD3,CD14,CD16,CD19,CD20,CD56) negative and HLA-DR<sup>+</sup> cells, and according to their expression of CD123 and CD11c. Apoptosis assay with annexin V was performed in T cells subpopulations of same patients.  
**Results:** Our results show a dominance of myeloid dendritic cells (mDCs) with a significant decrease in chronic HP and IPF patients compared with controls and subacute patients (p< 0.01).



[Dendritic cells subpopulations identified in BAL]

Activation markers (CD40 and CD80) were lower in HP patients. MDSC (CD11b+CD33+CD11c-HLA-DR-) were lower in chronic patients compared with subacute HP patients and controls.

Subpopulation	Controls	Subacute HP	Chronic HP	IPF
%LIN-HLA-DR+ CD11c+	32.2±6.6	5.3±1.1	12.4±4.9	23.4±9.3
%LIN-HLA-DR+ CD123+	6.2±3.8	0.5±0.3	1.1±1.6	0.43±0.3
mDCs:pDCs ratio	6.0±2.6	7.6±1.6	61.4±28	91.5±30.2
%LIN-HLA-DR+ CD11c+CD40+	72.6±5.2	1.3±0.4	1.4±0.1	2.4±0.7
%LIN-HLA-DR+ CD11c+CD80+	70.9±16.15	0.35±0.2	0.65±0.1	2.05±1.1
%CD11b+CD33+	44.3±8.7	16.4±3.8	7.9±6.3	5.8±2.1
%CD11b+CD33+ CD11c-HLA-DR-	5.2±1.2	6.44±3.1	2.1±0.8	1.1±1.1

[Dendritic cells and MDSCs identified in BAL of HP]

Apoptosis assay showed an increase of annexin V expression on CD4+ and CD8+T cells in chronic versus subacute HP patients (CD4+;53.5±4.9 vs 28±4.2;CD8+;45±4.2 vs 28±3.7 p< 0.01).

**Conclusions:** Our findings indicate that mDCs are decreased in HP patients that evolve to fibrosis supporting that a switch from Th1 to Th2 is playing a role in this process. MDSC were identified for the first time in HP and their decrease may be related to the anergic state of chronic patients.

#### PA11/76 EXPRESSION OF SIALYL LEWIS X IN HUMAN MONOCYTE-DERIVED DENDRITIC CELLS AND ITS RELEVANCE TO SELECTIN BINDING

Z. Silva<sup>1</sup>, C. Martins<sup>1</sup>, R. Castro<sup>2</sup>, P.A. Videira<sup>1</sup>

<sup>1</sup>Faculdade de Ciências Médicas, Departamento de Imunologia, Lisbon, Portugal, <sup>2</sup>Faculdade de Farmácia da Universidade de Lisboa, Lisbon, Portugal

**Objectives:** This study aims to investigate the expression of sialyl Lewis X (sLex) in Human monocyte-derived dendritic cells (DCs) and its relevance for the capacity of these cells to bind to selectins expressed by endothelium. We also intended to identify the enzymes responsible for sLex synthesis, namely fucosyltransferases, and to study the effect of different maturation stimuli on the expression.

**Materials and methods:** DCs were obtained as described previously (1). The expression of sLex was evaluated in immature DCs (iDCs) and DCs matured with IFN-γ and LPS by flow cytometry using anti-CD15s monoclonal antibody.

Adhesion assays were performed under static conditions by overlaying iDCs or neutrophils onto confluent TNF-α stimulated Human umbilical vein endothelial cell (HUVEC) monolayers. After washing of non-adherent cells, the remaining cells were stained for flow cytometry analysis.

The genetic expression of fucosyltransferases was evaluated using Real-Time PCR.

**Results:** sLex is expressed by iDCs, although the expression is lower than in neutrophils. Maturation stimuli affected this expression: LPS stimulation lowers whereas IFN-γ slightly raises sLex levels, compared with iDCs.

Under static conditions, the percentage of DCs or neutrophils that adhered to HUVEC cells was of 31% and 42%, respectively. Sialic acid removal affected both interactions; with a reduction to 19% of DCs and to 25% of neutrophils in the mixture.

DCs express FucT-IV and FucT-VII but do not express FucT-IX, FucT-VI or FucT-III. Upon DC maturation with IFN-γ, FucT-IV and FucT-VII are slightly upregulated. Given the level of expression of FucT-VII, it may be considered a relevant fucosyltransferase for sLex synthesis in DC.

**Conclusions:** We observed that sLex is expressed by DCs and that the expression is improved by IFN-γ. We are now investigating the importance of sLex for DC interaction with the endothelium under static and flow conditions. Since the successful use of DCs as vaccines is hindered by poor migration capacities induced by standard maturation protocols, understanding the role and modulation mechanisms of sLex synthesis in these cells may open new possibilities to improve their migratory capacities.

(1) Videira et al. 2008. *Glycoconj J* 25(3):259-68

#### PA11/77 SWINE INFLUENZA VIRUS (H3N2) INFECTION AND POLY-IC STIMULATION DIFFERENTIALLY UP-REGULATE SURFACE MARKERS AND CYTOKINE SECRETION ON PORCINE DENDRITIC CELLS

T. Mussá<sup>1</sup>, M. Pujol<sup>1</sup>, C. Rodríguez<sup>1</sup>, E. Silva<sup>2</sup>, L. Córdoba<sup>1</sup>, E. Crisci<sup>1</sup>, N. Busquets<sup>1</sup>, J. Maldonado<sup>3</sup>, J. Hernández<sup>2</sup>, J. Domínguez<sup>4</sup>, L. Fraile<sup>1</sup>, M. Montoya<sup>1</sup>

<sup>1</sup>Centre de Recerca en Sanitat Animal (CRESA), UAB-IRTA, Campus de la Universitat Autònoma de Barcelona, Bellaterra, Spain, <sup>2</sup>Laboratorio de Inmunología, CIAD A.C. Hermosillo, Sonora, Mexico, <sup>3</sup>Laboratorios HIPRA, S.A., Girona, Spain, <sup>4</sup>INIA, Autopista A-6, Km 7, Dpt. of Biotecnología, Madrid, Spain

**Objective:** There is growing evidence that the so-called “early” cytokines play an important role in swine influenza virus (SIV) infection, implicating the cells involved in secreting these cytokines. Therefore, our main goal was to characterise dendritic cell (DC) interaction with SIV *in vitro*.

**Methods:** Porcine bone marrow-derived DC and monocyte-derived DC were generated as previously described<sup>1,2</sup>. Pellets of DC were evaluated at electron microscopy (EM) Jeol 1400. SIV H3N2 was isolated from a recent outbreak in Spain. DC were infected at MOI 0.01 or stimulated with TLR agonists (Poly-IC, LPS, R837 or CpG). DC phenotype was analysed by flow cytometry at 8, 16 and 24 hours. IFN-α, TNF-α and IL-18 secretion were analysed by ELISA. qRT-PCR was performed for IL-10, TGF-β and FOXP3 gene expression as previously described<sup>3</sup>.

**Results:** DC revealed a defined vacuolar aspect with several dendritic processes in the cells by EM. Control DC in our assays were CD172a<sup>+</sup>, SLAI<sup>+</sup>, SLAII<sup>+</sup>, CD4<sup>+</sup>, CD11R1<sup>+</sup>, CD14<sup>low</sup>, CD16<sup>low</sup>, CD80/86<sup>low</sup> and CD163<sup>low</sup>. After stimulation with TLR agonists, they exhibited a differential response, being high responders to Poly-IC and moderate responders to LPS and CpG by cytokine secretion. However, R837-stimulated cells produced more IL-18 compared with their counterparts. Infected-DC presented different phenotype by means of SLAII up-regulation. IFN-α production increased with time whereas TNF-α and IL-18 decreased. No induction of IL-10, TGF-β and FOXP3 mRNA was detected as compared with mock treated-DC.

**Conclusion:** Ultrastructural and phenotypical analysis of our DC corresponds with previously described data<sup>3</sup>. DC exhibited a differential response to TLR agonists, suggesting a specific function for pathogen detection. Up-regulation of surface markers and increased levels of innate cytokines after SIV infection *in vitro* correlate with data from an early SIV infection *in vivo*<sup>4</sup>. These data pave the way for understanding the intimate relation between SIV and porcine dendritic cells for triggering a protective immune response.

<sup>1</sup>Kekarainen, T. et al., *Vet Immunol Immunopathol* (2008).

<sup>2</sup>Flores-Mendoza, L. et al., *Clin Vaccine Immunol* (2008).

<sup>3</sup>Carrasco, C.P. et al., *Immunology* (2001); Summerfield, A. and McCullough, K.C. *Dev Comp Immunol* (2008).

<sup>4</sup>Van Reeth, K. et al., *Vet Immunol Immunopathol* (2002).

#### PA11/78 BONE MARROW DERIVED DENDRITIC CELLS FROM TRAUMATIZED MICE SHOW REDUCED CAPACITY IN INDUCING T CELL RESPONSES

H. Liang<sup>1</sup>, Z. Wang<sup>1</sup>

<sup>1</sup>Research Institute of Surgery, Daping Hospital, Third Military Medical University, Dept.1, Chongqing, China

The change of dendritic cells functions after trauma has been reported, but it is unknown if bone marrow derived dendritic cells (BMDC) change their functions following injury. Here we studied the capacity in inducing allogeneic T cell responses of bone marrow derived dendritic cells (BMDC) in mice after hemorrhage combined with closed femur fracture. DC were induced *in vitro* from bone marrow isolated 24 h after hemorrhage combined with closed femur fracture, then their capacity in inducing allogeneic T cell responses were determined by mixed lymphocytes reaction (MLR), expression of MHC class II, CD40, CD80, and CD86 on DC surface were measured using flow cytometry, and IL-12 and IL-10 levels in LPS-stimulated DC supernatants were detected by ELISA kits. The results showed that the ability of BMDC to stimulate allogeneic T cells proliferation was decreased after injury, and the expression of CD40 on DC surface was significantly lowered than that of controls, whereas the expression of MHC class II, CD80, and CD86 on DC surface unaltered. IL-12p40, IL-12p70 secretion in LPS-stimulated DC supernatants were reduced while IL-10 levels unchanged following injury. It is suggested that bone marrow derived dendritic cells from traumatized mice show reduced capacity in inducing T cell responses, which may be partially attributable to decreased expression of costimulatory molecule CD40 and inadequate IL-12 secretion.

#### PA11/79 DENDRITIC CELL-T CELL CYTOKINE RESPONSES TO DER P 1 ALLERGEN PRESENTED BY DENDRITIC CELLS IN THE PRESENCE OF BCG BACILLI IN ALLERGIC PATIENTS AND HEALTHY DONORS

M. Kowalewicz-Kulbat<sup>1</sup>, P. Szpakowski<sup>1</sup>, S. Kosinski<sup>2</sup>, M.L. Kowalski<sup>2</sup>, F. Biet<sup>3</sup>, J. Pestel<sup>4</sup>, W. Rudnicka<sup>1</sup>

<sup>1</sup>University of Lodz, Department of Immunology and Infectious Biology, Lodz, Poland, <sup>2</sup>Medical University of Lodz, Department of Immunology, Rheumatology and Allergy, Lodz, Poland, <sup>3</sup>INRA Centre de Tours, UR918, Nouzilly, France, <sup>4</sup>Universite des Sciences et Technique de Lille, CNRS FRE2933, Lille, France

**Background:** Dendritic cells (DC) play a key role in regulating Th1/Th2 immunity. Environmental allergen-driven diseases are mediated by the expansion of the CD4+ Th2 subset of T cells. Der p 1, the major allergen of *Dermatophagoides pteronyssinus* was used in the study as 80% of the asthmatic patients develop allergy to house dust mite.

**Objectives:** We asked a question whether *Mycobacterium bovis* BCG vaccine and a recombinant BCG producing human IL-18 (rBCG-IL-18) were able to polarize Th2 towards Th1 lymphocyte response in the presence of Der p 1.

**Methods:** Blood was collected from patients with asthma positive to Der p 1 in skin prick test and healthy BCG vaccinated donors. Immature monocyte-derived dendritic cells (MD-DCs) were obtained after 6 day culture in the presence of IL-4 and GM-CSF and stimulated for 24 h with Der p 1 in absence or presence of *M. bovis* BCG bacilli or rBCG-IL-18. The production of IL-10, IL-12p70 by MD-DCs was quantified by ELISA. The effect of pulsed MD-DCs on naive T cells was evaluated by determining the T cell cytokine profile (IFN-γ, IL-5 and IL-10). Statistical significance was estimated by using the Mann-Whitney test.

**Results:** Der p 1-pulsed DC in the presence of BCG but especially rBCG-IL-18 increased the IL-10 production compared with Der p 1-pulsed DC in the group of healthy donors and allergic patients. Moreover, rBCG-pulsed DC from healthy donors but not allergic patients produced IL-12p70. Autologous naive T cells cocultured with mycobacterial antigen alone or in the presence of Der p 1-pulsed MD-DCs from allergic patients produced significantly less IFN-γ and more IL-5 compared with identically stimulated naive T cells from healthy donors. Naive T cells from healthy compared with allergic donors produced significantly more IL-10 when stimulated with rBCG alone or in the presence of allergen-pulsed DC.

**Conclusions:** The BCG vaccine was shown to display *in vitro* the ability to stimulate the Th1 response in the presence of Der p 1 allergen. The stimulatory effect of BCG was significantly increased by IL-18 and additionally enhanced by IL-12 in healthy subjects. Supported by MNiSZW N N401015236



**PA11/80 PHENOTYPIC AND FUNCTIONAL CHARACTERISATION OF DENDRITIC CELLS IN SPONTANEOUS MOUSE LYMPHOMA**M. Naujoks<sup>1</sup>, C. D. Brenner<sup>1</sup>, M. Przewoznik<sup>1</sup>, G. W. Bornkamm<sup>2</sup>, R. Mocikat<sup>1</sup><sup>1</sup>Helmholtz-Zentrum München, Institut für Molekulare Immunologie, München, Germany, <sup>2</sup>Helmholtz-Zentrum München, Institut für Klinische Molekularbiologie, München, Germany

Dendritic cells (DCs) have been shown to activate natural killer cells and to induce tumour rejection and long-term cytotoxic T-cell memory. However, the mechanisms by which DCs can activate the innate as well as the acquired immune system are not fully understood. Interestingly, in some tumour patients and tumour mouse models, DCs are functionally impaired. We analysed the phenotype and functional capability of DCs in c-myc transgenic mice that develop spontaneous B-cell lymphoma. In spleens and lymph nodes from tumour-bearing c-myc mice, the percentage of CD11c-positive DCs was increased in comparison to wildtype mice suggesting an elevated migration of DCs to the tumour sites. Several DC activation markers, like CD80, CD83, CD86 and MHCII were up-regulated. In vitro differentiation of bone marrow precursor cells from c-myc mice to DCs was not impaired. The activation status of DCs was determined by quantifying IL-12 production by flow cytometry as well as by RT-PCR using positively selected DCs. Transcript levels were normalised to the expression of a housekeeping gene. Allo-T cell stimulatory activity of CD11c-positive DCs of both wildtype and lymphoma mice was determined by co-culture of BALB/c mice to cultures of bone marrow-derived DCs followed by quantitation of IFN- $\gamma$  production. Furthermore, stimulation assays were performed using naive OVA-specific T cells that were cocultured with DCs loaded with OVA-peptides.

**PA11/81 COMPARING THE UPTAKE OF THE MAJOR BIRCH POLLEN ALLERGEN BET V 1.0101 AND ITS HOMOLOGUE FROM CELERY, API G 1.0101, BY HUMAN IMMATURE DENDRITIC CELLS**U. Smole<sup>1</sup>, D. Mechtcheriakova<sup>1</sup>, C. Radauer<sup>1</sup>, N. Balazs<sup>1</sup>, M. Bublin<sup>1</sup>, S. Wagner<sup>1</sup>, K. Hoffmann-Sommergruber<sup>1</sup>, F. Ferreira<sup>2</sup>, M. Wallner<sup>2</sup>, E. Jensen-Jarolim<sup>1</sup>, H. Breiteneder<sup>1</sup><sup>1</sup>Medical University of Vienna, Department of Pathophysiology, Center of Physiology, Pathophysiology, and Immunology, Vienna, Austria, <sup>2</sup>University of Salzburg, Christian Doppler Laboratory of Allergy Diagnosis and Therapy, Department of Molecular Biology, Salzburg, Austria

**Objective:** While Bet v 1.0101 is the most relevant sensitizing protein for birch pollen (BP) allergic patients, its homologues from plant foods are involved in allergic reactions only as a consequence of IgE cross-reactivity. Dendritic cells (DCs) are critical in internalizing, processing and presenting antigens to allergen-specific T cells. The aim of this study was to investigate whether the different allergenic properties of Bet v 1.0101 compared to Api g 1.0101, its homologue from celery, are reflected in the capture and uptake by DCs.

**Methods:** Bet v 1.0101 and Api g 1.0101 were labeled with Alexa Fluor 488 and 610, respectively. To exclude effects of the labeling procedure on the secondary structure of the proteins circular dichroism (CD) analysis was performed. Functional activity of labeled Bet v 1.0101 and Api g 1.0101 was tested by competitive IgE ELISA with unlabeled proteins. Immature DCs were generated from peripheral blood monocytes of BP allergic and healthy donors by *in vitro* culture with GM-CSF and IL-4. Labeled allergens (25  $\mu$ g/ml) were added to immature DCs on day 6 of culture. Internalization of labeled allergens was followed using live cell imaging.

**Results:** Labeled Bet v 1.0101 and Api g 1.0101 displayed native protein conformations as confirmed by CD measurements and retained their full IgE binding capacity. The proteins were taken up by DCs of both healthy and BP allergic donors in a time-, dose-, and temperature-dependent manner. Protein binding to the surface of DCs occurred within 10-20 minutes of exposure and internalization was observed after 30-40 minutes. Similar kinetics of Bet v 1.0101 and Api g 1.0101 uptake were observed for both allergic and healthy donors with some donor dependent variations.

**Conclusion:** We demonstrated that DCs of both BP allergic and healthy donors are able to take up Bet v 1.0101 and its homologous food allergen Api g 1.0101. Ongoing studies will clarify whether different mechanisms are involved in Bet v 1.0101 uptake in allergic individuals possibly leading to the induction of signals for Th2 polarization. Study is supported by the Austrian Science Fund; grant SFB-F01802 and -08.

**PA11/82 FIRST ASPECTS OF THE TIME DEPENDENCY IN IMMUNE MODULATION BY DENDRITIC CELLS**R. Luger<sup>1</sup>, S. Valookaran<sup>1</sup>, A. M. Dohnal<sup>1</sup>, T. Felzmann<sup>1</sup><sup>1</sup>Children's Cancer Research Institute, Vienna, Austria

Tolerance, immune stimulation and suppression are induced by dendritic cells (DC) at different stages of maturation. After short exposure to Toll-like-receptor (TLR)-4 ligand Lipopolysaccharide (LPS) under pro-inflammatory conditions human monocyte derived DCs produce TH1 polarizing Interleukin (IL)-12 and express increased amounts of co-stimulatory surface-molecules. Shortly after the peak expression of IL-12 the immune-suppressive cytokine IL-10, and other immune regulatory molecules like thymic stromal lymphopoietin (TSLP), G-CSF and GM-CSF, start to be expressed (see Abstract Dohnal A.M. et al.). This indicates that after a short maturation dependent stimulatory window the DC shifts its phenotype from stimulation towards mainly regulatory/suppressive functions.

We investigated the effects of the key immune-modulatory mechanisms targeting T cell (TC) stimulation by performing allogeneic mixed lymphocyte reaction (alloMLR) for different DC:TC co-culture intervals at a 1:1 ratio (DC:TC). TC were separated from DCs after 6 h, 24 h and 48 h and re-cultivated until day 6 of alloMLR. As control classical alloMLR was performed by continuous co-culture of 6 h pre-matured DCs with TC in 1:1 ratio for 6 days without separation. We observed that DCs, early after LPS/Interferon (IFN)- $\gamma$  maturation, show a high immune stimulatory capacity, which later-on is down modulated by delayed regulatory and suppressive mechanisms. Also the feedback signals from TCs targeting DCs, like CD40-CD40L ligation, were investigated and their immune-modulatory capacity was determined by signalling blockade and spherical separation.

Compared to classical alloMLR a short DC:TC contact for only 6 h leads to increase of TC-proliferation in both CD4<sup>+</sup> and CD8<sup>+</sup> TC subsets, measured in percentage proliferation using carboxyfluorescein-succinimidyl-ester (CFSE) dilution and in absolute cell numbers on day 6 of alloMLR. Cytokine expression profiling under time dependent manners after activation provides evidence for our hypothesis that DCs have an early immune stimulatory and a late regulatory/suppressive function that additionally is modulated by diverse signals administered from activated TC in alloMLR.

**PA11/83 INTERACTION OF HUMAN CD8<sup>+</sup> T CELLS AND DENDRITIC CELLS RESULT IN DIFFERENTIATION OF MONOCYTES TO TH1 PROMOTING INFLAMMATORY CELLS**S. Z. Chong<sup>1</sup>, K. L. Wong<sup>1</sup>, G. Lin<sup>1</sup>, P. A. Macary<sup>1</sup>, D. M. Kemeny<sup>1</sup><sup>1</sup>National University of Singapore, Immunology Programme, Singapore, Singapore

CD8<sup>+</sup> T cells are important for combating viral and intracellular pathogens by eliminating infected cells through cytotoxic killing. They have also been shown to play a helper role by interacting with dendritic cells (DCs) to induce the production of IL-12p70, an important cytokine for TH1 immunity. Notably, the maintenance of this TH1 response may rely on the recruitment of other immune cells such as monocytes, the blood precursors of macrophages and DCs. However, how this may happen is still unclear.

Here, we show that activated human CD8<sup>+</sup> T cells were able to prime immature DCs in the presence of a toll-like receptor (TLR) agonist to produce IL-12p70, demonstrating the intergration of TLR and CD8<sup>+</sup> T-cell mediated signals which were independent on CD4<sup>+</sup> T cell help. This interaction of CD8<sup>+</sup> T cells and DCs also resulted in production of inflammatory cytokines such as GM-CSF, IL-1, IL-6, IFN- $\gamma$  and TNF- $\alpha$  as well as chemokine production of IP-10, MCP-1, MIP-1 $\alpha$ , MIP-1 $\beta$  and RANTES for the recruitment of inflammatory cells. Finally, monocytes that were exposed to the CD8-DC cytokine milieu displayed an inflammatory phenotype that had antigen-presenting capabilities demonstrated using a mixed lymphocyte reaction and induced TH1 polarization of naive CD4<sup>+</sup> T cells to produce IFN- $\gamma$ . This study demonstrates how CD8<sup>+</sup> T cells could influence the immune environment by interacting with DCs, thereby acerbating chronic TH1 mediated immunopathology or how they could be used to correct pathogenic TH2-bias atopy.

**PA11/84 HIGH MOLECULAR WEIGHT COMPONENTS FROM ASCARIS SUUM DOWN-MODULATE THE ANTIGEN PRESENTING CELLS ACTIVITY**B. C. Favoretto<sup>1</sup>, S. R. Silva<sup>2</sup>, J. F. Jacysyn<sup>2</sup>, N. O. S. Câmara<sup>2</sup>, E. L. Faquim-Mauro<sup>1</sup><sup>1</sup>Butantan Institute, São Paulo, Brazil, <sup>2</sup>São Paulo University, São Paulo, Brazil

**Introduction:** We have previously shown that high molecular-weight components (PI) obtained from *Ascaris suum* extract inhibit the Th1/Th2-related immune responses induced by ovalbumin (OVA)-immunization in mice. We also described that PI down-modulates the expression of MHC class II and costimulatory molecules and the function of APCs, mainly dendritic cells (DCs), via an IL-10-dependent mechanism. DCs are able to discriminate and to be activated by pathogenic compounds through of expression of distinct receptors (PRRs).

**Objectives:** To evaluate the role of the TLR4 in the suppressive effect induced by PI on OVA-response and also to investigate the ability of PI to interfere on the maturation process of iDCs induced by LPS.

**Method:** Wild type or TLR4KO mice were immunized with OVA (100mg) or OVA+PI (200 mg). After 5 days, lymph node cells from these mice were prepared and stained with anti-CD80, CD86, CD40 or MHC class II-FITC/PE mAbs. iDCs derived from bone marrow of BALB/c mice were incubated with LPS, PI or LPS+PI for 18 h and stained with anti-CD80, CD86, CD40 or MHC-II-FITC/PE MoAbs. IL-12 was also measured by ELISA in culture supernatants.

**Results:** The flow cytometry analysis showed reduction of MHC-II and costimulatory molecules expression by cells of OVA+PI-immunized WT mice compared to OVA-primed cells. In contrast, no difference was observed in these molecules expression in cells from TLR4KO mice immunized in the same way. Higher CD40, CD80, CD86 and MHC-II expression was verified in DCs incubated with LPS compared with iDCs. On the other hand, it was verified an inhibition of these molecules expression and IL-12 secretion in DCs incubated with LPS+PI.

**Conclusion:** The results show that TLR4 is not involved in the immunosuppression induced by PI. Moreover, the PI components are able inhibit the DCs maturation and IL-12 secretion induced by a potent TLR4 ligand, as the LPS. Financial support: CNPq and FAPESP.

**PA11/85 IMMUNOMODULATORY EFFECTS OF GANODERMA MICROSPORUM IMMUNOMODULATORY PROTEIN ON BONE MARROW-DERIVED DENDRITIC CELLS AND IMMUNE RESPONSE**Y.-S. T. Tseng<sup>1</sup>, C.-P. Liao<sup>1</sup>, B.-L. Chiang<sup>1</sup><sup>1</sup>National Taiwan University, Taipei, Taiwan, Republic of China

**Objectives:** GMI is a small protein derived from *Ganoderma microsporum*. It only has 18 amino acids different from LZ-8, which is an immunomodulatory protein proven in the previous reports. In this study, we like to study the effect of GMI on the BM-DCs and subsequent immune response.

**Methods:** DCs derived from bone marrow stem cells (BMDCs) of 4–6 week old female BALB/c mice are treated with GMI for 24 hrs. The maturation of BMDCs is assayed by flow cytometry and the cytokine levels are measured by ELISA kit. Moreover, we co-culture the BMDCs with the allogenic naïve CD4<sup>+</sup> T cells for 48 hrs. Then, cytokine secretion and proliferation level of T cells are assayed. Finally, we try to find out the possible stimulating pathway by anti-TLR neutralization assay.

**Results:** The level of IL-12 p40 secreted from GMI-induced BMDCs was increased in a dose dependent manner. Although the expressing pattern of CD11c and MHCII did not significantly increase after GMI stimulation, the proliferation of the naïve CD4<sup>+</sup> T cells still markedly increased. In addition, T cells tended to secrete higher IFN $\gamma$  after co-culturing with the GMI treated BMDCs.

**Conclusion:** GMI was found to induce activation and functional maturation of BMDCs. Though the maturation cannot obviously be detected by the expression of surface markers, GMI treated BMDCs can make the naïve CD4<sup>+</sup> T cells proliferate dramatically and secrete higher IFN $\gamma$ . In addition, we also perform *in vivo* study and clarify the immunomodulatory role of GMI in animal model.

PA11/86 Abstract withdrawn by author

**PA11/87 THE ROLE OF SWAP-70 IN ADHESION AND MIGRATION OF MURINE DENDRITIC CELLS**A. Götz<sup>1</sup>, C. Ocana-Morgner<sup>1</sup>, S. Braungart<sup>1</sup>, R. Jessberger<sup>1</sup><sup>1</sup>Dresden University of Technology, Medical School, MTZ, Institute of Physiological Chemistry, Dresden, Germany

Dendritic cells (DCs) reside in peripheral tissues or blood and migrate to secondary lymphoid organs upon encountering microbial products such as LPS to initiate immune responses by priming naïve T cells. This “maturation” process is typically accompanied by changes in DC morphology and behavior. For example, DCs rearrange their actin cytoskeleton to migrate rapidly through complex environments in the interstitium.

Murine SWAP-70 is expressed in hematopoietic cells including DCs obtained from bone marrow precursors (BMDCs) or spleen (SDCs). SWAP-70 regulates adhesion and migration in B cells<sup>2</sup> and mast cells<sup>3</sup>. SWAP-70 also controls surface localization of peptide-loaded MHCII on BMDCs<sup>1</sup> and interacts with F-actin, Rac and RhoA-GTP. Rho GTPases play important roles in regulating DC functions, e.g. macropinocytosis/endocytosis, upregulation of surface MHCII molecules, efficient T-cell priming and migration. Unlike wild-type (wt) BMDCs, *Swap-70*<sup>-/-</sup> BMDCs show constitutively active RhoA<sup>1</sup>.

Initial experiments on adhesion and migration showed that in transfer experiments *in vivo* SWAP-70 deficient LPS-matured BMDCs traffic less efficiently to the draining lymph node than wt BMDCs. Furthermore, *Swap-70*<sup>-/-</sup> BMDCs showed reduced velocity of non-directional movements and impaired polarization on FN *in vitro*. To investigate DC migration, we are analyzing the response to CCL19 and CCL21 in transwell assays. Adhesion features of *Swap-70*<sup>-/-</sup> BMDCs are studied in static adhesion assays using different ECM and adhesion components (Fibronectin, RGDS-peptide, ICAM-1, VCAM-1). Initial data suggest hyperadhesion to FN in the absence of SWAP-70. We are further analyzing transendothelial migration and formation and maintenance of specific F-actin-rich structures implicated in cell migration called podosomes.

Considering the features of SWAP-70 known from other cell types and the phenotypes seen in DCs, it is likely that SWAP-70 plays an important role in regulating adhesion and migration of DCs.

1: Ocana-Morgner et al., Blood. 2009 Feb 12;113(7)

2: Pearce et al., Nat Immunol. 2006 Aug;7(8)

3: Sivalenka et al., Mol Cell Biol. 2004 Dec;24(23)

**PA11/88 NEURON-INTERACTING SATELLITE GLIAL CELLS IN HUMAN TRIGEMINAL GANGLIA HAVE AN ANTIGEN PRESENTING CELL PHENOTYPE**M. van Velzen<sup>1</sup>, J.D. Laman<sup>2,3</sup>, A. Kleinjan<sup>4</sup>, A. Poot<sup>1</sup>, A.D.M.E. Osterhaus<sup>1</sup>, G.M.G.M. Verjans<sup>1</sup><sup>1</sup>Erasmus Medical Center, Virology, Rotterdam, Netherlands, <sup>2</sup>Erasmus Medical Center, Immunology, Rotterdam, Netherlands, <sup>3</sup>MS Center ErasMS, Rotterdam, Netherlands, <sup>4</sup>Erasmus Medical Center, Pulmonary Medicine, Rotterdam, Netherlands

Satellite glial cells (SGC) in sensory ganglia tightly envelop the neuronal cell body to form discrete anatomical units. This type of glial cell is considered neuroectoderm-derived and provides physical support to neuron somata. There are scattered hints in the literature suggesting that SGC have an immune-related function within sensory ganglia. Here, we addressed the hypothesis that SGC are tissue-resident APC. The immune phenotype and function of a large series (n=40) of human trigeminal ganglia (TG) were assessed by detailed flow cytometry, *in situ* analyses, and functional *in vitro* assays. Human TG-resident SGC (TG-SGC) uniformly expressed the common leukocyte marker CD45, albeit at lower levels compared to infiltrating T-cells, and the macrophage markers CD14, CD68 and CD11b. In addition, TG-SGC expressed the myeloid dendritic cell (mDC) marker CD11c, the T-cell co-stimulatory molecules CD40, CD54, CD80, and CD86 and MHC class II. However, the mature DC marker CD83 was absent on TG-SGC. Functionally, TG-SGC phagocytosed fluorescent bacteria, but were unable to induce an allogeneic mixed leukocyte reaction. Finally, TG-infiltrating T-cells expressed the T-cell inhibitory molecules CD94/NKG2A and PD-1, and the interacting TG-SGC expressed the cognate ligands HLA-E and PD-L1, respectively. In conclusion, the data demonstrate that human TG-SGC have a unique leukocyte phenotype, with features of both macrophages and immature mDC, indicating that they have a role as TG-resident APC with potential T-cell modulatory properties.

**PA11/89 MULTIPLE ROLES OF VITAMIN A IN IMPRINTING OF GUT TROPISM AND EFFECTOR T CELL FUNCTION**E. Jaensson<sup>1</sup>, K. Kotarsky<sup>1</sup>, F. Zapata<sup>1</sup>, W. W. Agace<sup>1</sup><sup>1</sup>Lund University, Immunology, Lund, Sweden

**Objectives:** Dendritic cells (DCs) play a key role in antigen presentation and in priming of T cells. In addition, DCs induce tissue homing receptors on T cells. We have previously identified a subset of DCs in mesenteric lymph nodes (MLN) and small intestinal lamina propria (SILP) that express the integrin alpha chain CD103, and which show a unique ability to induce the gut homing receptor CCR9 on responding T cells. This ability is driven, at least in part, by a common underlying ability of intestinal DCs to generate the Vitamin A metabolite, retinoic acid (RA). In agreement with this, CD103<sup>+</sup> dendritic cells from MLN and SILP expressed higher levels of *aldh1a2*, the gene encoding RALDH2 (the key enzyme involved in the generation of RA from vitamin A derivatives) compared to CD103<sup>-</sup> dendritic cells. In this study we wanted to examine the role of Vitamin A for imprinting of DCs and in the generation and effector functions of T cells.

**Methods:** In the current study we have used *in vitro* DC:T cell co-culture systems and adoptive transfer models which have been analyzed using mainly microarray and flow cytometry.

**Results and conclusions:** While short term deprivation of dietary Vitamin A does not impede on the generation of gut tropic T cells, long term deprivation reduces the ability of DCs to generate gut tropic T cells in co-cultures even when Vitamin A metabolites are present in these co-cultures. Further, using microarray studies Vitamin A metabolites were found to regulate the expression of more than 500 genes in effector T cells. This indicates that apart from having a role in imprinting of gut tropism on T cells, Vitamin A derivatives have a role in imprinting DCs as well as effector T cell function.

**PA11/90 EXAGGERATED INFLAMMATORY RESPONSE OF PRIMARY HUMAN MYELOID DENDRITIC CELLS TO LIPOPOLYSACCHARIDE IN PATIENTS WITH INFLAMMATORY BOWEL DISEASE**D.C. Baumgart<sup>1</sup>, S. Thomas<sup>1</sup>, I. Przesdzin<sup>1</sup>, D. Metzke<sup>1</sup>, C. Bielecki<sup>1</sup>, S.M. Lehmann<sup>2</sup>, S. Lehnardt<sup>2</sup>, Y. Dörfel<sup>1</sup>, A. Sturm<sup>1</sup>, A. Scheffold<sup>3</sup>, J. Schmitz<sup>3</sup>, A. Radbruch<sup>3</sup><sup>1</sup>Charité Campus Virchow Klinikum, Medizinische Klinik mit Schwerpunkt Hepatologie und Gastroenterologie, Berlin, Germany, <sup>2</sup>Cecilie Vogt Klinik für Neurologie, Berlin, Germany, <sup>3</sup>Deutsches Rheumaforschungszentrum, Berlin, Germany

Inflammatory bowel disease (IBD) results from a breakdown of tolerance towards the indigenous flora in genetically susceptible hosts. Failure of dendritic cells to appropriately interpret molecular microbial patterns when directing innate and adaptive immune responses is conceivable. Primary (conventional, non-monocyte generated) CD1c+CD11c+CD14-CD16-CD19- myeloid blood or mucosal dendritic cells (mDC) from 76 patients with Crohn's disease (CD) or ulcerative colitis (UC) in remission, during flare-ups (FU) and 76 healthy or non-IBD controls were analyzed by flowcytometry (FACS) and real time polymerase chain reaction. Cytokine secretion of freshly isolated, cultured and LPS stimulated highly purified mDC (purity >95%) was assessed using cytometric bead arrays (CBA). More cultured and stimulated circulating mDC express CD40 in IBD patients. Stimulated circulating mDC from IBD patients secrete significantly more TNF- $\alpha$  and IL-8. TLR4 expression by mDC was higher in remission and significantly increased in flaring UC and CD patients compared with remission (p< 0.05) and controls (p< 0.001). Fluorochrome labeled LPS uptake by mDC was evaluated at different time points over 24h by measuring mean fluorescence intensity (MFI). Circulating mDC from IBD patients take up more LPS and the uptake begins earlier compared with controls (p< 0.05 in CD-FU and UC-FU at 24h). The frequency of mucosal mDC (p< 0.05) and the number of CD40 expressing mucosal mDC, is significantly greater in UC and CD compared with non-IBD controls (p< 0.001 vs. p< 0.01 respectively). Our data suggests an aberrant LPS response of mDC in IBD patients resulting in an inflammatory phenotype and possibly intestinal homing in acute flares.

**PA11/91 CYCLOPHOSPHAMIDE TREATMENT SPARES DC PRECURSORS IN THE BONE MARROW STIMULATING THEIR MOBILIZATION, PERIPHERAL DIFFERENTIATION AND TUMOR-INFILTRATION**L. Bracci<sup>1</sup>, G. Schiavoni<sup>1</sup>, A. Sisti<sup>1</sup>, S. Lorenzi<sup>1</sup>, F. Mattei<sup>1</sup>, M. Valentini<sup>1</sup>, M.T. D'Urso<sup>1</sup>, L. Gabriele<sup>1</sup>, E. Proietti<sup>1</sup>  
<sup>1</sup>Istituto Superiore di Sanità, Rome, Italy

Many clinical studies based on the combination of chemotherapy and other anticancer treatments have been published over the years showing variable responses. Recently, new knowledge has been generated on the immunomodulatory properties of some chemotherapeutic agents, such as cyclophosphamide (CTX), leading to a renewed interest in combination therapy regimens for cancer. In previous studies we observed that CTX can exert, on one hand, a direct effect on the tumor mass leading to the control of tumor growth and, on the other hand, an immunostimulatory activity through the modulation of the expression of various soluble factors (cytokine storm). Since the induction of an effective antitumor response requires the active participation of host APCs responsible for adequate antigen presentation and lymphocyte priming, we investigated the effects of CTX treatment on dendritic cells (DCs) *in vivo*.

We show that in mice implanted with EG7.OVA thymoma, CTX injection induced a transient reduction of total bone marrow cells, but not of DC precursors, which, instead, display enhanced proliferation and DC generation capabilities *in vitro*. Accordingly, the turnover rate of all DC subsets was markedly increased in CTX-treated mice as compared to controls. In particular, CD8α<sup>+</sup> conventional DCs, the key DC subset specialized in the cross-presentation of cell-associated antigens, undergo a transient and selective depletion followed by a rebound phase, in a way similar to what previously described for lymphocytes. In addition, CTX administration rendered the tumor mass more permeable to DC infiltration.

Currently, studies are being performed aimed at clarifying the function of the different DC subsets during chemotherapy, with the final aim of optimizing combination therapy protocols against malignancies.

**PA11/92 REGULATION OF TRAIL-MEDIATED APOPTOSIS IN PLASMACYTOID DENDRITIC CELLS**M. Balzarolo<sup>1</sup>, B. Blom<sup>2</sup>, J.P. Medema<sup>1</sup>, M.C. Wolkers<sup>1</sup>

<sup>1</sup>Academic Medical Center, University of Amsterdam, Laboratory for Experimental Oncology and Radiobiology (LEXOR), Center for Experimental and Molecular Medicine, Amsterdam, Netherlands, <sup>2</sup>Academic Medical Center, University of Amsterdam, Department of Cell Biology and Histology, Amsterdam, Netherlands

**Objectives:** Plasmacytoid dendritic cells (pDCs) are key players in antiviral immunity. When pDCs are activated through the Toll-like Receptor (TLR) 7 and 9, they exert the ability to clear infected cells through TNF-related apoptosis inducing ligand (TRAIL/Apo-2L)-mediated apoptosis. To date, the mechanisms that regulate TRAIL expression in pDCs are not fully understood. In this study we investigate the role of NGFI-A-binding protein 2 (Nab2), a transcriptional co-repressor that has been shown to control the expression of TRAIL in other cell types, for the regulation of TRAIL in pDCs.

**Methods:** We utilize a pDC-like cell line that expresses TRAIL upon TLR-mediated stimulation and that induces apoptosis of TRAIL-sensitive target cells.

**Results:** We show that TLR-mediated stimulation induces the expression of Nab2 as well as its cofactors Early growth genes1-3, and suggest a potential role of these factors for TRAIL regulation upon pDC stimulation. We are currently investigating which signals downstream of TLR signalling control the induction of Nab2.

**Conclusion:** Understanding the regulation of TRAIL in pDCs via Nab2 will provide insights in the molecular mechanisms that control cell fate and help us to comprehend how TRAIL-mediated killing can be exploited for therapeutic purposes.

**PA11/93 DENDRITIC CELL ACTIVATION REQUIRES ONLY FEMTOMOLAR CONCENTRATIONS OF LPS**G.A. Tynan<sup>1</sup>, E.C. Lavelle<sup>1</sup><sup>1</sup>Trinity College, Dublin, Ireland

**Objectives:** The activation of dendritic cells (DCs) is commonly used as a measure of the immunostimulatory activity of pathogen derived and endogenous proteins. However, many of these proteins are purified from bacterial expression systems. As a result, residual LPS contamination is a recurring theme and the potency of LPS is not always fully appreciated. To address this, polymyxin B is often used to neutralise contaminating LPS. The objective of this work was to determine the minimum LPS concentration required to induce DC maturation and cytokine secretion, and to assess the ability of polymyxin B to inhibit these processes.

**Methods and Results:** Murine BMDCs were stimulated with concentrations from 1pg/ml to 10µg/ml LPS, which was incubated alone or in the presence of polymyxin B (100µg/ml) for 2 hours at 37°C. Supernatants were collected 24 hours later and tested for the cytokines IL-6, TNF-α, IL-12p40 and IL-12p70 by ELISA. LPS concentrations as low as 20pg/ml induced secretion of IL-6 and TNF-α, while concentrations of 50pg/ml and 500pg/ml LPS were required to promote secretion of IL-12p40 and IL-12p70 respectively. The efficacy of polymyxin B varied for different cytokines and it was ineffective for LPS concentrations greater than 20ng/ml in the case of IL-6 and TNF-α. However, in the case of IL-12p40 and IL-12p70, the inhibitory properties of polymyxin B were preserved until LPS concentrations of 0.5µg/ml and 10µg/ml were reached respectively. DC maturation was assessed by expression of CD40, CD80 and CD86 by flow cytometry. A concentration of 50pg/ml LPS was enough to induce DC maturation, while LPS-driven DC maturation was no longer completely inhibited by polymyxin B at concentrations of greater than 20ng/ml.

**Conclusion:** Extremely low concentrations of LPS can promote DC activation. Interestingly, there are different thresholds for secretion of specific cytokines by DCs in response to LPS. Moreover, the assumption that the presence of polymyxin B eliminates LPS-mediated effects on DCs is inaccurate, and its effects are dependent on both the concentration of LPS present and the particular cytokines under investigation.

**PA12 – DENDRITIC CELLS IN PATHOLOGY****PA12/1 LAG-3: A NEW IMMUNE ESCAPE STRATEGY OF CYTOMEGALOVIRUS INFECTED DENDRITIC CELLS?**F. Haspot<sup>1</sup>, F. Halary<sup>1</sup><sup>1</sup>INSERM, U643, Nantes, France

In most healthy people, Human Cytomegalovirus infection (CMV) results in chronic but latent asymptomatic infection while in high-risks groups such as neonates, transplanted or HIV-infected patients CMV (re)activation is a major cause of disease and death. In order to highlight new molecular mechanisms by which CMV infections can escape the immune system and lead to chronic infections, we analyzed the gene expression pattern of CMV-infected (VHL-E strain, low passage) immature monocytes-derived dendritic cells (moDC). Using pangenomic DNA chip, we compared the gene expression of moDC infected with CMV during 6 and 48 hours to their non-infected counterpart. Out of the 40000 genes analyzed, more than 3000 genes were at least up- or down-modulated with a 2 fold change. Forty-eight hours following CMV infection, lymphocyte activation gene 3 (LAG-3; CD223) mRNA showed one of the highest fold difference (fd = +45). LAG-3 as CD4 is a member of the immunoglobulin superfamily and is also a MHC Class II high affinity ligand. While LAG-3 was known to be expressed on T, B and NK cells, it was only recently shown to be expressed on plasmacytoid dendritic cells. The up-regulation of mRNA encoding LAG-3 on CMV<sup>+</sup> moDC was confirmed by real-time quantitative PCR. Although TLR2 homodimer and TLR1/TLR2 heterodimer were shown to be functional sensor of CMV, their stimulation by Pam3CSK4 and HKLM (respectively) did not induced LAG-3 mRNA up-regulation. Therefore we hypothesize that LAG-3 mRNA upregulation is independent of signals provided by the interaction of the virus envelope with the DC membrane. Interestingly, stimulation with poly(I:C) and LPS (in a less extent) results in an increase of mRNA encoding LAG-3. An alternative membrane bound splicing variant (LAG-3V2) was detected after poly(I:C) treatment and the soluble variant of the molecules are now under investigation. LAG-3 upregulation following CMV infection might be a strategy by which CMV-infected cells escape the immune system thus contributing to a chronic infection setting.

**PA12/2 PROSTAGLANDIN E2 AND ANALOGS POTENTLY INHIBIT INTERFERON-ALPHA SECRETION BY PLASMACYTOID DENDRITIC CELLS VIA EP2 AND EP4 RECEPTOR ENGAGEMENT**D. Fabricius<sup>1</sup>, M. Neubauer<sup>1</sup>, B. Mandel<sup>1</sup>, C. Schütz<sup>1</sup>, A. Viardot<sup>2</sup>, A. Vollmer<sup>1</sup>, K.M. Debatin<sup>1</sup><sup>1</sup>Ulm University, Pediatrics, Ulm, Germany, <sup>2</sup>Ulm University, Internal Medicine, Ulm, Germany

**Objectives:** Plasmacytoid dendritic cell (pDC)-derived IFN-α plays a central role in anti-viral defense, as well as in certain autoimmune diseases such as systemic lupus erythematosus (SLE). Current information about the regulation of IFN-α and how it may be modulated therapeutically is limited. We therefore studied how pDC IFN-α secretion is affected by the immunomodulatory agents prostaglandin E2 (PGE2), the neuropeptide vasoactive intestinal peptide (VIP) and interleukin (IL)-10.

**Methods:** PBMC were acquired from healthy subjects or patients with active SLE (SLEDAI score > 8). Whole PBMC or isolated pDC were treated with various doses and combinations of PGE2, VIP and IL-10. Supernatants from isolated pDC were used for IFN-α ELISA. PBMC and isolated pDC were stained for surface markers and intracellular IFN-α and analyzed by flow cytometry.

**Results:** We found that PGE2, VIP and IL-10 were independent inhibitors of IFN-α secretion by pDC from healthy individuals. The inhibitory effect of VIP was enhanced by PGE2 and IL-10. Importantly, we were able to demonstrate potent inhibitory effects of PGE2 and VIP also on IFN-α secretion by pDC from SLE subjects. This inhibition was independent of age, disease activity and therapy, and was significantly stronger than that observed in healthy pDC. Since PGE2 showed the strongest effect, we studied several prostaglandin (PG) analogs and found that IFN-α inhibition was most potent with the analogs alprostadil and butaprost, whereas sulprostone had no effect. This showed that IFN-α inhibition of PGE2 was predominantly mediated via the PG receptors EP2 and EP4, which was confirmed by PCR and immunofluorescence.

**Conclusion:** We demonstrate that PGE2, VIP and IL-10 are potent inhibitors of pDC IFN-α secretion in healthy and SLE individuals. Particularly the PGE2 analogs alprostadil and misoprostol, which are already approved for the treatment of various indications, showed very strong effects in this context. Our results are therefore suggestive for their evaluation as treatment option for SLE and other IFN-α-dependent autoimmune diseases. Our data may also explain reports about the induction of SLE-like symptoms by cyclooxygenase inhibitors.



## PA12/3 GALECTIN-3 IS REQUIRED FOR THE AUTOIMMUNE INFLAMMATION IN THE ISLETS AND CNS

M.L. Lukic<sup>1,2</sup>, H.R. Jiang<sup>1</sup>, E. Mensah-Brown<sup>1</sup>, A. Shahin<sup>1</sup>, F.Y. Liew<sup>3</sup><sup>1</sup>Faculty of Medicine and Health Sciences, UAE University, Microbiology and Immunology, Al Ain, United Arab Emirates, <sup>2</sup>University of Kragujevac, Faculty of Medicine, Microbiology and Immunology, Belgrade, Serbia, <sup>3</sup>Glasgow Biomedical Research Center, Division of Immunology, Infection and Inflammation, Glasgow, United Kingdom

**Background:** Galectin-3 (Gal-3) is a member of the b-galactoside-binding lectin family and play an important role in inflammation. It is overexpressed in CNS after EAE induction and in the islet cells exposed to proinflammatory cytokines. However, the role of Gal-3 in autoimmune diseases is unclear. The aim of this study was to determine cellular and molecular basis of Galectin-3 involvement in the development of multiple low dose streptozotocin (MLD-STZ) induced diabetes and experimental allergic encephalomyelitis (EAE), two disease models which have similar immunopathogenesis.

**Methods:** MLD-STZ diabetes (5 × 40 mg STZ daily) and EAE (100 µg MOG<sub>35-55</sub> peptide in CFA + 2 × 100 µg pertussis toxin) were induced in male Gal-3 deficient (−/−) and “wild-type” C57BL/6 mice and disease evaluated by clinical and histological criteria. Infiltrating cells in the target tissue were evaluated by FACS analyses and cytokine expression by RT-PCR. Additionally capacity of dendritic cells to produce pro and anti-inflammatory cytokines and to induce lymphocyte proliferation and cytokine production after disease induction was also tested.

**Results:** We have demonstrated that in both experimental models Galectin-3 deficiency reduces the severity of diseases. The difference in clinical score (EAE) and glycemia (MLD-STZ-diabetes) was confirmed by quantitative histopathological analysis of the target tissue. Further, number and survival of CD11b<sup>+</sup> and CD11c<sup>+</sup> cells and the expression of TNF-α, IFN-γ, IL-17 and iNOS was significantly reduced in Gal-3<sup>−/−</sup> mice. After disease induction Gal-3<sup>−/−</sup> mice had significantly more IL-5 and IL-10 in the serum and more CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> cells in the spleen and target tissue. Finally, dendritic cells from gal-3<sup>−/−</sup> mice produced more IL-10 and enhanced Th-2 polarization.

**Conclusion:** Taken together our results demonstrate that galectin-3 plays an influential role in promoting MLD-STZ-induced diabetes and EAE and suggest it as possible target in therapy of organ-specific autoimmunity.

## PA12/4 INFLAMMATION INDUCES FURTHER DIFFERENTIATION OF MYELOID-DERIVED SUPPRESSOR CELLS AND CHANGE OF FUNCTION

D. Labrousse<sup>1</sup>, O. Cexus<sup>1</sup>, T. Elliott<sup>1</sup>, S. Quarantino<sup>1</sup><sup>1</sup>University of Southampton, Cancer Sciences Division – MP 824, Southampton, United Kingdom

Myeloid Derived Suppressor Cells (MDSC) are immature myeloid precursors that express Gr1 and CD11b markers. Their expansion has been largely described in cancer patients and in tumour-bearing mice. MDSC accumulate in the tumours and peripheral tissues where they inhibit the activity of anti-tumour T cells.

Cancer and autoimmune diseases (AID) are both chronic inflammatory diseases and have in common the infiltration of tissues by monocytic immune cells, the release of soluble factors such as cytokines (IL-1b, IL-6, TNFα) and the activation of p38 MAPK and NF-κB. The aim of this project is to investigate the effect of the inflammation in AID on the phenotype and function of MDSC.

The TAZ10 TCR transgenic mouse model develops spontaneous autoimmune thyroiditis with clinical, hormonal and histological signs comparable to the human disease. In this model, MDSC accumulate in both lymphoid and non-lymphoid organs at the onset and initial phase of the disease and in-vitro they strongly inhibit T cell responses against the specific antigen as well as against αCD3/CD28 stimulation. Strong of these results, we then tried to implement the use of MDSC in-vivo as a potential tool to provide protection and block disease progression by inhibiting the activation of self-reactive T cells.

Contrary to our expectations, adoptive transfer of purified MDSC into TAZ10 mice had a detrimental effect, as adoptively transferred mice all died prematurely within 6 weeks due to a very aggressive autoimmune condition. These results strongly suggested a switch in the function of MDSC from inhibitory to activatory. We used B6.eYFP mice expressing ubiquitously YFP to track down MDSC in-vivo and help understanding the role played by MDSC. These cells showed a significant increased expression of CD11c, F4/80 and MHC class II molecules, all specific markers defining professional Antigen Presenting Cells (APC) like dendritic cells and macrophages. Therefore, we showed that upon inflammation, MDSC do not retain their suppressive phenotype but start differentiating into mature cells that promote inflammation.

## PA12/5 CHARACTERIZATION OF DENDRITIC CELL FUNCTIONAL DEFECTS IN WISKOTT-ALDRICH SYNDROME

M. Catucci<sup>1,2</sup>, P. Larghi<sup>3</sup>, M. Bosticardo<sup>2</sup>, A. Doni<sup>4</sup>, A. Sica<sup>3</sup>, M.G. Roncarolo<sup>1,2</sup>, A. Villa<sup>1,5</sup><sup>1</sup>San Raffaele Telethon Institute for Gene Therapy, Milan, Italy, <sup>2</sup>Vita-Salute San Raffaele University, Milan, Italy, <sup>3</sup>Fondazione Humanitas per la Ricerca, Rozzano (Milan), Italy, <sup>4</sup>Istituto Clinico Humanitas IRCCS, Rozzano (Milan), Italy, <sup>5</sup>Istituto di Tecnologie Biomediche, CNR ITB, Human Genome Department, Segrate (Milan), Italy

Wiskott-Aldrich Syndrome (WAS) is a rare X-linked primary immunodeficiency caused by defective expression of the WAS protein (WASP) in haematopoietic cells. Several immune functions in T and B lymphocytes, platelets, dendritic cells (DCs), macrophages, natural killer (NK) cells and neutrophils of WAS patients are altered, thus resulting in a complex clinical phenotype. Clinical manifestations include recurrent infections, eczema, thrombocytopenia, autoimmune disorders and lymphomas. Increasing evidences show the contribution of DC defects in WAS pathogenesis. Reduction of WASP expression impairs cytoskeleton reorganization and inhibits podosome formation in DCs, dramatically reducing their motility and functionality. As a consequence of such defects, an impaired T and NK cell priming by WASP-deficient DCs has been described. It is well known that DCs recognize pathogen antigens mainly through Toll-like receptors (TLRs). In our work, we hypothesize that defective *was*<sup>−/−</sup> DC capacity to trigger immune cells may be also due to an inefficient antigen uptake and TLR signalling. To evaluate TLR response in *was*<sup>−/−</sup> murine model, we challenged bone marrow-derived DCs (BMDCs) with different TLR agonists. Our data show that upon stimulation with LPS or CpG, the transcription of some cytokine specific genes, such as *il-12*, *tnf-α*, *il-1b* and *ifn-γ*, by *was*<sup>−/−</sup> BMDCs is defective. Moreover, upon stimulation with LPS, the release of TNF-α and IL-10 by *was*<sup>−/−</sup> BMDCs is reduced. Intracellular staining for IL-12 on BMDCs stimulated with LPS showed that *wt* BMDCs accumulate IL-12 at early time points (12 hours) and release it at later time points (24 and 48 hours). On the contrary, *was*<sup>−/−</sup> BMDCs accumulate more IL-12 than *wt* BMDCs, at every time point assayed (from 3 hours to 48 hours). Such observation is consistent with the hypothesis that cytoskeleton defects in WASP-deficient DCs may also impair cytoplasmic vesicle trafficking and cytokine release. To better characterize DC response to TLR triggering we are performing imaging analyses of specific agonist uptake (fluorescent CpG and LPS), in parallel with TLR recruitment and endosome recycling. Our data show that DC response upon TLR triggering is impaired in *was*<sup>−/−</sup> murine model, but further analyses are still ongoing to clarify the mechanisms underlying *was*<sup>−/−</sup> DC defects.

## PA12/6 UNIQUE POPULATIONS OF DENDRITIC CELLS GENERATED FROM SEPTIC PATIENTS INDUCE ANERGIC AND IMMUNOSUPPRESSIVE T CELLS

V. Faivre<sup>1,2,3</sup>, A.C. Lukaszewicz<sup>2,3</sup>, A. Alves<sup>4</sup>, D. Charron<sup>1</sup>, D. Payen<sup>2,3</sup>, A. Haziot<sup>1</sup><sup>1</sup>INSERM U940, IUH, Université Paris Diderot, Paris, France, <sup>2</sup>Laboratoire d'Anesthésiologie EA322 Université Paris Diderot, Paris, France, <sup>3</sup>Départ Anesthésie Réanimation SMUR, Hôpital Lariboisière, AP-HP, Paris, France, <sup>4</sup>Service de Chirurgie Générale et Digestive, Hôpital Lariboisière, Paris, France

**Objectives:** Sepsis is a complex, multifactorial pathology with high susceptibility to secondary infections. Alterations in innate immune responses affect monocytes with a modified phenotype and selective low responsiveness to microbial stimulation. Impaired adaptive immune responses have also been described with lymphocyte depletion and defective T cell proliferation and IL-2 secretion. To determine the contribution of dendritic cells to the immune dysregulation in sepsis, we analyzed the differentiation and the functions of dendritic cells generated from monocytes obtained from septic patients.

**Methods:** Blood samples were collected over a 28 day period from peritonitis patients (n=24, with 0 to 4 organ failures) and we studied the ex vivo differentiation of monocytes into dendritic cells and the functional properties of the generated cells (phenotyping, analysis of internalization and responsiveness to TLR ligands, mixed leukocyte reaction, cell sorting, T cell proliferation assays, cytokines assays).

**Results:** Our results showed the prominent development of an atypical CD1a<sup>−</sup> negative (CD1a<sup>−</sup>) dendritic cell population in septic patients that peaked in samples collected at day 7, in sharp contrast to the minor percentage these cells represented in control cultures. The expression of dendritic cell specific markers confirmed the identity of these cells. The analysis of purified CD1a<sup>−</sup> and CD1a<sup>+</sup> dendritic cell populations generated from patients showed that

- (i) CD1a<sup>−</sup> dendritic cells did not induce T cell proliferation
- (ii) CD1a<sup>−</sup> dendritic cells from patients induced a weak but significant Th1-like cytokine T cell profile, contrasting with the regulatory properties of CD1a<sup>−</sup> dendritic cells from controls
- (iii) compared to control cells, CD1a<sup>+</sup> dendritic cells derived from septic patients enhanced the expression of Foxp3 in proliferating T cells.

**Conclusion:** Together, these results indicate a redistribution of stimulatory and regulatory immune functions among dendritic cell populations cultured from septic patients and suggest novel and complex control mechanisms of responses to infection that may result from the reprogramming of monocytes.

## PA12/7 DITRIMENTAL ROLE OF DENDRITIC CELLS DURING Y. ENTEROCOLITICA INFECTION IN VIVO

P. Warnke<sup>1</sup>, T.-R. Linzer<sup>2</sup>, S. Schmitz<sup>2</sup>, G. Hämmerling<sup>3</sup>, I.B. Autenrieth<sup>1</sup>, S.E. Autenrieth<sup>1,2</sup><sup>1</sup>Universitätsklinik Tübingen, Institut für Medizinische Mikrobiologie und Hygiene, Tübingen, Germany, <sup>2</sup>Universität Tübingen – Institut für Zellbiologie, Immunologie, Tübingen, Germany, <sup>3</sup>DKFZ Heidelberg, Molekulare Immunologie, Heidelberg, Germany

*Yersinia enterocolitica* (*Y. enterocolitica*) blocks T cell priming, required for the control of *Y. enterocolitica* infection, by inhibiting maturation and antigen uptake as well as the induction cell death of bone marrow-derived DCs (DCs). Here we investigated the role of dendritic cells during an infection with *Y. enterocolitica* in vivo using C57BL/6 wild type and transgenic mice expressing the diphtheria toxin (DT) receptor under the control of the CD11c promoter (CD11c.DOG mice). Repetitive application of DT led to a long term ablation of DCs.

Analyzing the course of infection we observed less bacterial load in the spleen one to 7 days after infection in the CD11c.DOG mice compared to wild type mice. This led to a better survival of the CD11c.DOG mice compared to the wild type mice. In addition, we observed less viable intracellular *Yersinia* in CD11b<sup>+</sup> and Gr-1<sup>+</sup> cells one day post infection in the CD11c.DOG mice compared to wild type mice. No difference in the cell populations in the spleen could be observed one day post infection between the two mouse strains. Later on the number of CD11b<sup>+</sup> and Gr-1<sup>+</sup> cells was doubled in the spleen of CD11c.DOG mice compared to wild type mice. One day post infection the amount of CCL2, CCL4, CCL7, and IFN- $\gamma$  was increased 12 to 35 fold in wild type mice compared to CD11c.DOG mice as analyzed by RT-PCR. Furthermore, adoptive transfer of CFSE-labeled OVA-specific CD4<sup>+</sup> T cells into CD11c.DOG mice treated with DT and infected with *Y. enterocolitica* revealed reduced proliferation rates compared to these of infected CD11c.DOG mice not treated with DT. Taken together, our data demonstrate that DCs play an adversary role during infection with *Y. enterocolitica* by increasing cytokine and chemokine production as well as the bacterial load.

#### PA12/8 A CRUCIAL ROLE OF THE SPLEEN IN THE INDUCTION OF PATHOGENIC HOST RESPONSES TOWARDS P. BERGHEI ANKA INFECTION

B. Schumak<sup>1</sup>, K. Schmidt<sup>2</sup>, S. Specht<sup>2</sup>, F. Juengerkes<sup>1</sup>, G.J. Haemmerling<sup>3</sup>, A. Hoerauf<sup>2</sup>, A. Limmer<sup>1</sup>

<sup>1</sup>University of Bonn, IMMEI, Bonn, Germany, <sup>2</sup>University of Bonn, IMMIP, Bonn, Germany, <sup>3</sup>German Cancer Research Center (DKFZ), Tumor Immunology, Heidelberg, Germany

Cerebral malaria is a severe complication of *Plasmodium* infection and is generally assumed to be an immune-mediated pathology as a result of excessive inflammation. Interferon gamma and activated T cells are among the few factors that are known to be essential for the development of experimental CM (ECM) in susceptible mouse strains upon infection with *P. berghei* ANKA. It has been long speculated where pathogenic T cells and inflammatory cytokines causing ECM are generated. Among direct effects on the brain, the spleen has been taken into consideration. We show that splenectomized animals were protected against ECM; in addition, our data demonstrate the relevance of an intact micro-architecture of the spleen. Incidence of ECM was significantly increased if splenectomized mice received a splenic auto-transplant. PbA infected RAG-deficient mice developed ECM only if they received effector cells from PbA infected wildtype donors but not after transfer of lymphocytes from naïve donors. Furthermore, we determined a crucial role for dendritic cells in the detection of Plasmodium parasites and subsequent induction of detrimental parasite specific immune responses. Mice depleted of DCs or deficient in IL-12p35 were protected against ECM and lacked PbA specific T cells, which emphasizes the importance of these antigen presenting cells in the induction of parasite-specific T cell responses. In addition, support of T cell priming and induction of interferon gamma belong to the most prominent biological functions of IL-12, a cytokine which is almost exclusively produced by DCs. Taken together, our data show the importance of the spleen in the induction of pathological host responses towards blood stage PbA infection in susceptible mice.

#### PA12/9 DENDRITIC CELLS CAPTURE OXLDL THROUGH DC-SIGN: A POSSIBLE ROLE IN ATHEROSCLEROSIS

S.M. Galama<sup>1</sup>, E. Kanters<sup>1</sup>, M. Sanchez Hernandez<sup>2</sup>, G. Kraal<sup>1</sup>, T.B.H. Geijtenbeek<sup>1</sup>

<sup>1</sup>VU University Medical Center, Department of Molecular Cell Biology and Immunology, Amsterdam, Netherlands

**Introduction:** Major cause of cardiovascular disease is the narrowing and occlusion of the arteries as a result of atherosclerosis. Oxidation of low density lipoproteins (oxLDL) in the vessel wall drives the chronic inflammation in atherosclerotic sites. Little is known about the role of dendritic cells (DCs) in oxLDL uptake and subsequent inflammatory responses. Here we have investigated the interaction oxidized LDL (oxLDL) with DCs and the role of these DCs in the progression of the vessel wall inflammation.

**Methods:** Immature DCs were exposed to labelled LDL and oxLDL and uptake was determined by flow cytometry. The receptors were identified by using inhibitors such as blocking antibodies and carbohydrates. A DC-SIGN-Fc binding assay was performed to investigate interactions with LDL and oxLDL. DC function after oxLDL uptake was investigated by determining DC maturation and cytokine expression.

**Results:** Our data show that DC-SIGN specifically binds oxLDL, but not non-modified LDL. Interactions of DC-SIGN with oxLDL occur at the primary C-type lectin binding site, in a calcium dependent manner. On immature DCs DC-SIGN mediates a major part of the oxLDL interactions, as shown by antibodies against DC-SIGN. DC-SIGN is indicated to be involved in oxLDL uptake. Upon binding to DCs oxLDL is rapidly internalized into lysosomes and induces a specific cytokine profile.

**Conclusion:** Our results indicate that DC-SIGN is involved in the uptake of oxLDL by immature DCs. Thus DC-SIGN may be involved in antigen presentation of lipids or associated proteins of oxLDL, thereby contributing to the inflammatory process in atherosclerosis.

**Funding:** This project is funded by a TOP Grant (NWO 91208001) provided by the Dutch organisation for health research and health innovation (ZonMw).

#### PA12/10 THE IMMUNOSUPPRESSIVE EFFECTS OF INDOLEAMINE 2,3-DIOXYGENASE (IDO) IN HUMAN MYCOBACTERIUM TUBERCULOSIS INFECTION

C.A. Ganoza<sup>1</sup>, K.C. Faé<sup>1</sup>, R. Hurwitz<sup>2</sup>, S.H.E. Kaufmann<sup>1</sup>

<sup>1</sup>Max-Planck-Institute for Infection Biology, Department of Immunology, Berlin, Germany, <sup>2</sup>Max-Planck-Institute for Infection Biology, Biochemistry Core Facility, Berlin, Germany

Dendritic cells (DCs) are antigen presenting cells (APCs) that play an important role in the regulation of immunity. DCs accomplish this by promoting or curtailing T-cell responses by the integration of a diverse array of incoming signals and by directing an appropriate T-cell response. Indoleamine 2,3-dioxygenase (IDO) is an enzyme that degrades the essential amino acid tryptophan. APCs expressing IDO can suppress T-cell responses and promote tolerance. Considerable evidence supports this hypothesis, including studies of mammalian pregnancy, tumour resistance, chronic infections and autoimmune diseases. This enzyme is critically involved in CD4- and CD8-T cell suppression, as well as in activation of regulatory T-cells. Existing evidence suggests that immunosuppressive mechanisms affect the outcome of tuberculosis, therefore we hypothesize that IDO plays a role in disease progression.

Human monocyte-derived DCs and macrophages strongly upregulated the expression of IDO after *Mycobacterium bovis* BCG and both virulent and avirulent strains of *M. tuberculosis* (H37Rv and H37Ra). These data further support the role of the tryptophan catabolism pathway during tuberculosis infection. Furthermore, this upregulation was highest with the virulent strain (H37Rv), and live bacteria induced stronger expression compared to killed bacteria or their cell wall components. The enzymatic activity of IDO, measured by the conversion of tryptophan to the active catabolites (kynurenines) by HPLC, showed that live bacteria induce higher enzymatic activity in APCs. Using a T-cell proliferation assay we observed that inhibition of IDO's enzymatic activity (using the specific inhibitor 1-methyl-L-tryptophan) in infected DCs reversed the kynurenine-induced CD8 T-cell proliferation arrest, thus supporting the immune regulatory function of the active catabolites generated by the increased degradation of tryptophan. Taken together, these results indicate that the immune regulatory function of the tryptophan catabolism in APCs is involved in the pathogenesis of human *M. tuberculosis* infection.

#### PA12/11 POSTOPERATIVE ILIEUS DEPENDS ON DC-DEPENDENT TH1, BUT NOT TH17 CELL STIMULATION

D.R. Engel<sup>1</sup>, A. Koscielny<sup>2</sup>, J. Maurer<sup>1</sup>, J. Kalff<sup>2</sup>, C. Kurts<sup>1</sup>

<sup>1</sup>Institute for Molecular Medicine, Bonn, Germany, <sup>2</sup>Department of Surgery, Bonn, Germany

Intestinal manipulation during abdominal surgery leads to local bowel dysfunction and inflammation, which subsequently spreads over the entire gastrointestinal tract. Such dysfunction is associated with high morbidity. It has been shown previously that resident macrophages are involved in bowel dysfunction, suggesting involvement of the immune system, but the underlying mechanisms are unknown.

Here we show that such intestinal dysfunction is reduced in CD4 deficient mice indicating a role for T cells. Also depletion of CD25<sup>+</sup> cells, but not of Foxp3 positive cells ameliorated dysfunction, indicating that T effector cells are important. RNA for the Th1 cytokines IL12 and IFN $\gamma$ , but not of the Th17 cytokines IL23 and IL17 were upregulated after manipulation. Furthermore deficiency for IFN $\gamma$ , the IL12 subunit p35 and for the Th1 transcription factor T-bet reduced gut pathology significantly indicating that the bowel trauma is triggered by a Th1 response. The Th1 inducing cytokine IL12 was mainly produced by DC and depletion of these cells using CD11c-DTR mice or administration with an anti-IL12 antibody prevent bowel dysfunction as well. These results indicate that DCs induce a Th1 immune response after abdominal surgery, which subsequently mediates bowel dysfunction.

#### PA12/12 CHARACTERIZATION OF ANTIGEN PRESENTING CELL SUBSETS IN PLASMODIUM FALCIPARUM INFECTED AND NON INFECTED CHILDREN BELONGING TO DIFFERENT SYMPATRIC ETHNIC GROUPS IN MALI

C. Arama<sup>1,2</sup>, P. Giusti<sup>1</sup>, S. Boström<sup>1</sup>, V. Dara<sup>2</sup>, B. Traoré<sup>2</sup>, A. Dolo<sup>2</sup>, O. Doumbo<sup>2</sup>, S. Varani<sup>1</sup>, M. Troye-Blomberg<sup>1</sup>

<sup>1</sup>Stockholm University, Wenner-Gren Institute, Department of Immunology, Stockholm, Sweden, <sup>2</sup>University of Bamako, Malaria Research and Training Centre FMPOS, Bamako, Mali

**Objectives:** Interethnic studies have shown that adult Fulani are more resistant to *Plasmodium falciparum* malaria than sympatric ethnic groups as reflected by less clinical symptoms, lower parasite rates and densities. In addition, Fulani have higher levels of anti-malarial antibodies, higher numbers of T-helper cells, lower expression of FOXP3 and CTLA4 indicating differences in immune regulation. Dendritic cells (DC) are important in the crosstalk between cells of innate and acquired immune response. Here, we hypothesize that the differences seen between Fulani and Dogon are at the level of DC subsets.

**Methods:** To address the role of DC subtypes in the Fulani's relative immunity to malaria as compared to the Dogon, an immunological study was performed in Mali during the malaria transmission season of 2008. Children aged 2-10 years with and without active *P.falciparum* infections belonging to the Fulani and Dogon sympatric ethnic groups were enrolled. Peripheral blood mononuclear cells were collected, fixed and frozen immediately in the field. The cells were then stained for the different DC subtype markers BDCA1, BDCA2, BDCA3 and activation markers for FACS analysis.

**Results:** The study revealed that infected Fulani children exhibited increased expression of HLA-DR on circulating BDCA2<sup>+</sup> and BDCA3<sup>+</sup> cells as compared to all other groups. The infected Fulani children also had lower numbers of these DC subsets in circulation as compared to the uninfected Fulani. Finally, the serum levels of IFN- $\alpha$  was higher in infected Fulani than uninfected children of both Dogon and Fulani.

**Conclusion:** Lower levels of BDCA2<sup>+</sup> cells were observed in infected Fulani children as compared to uninfected, the cells were also more activated as evidenced by increased HLA-DR expression. We propose that the activation of BDCA2<sup>+</sup> cells in the infected Fulani might cause an increased migration of these cells to lymphoid organs with a drastic reduction of their number in the blood. In addition, the increased secretion of IFN- $\alpha$  would fit well with the activated status of BDCA2<sup>+</sup> cells in the infected Fulani. Taken together, this data suggest that difference in DC activation may be a contributing cause to the different susceptibility to malaria in these sympatric ethnic groups.

**PA12/13 CHARACTERIZATION OF ANTIGEN PRESENTING CELL SUBSETS IN *PLASMODIUM FALCIPARUM* INFECTED AND NON INFECTED CHILDREN BELONGING TO DIFFERENT SYMPATRIC ETHNIC GROUPS IN MALI**

C. Arama<sup>1,2</sup>, P. Giusti<sup>1</sup>, S. Boström<sup>1</sup>, V. Dara<sup>2</sup>, B. Traoré<sup>2</sup>, A. Dolo<sup>2</sup>, O. Doumbo<sup>2</sup>, S. Varani<sup>1</sup>, M. Troye-Blomberg<sup>1</sup>  
<sup>1</sup>Stockholm University, Immunology, Stockholm, Sweden, <sup>2</sup>University of Bamako, Malaria Research and Training Centre FMPOS, Bamako, Mali

**Objectives:** Interethnic studies have shown that adult Fulani are more resistant to *Plasmodium falciparum* malaria than sympatric ethnic groups as reflected by less clinical symptoms, lower parasite rates and densities. In addition, Fulani have higher levels of anti-malarial antibodies, higher numbers of T-helper cells, lower expression of FOXP3 and CTLA4 indicating differences in immune regulation. Dendritic cells (DC) are important in the crosstalk between cells of innate and acquired immune response. Here, we hypothesize that the differences seen between Fulani and Dogon are at the level of DC subsets.

**Methods:** To address the role of DC subtypes in the Fulani's relative immunity to malaria as compared to the Dogon, an immunological study was performed in Mali during the malaria transmission season of 2008. Children aged 2–10 years with and without active *P. falciparum* infections belonging to the Fulani and Dogon sympatric ethnic groups were enrolled. Peripheral blood mononuclear cells were collected, fixed and frozen immediately in the field. The cells were then stained for the different DC subtype markers BDCA1, BDCA2, BDCA3 and activation markers for FACS analysis.

**Results:** The study revealed that infected Fulani children exhibited increased expression of HLA-DR on circulating BDCA2<sup>+</sup> and BDCA3<sup>+</sup> cells as compared to all other groups. The infected Fulani children also had lower numbers of these DC subsets in circulation as compared to the uninfected Fulani. Finally, the serum levels of IFN- $\alpha$  was higher in infected Fulani than uninfected children of both Dogon and Fulani.

**Conclusion:** Lower levels of BDCA2<sup>+</sup> cells were observed in infected Fulani as compared to uninfected, the cells were also more activated as evidenced by increased HLA-DR expression. We propose that the activation of BDCA2<sup>+</sup> cells in the infected Fulani might cause an increased migration of these cells to lymphoid organs with a drastic reduction of their number in the blood. In addition, the increased secretion of IFN- $\alpha$  would fit well with the activated status of BDCA-2<sup>+</sup> cells in the infected Fulani. Taken together this data suggest that difference in DC activation may be a contributing cause to the different susceptibility to malaria in these sympatric ethnic groups.

**PA12/14 THYMUS DESTRUCTION AFTER INFECTION WITH HIGHLY PATHOGENIC AVIAN INFLUENZA VIRUS IS ORIGINATED FROM INFECTED DCS**

A. Vogel<sup>1</sup>, E. Haasbach<sup>1</sup>, S. Reiling<sup>1</sup>, K. Droebner<sup>1</sup>, K. Klingel<sup>2</sup>, O. Planz<sup>1</sup>  
<sup>1</sup>Friedrich-Loeffler-Institut Tübingen, Institute of Immunology, Tübingen, Germany, <sup>2</sup>Eberhard-Karls-Universität Tübingen, Institute of Pathology, Tübingen, Germany

Highly pathogenic avian influenza A viruses (HPAIV) cause severe disease in humans and could be the source for the next pandemic influenza. The basis for the increased pathogenesis of these influenza viruses remains unclear. Various investigations suggest that virus-induced hypercytokinemia and reduction of the lymphocyte population may contribute to disease severity. In addition, the presence of cytokines and chemokines in the lung of infected individuals results in an uncontrolled influx of immune mediators and consequently to a massive lung damage.

In this study we demonstrated that HPAIV infection of mice leads to infection of dendritic cells (DCs) in the lung. These influenza viruses activated DCs migrated afterwards into secondary lymphoid organs, namely lymph node (LN) and thymus.

Our findings showed infectious virus, viral antigen and virus specific nucleic acid in the LN but also in the thymus of HPAIV infected mice. Consequently, we presumed that influenza virus influences maturation and activation of T-cells. Adoptive transfer of lung DCs from HPAIV infected GFP-transgenic mice into wild-type mice further indicated that DCs carry HPAIV from the lung into the thymus. The presence of HPAIV could lead to infection of the CD45-negative epithelial cells which can interfere with thymic T-cell selection. Influenza virus infection of lung specific DCs was only found after HPAIV infection but not after infection with human H1N1 influenza virus. Therefore, we conclude that the increased pathogenesis of HPAIV infection including lymphopenia is due to the fact that these viruses are able to infect DCs in the lung and that infected DCs transport influenza virus to the thymus. Here, the infection leads to functional damage of the thymus and the cellular immune response.

**PA12/15 DIFFERENT PROPERTIES OF CORD BLOOD CELLS OF NEWBORNS OF HEALTHY AND ALLERGIC MOTHERS**

J. Hrdý<sup>1</sup>, O. Novotná<sup>1</sup>, I. Kocourková<sup>2</sup>, I. Sterzl<sup>1</sup>, L. Prokešová<sup>1</sup>  
<sup>1</sup>Charles University in Prague, First Faculty of Medicine, Institute of Immunology and Microbiology, Prague, Czech Republic, <sup>2</sup>Institute for the Care of Mother and Child, Prague, Czech Republic

**Objectives:** Allergic diseases belong to common illnesses with continuously increasing incidence. We tried to identify some early prognostic markers in cord blood cells pointing to increased risk of future allergy development which could serve to early introduction of preventive measures.

**Methods:** Properties of cord blood cells of children with high risk of allergy (children of allergic mothers) were compared with cord blood cells of children of healthy mothers. We tested *in vitro* reactivity of cord blood mononuclear leukocytes (CBML) by <sup>3</sup>H thymidine incorporation. Expression of cytokines (IL-1 $\beta$ , IL-2, IL-4, IL-8, IL-10, IL-13, IFN- $\gamma$ , TNF- $\alpha$ , TGF- $\beta$  and EGF) in CBML was measured by real-time PCR and their concentration in cord blood sera by ELISA. Cord blood mDC (grown from CD14 positive monocytes) were characterized by gene expression of activation markers (CD80, CD83, CD86) and cytokines of IL-12 family. Proportion of Tregs in cord blood of children of healthy and allergic mothers was compared by flow cytometry. T-test was used for statistical analyzing normally distributed data otherwise Mann-Whitney test was exploited.

**Results:** CBML of children of allergic mothers have significantly higher both spontaneous and polyclonally stimulated proliferation activity pointing to possible increased promptness of future allergy onset. Cord blood cells of allergic group are characterized by increased expression of IL-10, IL-13, EGF and decreased expression of other cytokines tested. Expression of cytokines after *in vitro* stimulation of CBML is generally increased in allergic group in comparison with healthy one. These two groups differ also in serum cytokine levels. Increased proportion of mDC expressing CD83 after LPS stimulation was found in allergic group.

**Conclusion:** Allergic phenotype was apparent already on the level of cord blood cells. Higher lymphocyte proliferation activity and higher stimulation readiness of mDC of cord blood of children of allergic mothers imply easier allergy induction. Also, significantly decreased serum levels of EGF in newborns of allergic mothers could negatively influence maturation and permeability of mucosal membranes of these children and support thus allergen penetration.

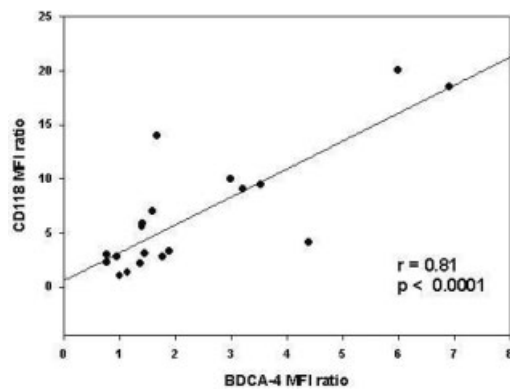
This work was supported by grants of Ministry of Education, Youth and Sports of the Czech Republic MSM0021620806 and Grant Agency of the Czech Republic 310/08/H077.

**PA12/16 THE EXPRESSION OF C-TYPE LECTIN RECEPTOR BDCA-4, BUT NOT BDCA-2 CORRELATES WITH TYPE I IFN RECEPTOR CD118 EXPRESSION ON PLASMACYTOID DENDRITIC CELLS IN BOTH PATIENTS SUFFERING FROM INFLAMMATORY SYSTEMIC AUTOIMMUNE DISEASES AND HEALTHY SUBJECTS**

X. Ruijing<sup>1</sup>, M.-C. Béné<sup>1</sup>, G. Faure<sup>1</sup>, J.-D. De Korwin<sup>2</sup>, M. De Carvalho Bittencourt<sup>1</sup>  
<sup>1</sup>University Hospital Nancy, Laboratory of Immunology, Vandoeuvre-les-Nancy, France, <sup>2</sup>University Hospital Nancy, Internal Medicine Department, Vandoeuvre-les-Nancy, France

IFN- $\alpha$  production by plasmacytoid dendritic cells (pDCs) is inhibited by cross-linking C-type lectin receptors (CLRs) BDCA-2 and BDCA-4, making them attractive targets for immunotherapy in autoimmune (AI) patients. Yet, very few studies have so far studied the expression levels of these markers in circulating pDCs in AI patients. Using multiparameter flow cytometry, we compared whole blood pDCs levels from 9 AI patients (6 scleroderma and 3 Sjögren's Syndrome patients) with a group of 6 healthy control (HC) subjects. pDCs were identified by a CD45<sup>+</sup> Lin<sup>neg</sup> HLA DR<sup>+</sup> BDCA-2<sup>+</sup> phenotype. Expression of BDCA-4 and the type I IFN receptor CD118 on pDCs were determined independently and the respective medians of fluorescence intensity ratios (MFI-R) were calculated according to fluorescence minus one control. As pDCs have been shown to be implicated in regulatory T cells (Tregs) generation, we also measured Tregs levels, identified by the CD4<sup>+</sup> CD25<sup>+</sup> CD127<sup>low/neg</sup> phenotype. AI patients had decreased levels of pDCs compared to HC (0.21  $\pm$  0.02 and 0.4  $\pm$  0.1% of PBMC respectively,  $p < 0.05$ , Student's *t* test). No significant differences in Tregs levels were observed between AI patients and HC (6.9  $\pm$  0.3 vs 8.3  $\pm$  1.2% of CD4 lymphocytes respectively,  $p = NS$ , Student's *t* test). No correlation was found between pDCs and Tregs levels. BDCA-4 expression were higher in HC (3.6  $\pm$  1) than in AI patients (1.5  $\pm$  0.2, MFI-R,  $p < 0.05$ , Student's *t* test) as well as the expression of CD118 (11  $\pm$  2.8, HC vs. 5.4  $\pm$  1.3 MFI-R, AI patients,  $p = 0.06$ , Student's *t* test). In contrast, BDCA-2 expression was higher in AI patients (4  $\pm$  0.6 vs 2.1  $\pm$  0.1 MFI in HC,  $p = 0.05$ , Student's *t* test). Most interesting, a strong positive correlation between BDCA-4 and CD118 expression in HC ( $p < 0.0001$ , Pearson correlation;  $r = 0.97$ , regression analysis) and in AI patients ( $p < 0.05$ , Spearman's rank;  $r = 0.51$ , regression analysis) was found. BDCA-2 MFI and CD118 MFI did not correlate. These combined results suggest that BDCA-4 and CD118 expression in pDCs are cross regulated. Their implication in the exacerbated IFN- $\alpha$  pathway of AI patients deserves further investigation for identifying new targets for immunotherapy in autoimmunity.





[BDCA4 CD118]

#### PA12/17 THE RELATIONSHIP BETWEEN THE PRESENCE MYELOID DENDRITIC CELL AND THE PROGRESSION OF CERVICAL INTRAEPITHELIAL NEOPLASIA

S. F. Velasco Ramírez<sup>1</sup>, A. Del Toro Arreola<sup>1</sup>, M. Jiménez Pérez<sup>2</sup>, R. Robles García<sup>3</sup>, A. Suarez Rincon<sup>4</sup>, R. A. Franco Topete<sup>5</sup>, L. Flores Romo<sup>6</sup>, R. Hernández Pando<sup>7</sup>, A. Daneri Navarro<sup>1</sup>

<sup>1</sup>Universidad de Guadalajara, Fisiología, Guadalajara, Mexico, <sup>2</sup>Universidad de Guadalajara, Salud Pública, Guadalajara, Mexico, <sup>3</sup>Instituto para el Fortalecimiento de las Capacidades en Salud/ Dirección de Desarrollo, Centro de Estudios Especializados ARG, México, Mexico, <sup>4</sup>Hospital General Regional #45. IMSS, Clínica de Colposcopia, Guadalajara, Mexico, <sup>5</sup>OPD Hospital Civil de Guadalajara, Servicio de Patología, Guadalajara, Mexico, <sup>6</sup>Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, Biología Celular, México, Mexico, <sup>7</sup>Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, México, Mexico

Carcinoma of the cervix is the second most frequent malignant tumor in women worldwide. The development of cervical cancer is a multi-step process that involves a precursor pre-invasive stage. There is short information on possibly changes in the mucosal immunity specifically the role that plays the dendritic cells (DCs) for the development, maintenance, and progression in women with cervical intraepithelial neoplasia (CIN) of low or high grade and cancer. Since the presence of DCs or Langerhans cells which form a three-dimensional network in epithelial layer of the cervix is essential in producing immune response, we compared the infiltrating cell density of mature (CD83<sup>+</sup>) and immature (Langerin<sup>+</sup> and CD1a<sup>+</sup>) DCs in samples from three groups: normal cervix, CIN of low and high grade. Inclusion of women at cohort was made at the moment of CIN diagnosis at Gynaecology Department (Instituto Mexicano del Seguro Social). Our study enrolled 73 patients diagnosed by histopathological criteria as CIN. Langerin<sup>+</sup>, CD1a<sup>+</sup> and CD83<sup>+</sup> expression in cervical tissue paraffin-embedded was measured by immunohistochemistry. The majority of Langerin<sup>+</sup> and CD1a<sup>+</sup> DCs were identified at the basal and midzone region of the epithelial layer in the normal cervix. Stromal layer of normal cervix showed fewer cells Langerin<sup>+</sup>, CD1a<sup>+</sup> and CD83<sup>+</sup> staining. Variations in the expression of Langerin<sup>+</sup>, CD1a<sup>+</sup> and CD83<sup>+</sup> cells in CIN of low and high grade were observed in epithelial layer as stroma than normal cervix. These data may help to understanding the nature of the immune response in CIN and the progression to cancer.

#### PA12/18 DENDRITIC CELLS (DCS) MATURATION AND HYALURONAN (HA) LEVELS IN MICE INJECTED WITH CELL LINES DERIVED FROM A T CELL LYMPHOMA

R. Cordero Russo<sup>1</sup>, S. M. Blois<sup>2</sup>, G. Blanco<sup>1</sup>, E. Alvarez<sup>1</sup>, M. G. García<sup>3</sup>, S. Hajos<sup>1</sup>

<sup>1</sup>Universidad de Buenos Aires, Facultad de Farmacia y Bioquímica, IDEHU-CONICET, Buenos Aires, Argentina, <sup>2</sup>Universitätsmedizin Berlin, Charité Centrum 12 für Innere Medizin und Dermatologie, Berlin, Germany, <sup>3</sup>Universidad Austral, Laboratorio de Terapia Génica, Derqui, Argentina

**Objectives:** The existence of tolerance mechanisms allows tumour cells to grow in immunocompetent host. DCs, influenced by their microenvironment, may tolerate or stimulate the immune system against cancer. HA is a glycosaminoglycan increased in the extracellular matrix within tumours, related with tumour progression. HA fragments, but not native HA, induce DC maturation, generating an effective immune response. The aim of this work was to analyse the maturational state of DCs in the organs infiltrated by lymphoid tumours and its relationship with HA levels. For this purpose, we used murine lymphoma cell lines resistant to doxorubicin (LBR-D160), vincristine (LBR-V160), and a sensitive line (LBR-) with dissimilar invasive behaviour *in vivo*, being LBR-V160 the less aggressive one.

**Methods:** BALB/c mice were injected with tumour cells or RPMI-1640, sacrificed at day 10 (non terminal state) and infiltrated liver, lung and lymph nodes were removed for the analysis of DCs presence (CD11c<sup>+</sup> cells) and maturation (expression of CMH-II and CD80) by flow cytometry. Besides, serum HA was measured by ELISA. Data was analysed by one-way ANOVA and Dunnett's post test.

**Results:** Analysis of CD11c expression in liver showed that percentage of CD11c<sup>+</sup> cells in mice inoculated with LBR- and LBR-D160 ( $5.3 \pm 0.7\%$  and  $5.9 \pm 0.9\%$ ) was significantly decreased compared with control mice ( $9.0 \pm 1.7\%$ ;  $P < 0.05$ ), observing no differences in mice inoculated with LBR-V160 ( $11.7 \pm 2.6\%$ ). Besides, no changes in CD11c levels between the groups were observed in lymph nodes and lung. Analysis of CMH-II and CD80 expression in CD11c<sup>+</sup> cells in liver showed a decrease in double positive cells in LBR- and LBR-D160 injected mice ( $11.3 \pm 4.4\%$  and  $17.6 \pm 5.2\%$ ) compared with control ( $37.1 \pm 10.1\%$ ;  $P < 0.05$ ), finding no differences in LBR-V160 injected mice ( $35.8 \pm 14.9\%$ ). In lymph nodes, but not in lung, similar results were observed. Serum HA levels were increased in LBR- and LBR-D160 injected mice compared with LBR-V160 injected and control mice ( $P < 0.05$ ).

**Conclusion:** We conclude that mice injected with the cell lines with major invasive capacity (LBR- and LBR-D160) presented decreased DCs maturation in liver and lymph nodes as well as increased serum HA levels. We consider that this could be an obstacle in the induction of an effective antitumoral immune response.

#### PA12/19 DENDRITIC CELLS PRODUCE IL-12 EARLY AFTER NEOSPORIA CANINUM INFECTION IN BALB/C MICE

L. Teixeira<sup>1</sup>, S. Dá Mesquita<sup>1</sup>, S. Botelho<sup>1</sup>, A. Correia<sup>2</sup>, M. Vilanova<sup>1,3</sup>

<sup>1</sup>ICBAS – University of Porto, Porto, Portugal, <sup>2</sup>Centro de Biologia Molecular e Ambiental (CMBA), Departamento de Biologia, Universidade do Minho, Braga, Portugal, <sup>3</sup>IBMC – Instituto de Biologia Molecular e Celular, Porto, Portugal

*Neospora caninum* is a cyst-forming coccidian parasite causative of clinical infections in a wide range of animal hosts. Here, the maturation and activation of splenic conventional (cDCs) and plasmacytoid (pDCs) dendritic cells was studied in BALB/c mice challenged i.p. with *N. caninum* tachyzoites. Upon infection, the number of spleen cDCs was found to initially decrease, as observed 12h and 2 days after the parasitic challenge. At day 5, however, the number of these cells was observed to be above that of controls. In contrast, the number of splenic pDCs did not significantly change after infection. Both cell subtypes displayed up-regulation of co-stimulatory and MHC-class II molecules. This stimulatory effect was more marked at 12h after infection, when an increased proportion of cDCs and pDCs producing Interleukin-12 (IL-12) was also observed. At this time point after infection *N. caninum* tachyzoites could be observed in close association with splenic sorted CD11c<sup>+</sup> cells. Overall these results show that cDCs and pDCs are both involved in the innate immune response to *N. caninum* infection. Moreover, they also show that early upon infection pDCs are a preferential source of IL-12, an important cytokine promoting host resistance to neosporosis.

This work was supported by Fundação para a Ciência e a Tecnologia (FCT) grant n°POCTI/CVT/38791/MGI/2001 and FEDER. Luzia Teixeira was financed by FCT fellowship SFRH/BD/12983/2003.

#### PA12/20 DENDRITIC CELLS IN THE LARYNGEAL CARCINOMA

H. F. Samara<sup>1</sup>, A. Kruk – Zagajewska<sup>2</sup>, J. Ueromski<sup>1</sup>, G. Dworacki<sup>1</sup>, M. Harasymczuk<sup>2</sup>

<sup>1</sup>Poznan University of Medical Sciences, Chair and Department of Clinical Immunology, Poznan, Poland, <sup>2</sup>Poznan University of Medical Sciences, Clinic of Otolaryngology and Laryngological Oncology, Poznan, Poland

**Background:** Dendritic cells (DCs) are one of the most important elements of antitumor immune response. They are responsible for recognition of tumor associated antigens, their presentation and initiation of specific adaptive response. In the microenvironment of tumor the presence and function of DCs are influenced by various factors. It has been found that their presence is a positive prognostic factor in various types of neoplasias.

Our aim in this study was to assess myeloid and plasmacytoid subpopulations of DCs in the tumor tissue, regional lymph nodes and peripheral blood taken from patients in advanced stages (T3 and T4) of laryngeal squamous cell carcinoma treated by total laryngectomy (n=25).

**Materials and methods:** Tumor and lymph node fragments were frozen for immunohistochemical reaction with monoclonal antibodies against CD1a, S100, HLA-DR, and CD11c for the detection of mature and immature myeloid DCs. Semi-quantitative scale was used to count cells in infiltrations. Peripheral blood samples and lymph node cell suspension were examined by means of flow cytometry for the detection of myeloid DCs and plasmacytoid DCs. For this purpose a cocktail

of anti-lineage monoclonal antibodies and anti-HLA-DR, -CD11c, -CD123 were used to identify DCs. The percentage of each subpopulation was obtained. Comparative statistics were performed between groups with or without lymph node metastases.

**Results and conclusions:** Mature myeloid DCs were present mainly within cell infiltrations, while immature cells were preferably in cancer nests. In cases with lymph node metastases the intensity of DC infiltrations was less compared to non-metastatic cases. In the peripheral blood and lymph nodes there was similar tendency of lower percentage values of myeloid and plasmacytoid DCs. However, in cases with lymph node metastases the expression of surface HLA-DR was relatively higher. These observations suggest a correlation between lower numbers of DCs and more aggressive growth of tumors.

#### PA12/21 EFFECTS OF IRON OVERLOAD ON DENDRITIC CELLS DIFFERENTIATION AND FUNCTION

C. Ka Wai<sup>1</sup>, C. G. Chi-Fung<sup>1</sup>

<sup>1</sup>The University of Hong Kong, Department of Paediatric and Adolescent Medicine, Pokfulam, Hong Kong

**Background and objectives:** Infection gradually is emerging as the leading cause of mortality and morbidity among thalassemic patients in developed countries. Since most patients have iron overload and how excessive iron affects dendritic cell (DC), the professional antigen presenting cells, function remains unexplored. Thus, we hypothesize that the immune function of DC may be disturbed by iron overload in thalassemic patients.

**Method:** Monocyte derived dendritic cells (DC) were induced from CD14+ positively selected monocytes with GM-CSF and IL-4 for 7 days. 100ng Lipopolysaccharide (LPS) was added to the cell cultures on day 5 to induce dendritic cell maturation. Cells without addition of LPS were considered to be immature DC (iDC). To mimic the situation of iron overload, 50µM Iron (III) chloride was added to the above culture conditions on day 0 and re-constituted on day3 with medium change. Differentiation, maturation status and immuno-modulatory function of DCs from these conditions were analyzed by immunophenotypic changes using flow cytometer and allogeneic activation with CD4+ T cells.

**Results:** Down-regulation of CD1a and up-regulation of immunoglobulin like transcript 3 (ILT-3) were observed in both iron treated immature DC and iron treated LPS- induced mature DC (mDC). There were no significant changes in the expression of HLA-DR, CD40, CD80 and CD86. Though CD1a expression declined, > 90% of DCs were CD14 negative which meant iron had no effect on DC differentiation. Besides, iron treated iDC induced weaker CD4+ T cell proliferation while iron treated mDC treated no difference when compared to the iron free controls.

**Conclusion:** Sublethal iron overload might affect the DC immunostimulatory function but it did not interfere the process of DCs induction from monocytes or DCs maturation.

#### PA12/22 HHV-6A INFECTS DENDRITIC CELLS, MODULATES THEIR DIFFERENTIATION AND INDUCE TYPE I IFN SECRETION

R. Gustafsson<sup>1</sup>, S. Adikari<sup>2</sup>, M. Svensson<sup>3</sup>, A. Fogdell-Hahn<sup>1</sup>

<sup>1</sup>Karolinska Institutet, Clinical Neuroscience, Stockholm, Sweden, <sup>2</sup>University of Peradeniya, Colombo, Sri Lanka, <sup>3</sup>Karolinska Institutet, Center for Infectious Medicine, Stockholm, Sweden

**Objective:** Dendritic cells (DCs) are crucial components of the immune system. Previous studies have shown that HHV-6 can infect DCs and impair their ability to activate T cells. No one have, however looked at the role of type I IFN in HHV-6 infected DC. The aim of this study was to investigate the effect of HHV-6 on the phenotype and function of DCs, including their type I IFN response.

**Methods:** Monocyte derived dendritic cells (MDDCs) were infected with human herpes virus 6A (HHV-6A) GS strain at various multiplicity of infection (MOI). Viral replication was assessed using real time Q-PCR and immunofluorescence microscopy. Secretion of type I IFN was measured using a bioassay. MDDC phenotype was investigated by flow cytometry and their ability to activate CD4+ or CD8+ allo T cells was assessed by mixed lymphocyte reaction (MLR).

**Results:** Viral replication was supported by MDDCs as evident by Q-PCR and immunofluorescence microscopy. MDDCs responded to HHV-6A infection by secretion of type I IFN in a titre dependent manner. Flow cytometric analysis on cells 3 dpi, when the virus seemed to have the largest effect on the MDDC, suggests an activation of the cells by up-regulation of activation markers CD83 and CD86. The expression of HLA-DR seemed to be unaltered whereas the expression of HLA-ABC was slightly suppressed on virus infected cells. These modulations did, however not affect the ability of MDDCs to activate allo-T cells.

**Conclusion:** HHV-6 can infect MDDC that responds by type I IFN secretion (as described for the first time) and up-regulation of activation markers CD83 and CD86. HHV-6A seems to down-regulate the expression of HLA-ABC but this modulation does not affect the ability of MDDC to activate CD4+ or CD8+ allo-T cells.

#### PA12/23 DENDRITIC CELLS IN PATIENTS WITH TYPE 1 DIABETES (T1D) AND IN THEIR RELATIVES WITH GENETIC DEFINED RISK FOR T1D DEVELOPMENT

J. Kayserova<sup>1</sup>, K. Stechova<sup>2</sup>, T. Ulmannova<sup>2</sup>, A. Sediva<sup>1</sup>, S. Kolousova<sup>1</sup>, R. Spisek<sup>1</sup>

<sup>1</sup>Charles University, Department of Immunology, Prague, Czech Republic, <sup>2</sup>Charles University, Paediatrics, Prague, Czech Republic

**Introduction:** Dendritic cells (DC) are main antigen presenting cells which induce and regulate immune reactions. DC in perifer blood could be identified by monoclonal antibodies against several markers – DC are lineage negativ (CD3-, CD14-, CD19-, CD56-) and HLA-DR+. According to expresion of surface molecules CD11c and CD123 (interleukin 3 receptor alfa) DC are divided into CD11c+ myeloid DC (mDC) and CD11c- CD123 hi plasmacytoid DC (pDC) subgroup. We selected T1D (type 1 diabetes) as a representative of organ specific autoimmune diseases with well defined autoantigens to study the role of DC in autoimmunity development.

**Objective:** We compared a DC subtype count in peripheral blood of T1D patients, their first degree relatives, patients suffering from type 2 diabetes (T2D) and of healthy controls.

**Methods:** We examined 13 T1D patients (11/13 patients with recently diagnosed disease, 2/13 patients with long-lasting T1D), 9 patients with T2D, 17 first degree relatives and 6 healthy controls with negative family history of autoimmune disease. Blood samples of patients with recent diabetes onset were taken after stabilization of internal environment. DC in peripheral blood was determined by combination of monoclonal antibodies by flow cytometry.

**Results:** A significantly lower number of myeloid DC as well as of plasmacytoid DC was found in a group of first degree relatives in comparison with healthy controls (p< 0,01, resp. p< 0,05). We found significantly lower count of pDC (p< 0,01, resp. p< 0,05) in patients with T1D and also in patients suffering from T2D in comparison with controls.

**Conclusion:** Our study demonstrate numerical deficit in DC subpopulations (mainly pDC) in diabetic patients and in their relatives. It may indicate that also some defect in antigen presentation process may be important in diabetes development but further study concentrated also on functional capacity of DC is clearly necessary.

Supported by the project of the Czech Ministry of Education MSM0021620812 and by the project of the Czech Ministry of Health IGA MZCR NR/9355-3.

#### PA12/24 THYMIC CORTICAL DENDRITIC MACROPHAGES IN THE HUMAN NORMAL THYMUS AND CORTICAL THYMOMA

K. Takahashi<sup>1</sup>

<sup>1</sup>Okayama University Medical School, Faculty of Health Science, Okayama-City, Japan

**Objectives:** Little is known about the nature of thymic cortical dendritic macrophages (TCDM), which were recently identified by us (Immunology 213:837-847, 2008) as thymic cortex-specific macrophages in the human thymus exhibiting feature intermediate between dendritic cells (DC) and macrophages. We investigate the nature of TCDM in normal thymus and thymomas immunohistochemically.

**Methods:** Human normal thymuses and thymoma are studied. Formalin-fixed, paraffin-embedded or unfixed frozen sections are examined immunohistochemically. TCDM isolated from normal thymus are also examined under culture conditions immunocytochemically.

**Results:** TCDM are identified as large extensively dendriform cells with watery clear cytoplasm containing several to many nuclear debris, evenly scattered throughout the cortex. TCDM are fascin+ DC-SIGN+ HAM56+ CD134+ vimentin+ CD68+ HLA-DR- lysozyme- CD40- CD83- CD86- DEC205- DC-LAMP-. The most striking features of TCDM is to contain degraded nuclei of apoptotic thymocytes. Lysozyme+ ordinary macrophages, which are mainly distributed just beneath the capsule, do not contain apoptotic thymocytes. In contrast to ordinary macrophages and DC, TCDM frequently contact with small capillaries intimately. Cultured TCDM are identified as fascin+ HAM56+ large dendriform cells containing apoptotic thymocytes, while DC are identified as fascin+ HAM56- relatively small dendriform cells without apoptotic thymocytes. Interestingly, cultured TCDM are converted to be strongly positive for HLA-DR. Numerous living thymocytes are seen within the cytoplasm of thymic epithelial cells (emperipholes), which are cytokeratin+ large adherent cells. Thymic epithelial cells neither exhibit phagocytic activity nor phagocytose apoptotic thymocytes. TCDM are also found in cortical thymomas (type B1, B2, B3 thymomas in WHO classification) in large numbers, but not in medullary thymomas (type A and AB thymomas in WHO classification). In contrast to TCDM in normal thymus (n-TCDM), TCDM in these cortical thymoma (t-TCDM) scarcely contain apoptotic thymocytes. Interestingly, t-TCDM frequently contain living thymocytes within their cytoplasm (emperipholes), although such structures are rarely found in n-TCDM, suggesting that t-TCDM are functionally different from n-TCDM.

**Conclusion:** TCDM are suggested to be professional scavengers of apoptotic thymocytes in the human thymus. TCDM are also suggested to be fixed macrophages rather than migrating macrophages. The unusual function of t-TCDM may be related to cortical thymoma-associated immunological abnormalities.

#### PA12/25 DETECTION AND CHARACTERIZATION OF DENDRITIC CELLS SUBSETS BY EIGHT COLOUR FACS ANALYSIS

I. Zentsova-Jaresova<sup>1</sup>, J. Kayserova<sup>1</sup>, D. Rozkova<sup>1</sup>, A. Sediva<sup>1</sup>

<sup>1</sup>Charles University, 2nd Faculty of Medicine and University Hospital Motol, Dpt. of Immunology, Prague, Czech Republic

Dendritic cells (DCs) are specific antigen-presenting cells that play critical roles in the initiation and direction of immune responses. Subpopulations of DCs might be distinguished by different expression of their surface molecules. In the most studies, the DCs were characterized by expression of CD45, HLA-DR CD11c (mDC) and CD123 (pDC). Lately, DCs were subdivided into four groups: CD16+, CD1c (BDCA-1) +, BDCA-3+, CD123+ (BDCA-2+).

In this study we characterized these DCs subsets in peripheral blood and in tissue boundary of lung – in BAL (broncho-alveolar lavage) by 8 colour FACS analysis in patients with cystic fibrosis, allergic asthma and in controls (patients with malformations of respiratory tract with no lung tissue inflammation). Our aim was to prospect distribution of these subpopulations and their expression of PRRs (pathogen recognition receptors) in the blood and BAL respectively. We have generated a specific panel of mAbs that identify three human dendritic cell subsets at once. These subpopulations we characterise with common markers CD45, Lin- (CD3,CD19,CD20,CD56), HLA-DR, CD14, CD16, BDCA-1, BDCA-3. In these groups of cells we further detected PRRs with selected antibodies (DC-SIGN, MR, DEC-205, TLR2, 4). Preliminary data shows that all four subsets are present in both microenvironments. However, while the most represented population of mDC (CD11c+) are CD11c+CD16+ cells in blood, the dominant role play CD11cBDCA-1+ cells in lung. Interestingly, the smallest population in blood CD11cBDCA-3+ is the second most dominant subset in BAL. As we supposed, DCs subsets in BAL lavage show stronger expression of PRRs receptors comparing the blood. In this study we describe development of novel eight colours FACS analysis for reliable monitoring of different DCs subsets and define their role in pathogenesis of different pathological conditions and allergy diseases. The comparison of this analysis between allergy and autoimmune inflammation is ongoing. This study was supported by IGA NR 8458-5, VZ MZ ČR 00064203, GAUK 7840/2007 and VZ MŠMT MSM 0021620812.

## PA18 – CLASSICAL AND NON-CLASSICAL MHC GENES AND MOLECULES

### PA18/1 BIOCHEMICAL AND CELLULAR CHARACTERIZATION OF THE TRUNCATED ISOFORM OF MHC-RELATED 1, MR1B: EVIDENCES FOR A FUNCTIONAL ROLE

J. Lion<sup>1</sup>, C. Ligeonnet<sup>1</sup>, M. Plistat<sup>1</sup>, E. Treiner<sup>1,2,3</sup>

<sup>1</sup>Inserm U925, Amiens, France, <sup>2</sup>Université de Picardie Jules Verne, Amiens, France, <sup>3</sup>CHU Amiens, Amiens, France

MR1 (MHC-Related 1) is a monomorphic MHC class I-like gene encoded on chromosome 1 in mice and humans. It has been shown that the MR1 protein is the selecting/restricting element for a new unconventional T-cell subset, called MAIT (Mucosal-Associated Invariant T) cells. Although MR1 mRNA is ubiquitously transcribed, the parameters regulating the protein expression are still largely elusive. It has been proposed that MR1 surface expression is dependent upon the availability of a ligand able to bind to its antigen-binding groove. Interestingly, the human MR1 (hMR1) pre-mRNA yields various MR1 isoforms, including two putative membrane proteins: MR1A display all the usual MHC class I-like domains, while MR1B lacks the extracellular alpha3 domain. Although both isoforms are transcribed, only MR1A expression has been studied to date.

**Objectives:** To analyze, at the biochemical and cellular level, hMR1B expression in transfected cell lines.

**Methods:** The HT-1080 fibrosarcoma cell line was used to generate stable transfectants expressing hMR1A and/or hMR1B fused to EGFP or a FLAG-Tag. The expression and the trafficking of both isoforms were compared using biochemistry, immunofluorescence microscopy, and flow cytometry.

**Results:** Both hMR1A and hMR1B are expressed in the HT-1080 cell line as glycoproteins. Their intra-cellular expression pattern largely differs, as hMR1A localizes partially in vesicular compartments, whereas hMR1B mostly accumulates in the endoplasmic reticulum (ER). Interestingly, hMR1B is expressed at the cell surface, although it is not recognized by an anti-MR1A antibody. Moreover, hMR1A and hMR1B are able to interact in double transfection experiments.

**Conclusion:** These results show that the hMR1B mRNA isoform is able to yield a functional membrane protein. The hMR1B protein trafficking pattern suggests that it may survey different cellular compartments that hMR1A. It could also play a role in assisting hMR1A for expression. Finally, as hMR1B is not able to bind b2-microglobulin, it could replace for hMR1A when b2m is down-regulated. These data strongly suggest that hMR1B is a functional protein that could play a role in MAIT cells activation.

### PA18/2 HLA-B AND MICA INFLUENCE OUTBREAK OF CELIAC DISEASE IN DQ2 PATIENTS WITH TYPE 1 DIABETES

N. Bratanić<sup>1</sup>, N. Ursic-Bratina<sup>1</sup>, T. Battelino<sup>1</sup>, B. Vidan-Jeras<sup>2</sup>

<sup>1</sup>University Children's Hospital, Ljubljana, Slovenia, <sup>2</sup>Blood Transfusion Ctr of Slovenia, Tissue Typing Center, Ljubljana, Slovenia

**Objectives:** Celiac disease (CD) is the most common food-induced enteropathy with prevalence 1:200 in many populations. Patients are intolerant toward the gluten proteins in the wheat. Classical early childhood symptoms are mainly located in the small intestine. The primary HLA association in the great majority of patients is with HLA-DQ2 and in a minority of patients with HLA-DQ8. The opposite is with type 1 diabetes (T1D). The expression of the MICA is mainly restricted to the gastrointestinal epithelium. MICA molecules are recognized by  $\gamma\delta$  T lymphocytes and NK cells via the NKG2D. Due to these features MICA might be relevant in the immunopathogenesis of CD. The aim of our study was to confirm or exclude particular MICA alleles and extended haplotypes as risk factors for outburst of CD in patients with T1D and to determine the ratio between two haplotypes A\*01,B\*08,DQA1\*0501,DQB1\*0201,DRB1\*0301 and A\*26,B\*18,DQA1\*0501,DQB1\*0201,DRB1\*0301.

**Methods:** We compared control group of 70 individuals with a group of 36 children with T1D and CD that was already described in our study on the HLA-DRB1-QBP-DQB1 haplotypes. MICA alleles were determined using LAB Type SSO MICA (One Lambda). Statistical analysis was performed using two-tailed Fisher's exact test.

**Results:** Combination of T1D and CD was most significantly associated with B\*08 ( $p=10^{-10}$ ). MICA\*008 was found significantly overrepresented in patients ( $p=3 \times 10^{-9}$ ) and always linked to B\*08. While MICA\*008-HLA-A\*01, B\*08, DQ2, DR3 extended haplotype was strikingly over-represented ( $p=5, 3 \times 10^{-6}$ ) in patients, MICA\*018, HLA-B18, DQ2, DR3 was not found at all in the same group. In the control group MICA\*008 was preferably linked with other HLA-B-DR-DQ haplotypes.

**Conclusion:** We suggest a combined influence of alleles present in the extended haplotype MICA\*008, B\*08, DQ2.

### PA18/3 EXPRESSION PATTERN AT RNA AND PROTEIN LEVEL OF MHC CLASS-I RELATED CHAIN A AND CHAIN B IN A PANEL OF CELL LINES DERIVED FROM HUMAN CERVICAL CANCER

S. del Toro-Arreola<sup>1</sup>, N. Arreyguez-Garcia<sup>2</sup>, A. Aguilar-Lemarray<sup>2</sup>, A. Cid-Arregui<sup>3</sup>, M. Jimenez-Perez<sup>2</sup>, J. Haramati<sup>1</sup>, A. Daneri-Navarro<sup>1</sup>, A. del Toro-Arreola<sup>1</sup>, A. Bravo-Cuellar<sup>2</sup>, L. F. Jave-Suarez<sup>2</sup>

<sup>1</sup>Universidad de Guadalajara, Laboratorio de Inmunología, Guadalajara, Mexico, <sup>2</sup>Instituto Mexicano del Seguro Social, Centro de Investigación Biomédica de Occidente, Guadalajara, Mexico, <sup>3</sup>Deutsches Krebsforschungszentrum (DKFZ), Tumor Gene Therapy, Heidelberg, Germany

NK cells are an important arm of the innate immune system specialized for killing virus-infected and tumor cells. Their activity is tightly regulated by a balance of inhibitory and activating receptors. NKG2D, a major activating receptor, stimulates the natural cytotoxicity after binding to MHC-related molecules induced under stress conditions. The best characterized NKG2D ligands are represented by MHC class I-related chain A, and B (MICA and MICB). Under normal physiological conditions MIC expression is almost restricted to gastrointestinal epithelium, but they can be up-regulated by epithelial and hematological tumors. Despite the fact that MICA and MICB share a high homology at the DNA and protein level, there is evidence for differential regulation of their promoters indicating that these molecules could differentially respond to several damage stimuli. Therefore, the following research was focused to gain a better understanding of MICA and MICB expression at the molecular and cellular level in a panel of human cervical cancer cell lines. We used flow cytometry, Western blot, and ELISA to detect MICA and MICB at a protein level (in cell surface, in cell lysates or soluble molecules, respectively) in HeLa, SiHa and C-33 cells as well as in a non-tumorigenic keratinocyte cell line (HaCaT). By Real-Time PCR we quantified the MICA- and MICB-mRNA. MICA/B was found in all of the cell lysates. In cell surface we found a predominant expression of MICA in almost of the cell lines. However, when we looked for soluble proteins in supernatant we detected a higher concentration of soluble MICB. The molecular analysis did not show an important difference at the MICA- or MICB-mRNA level; however, when HeLa and HaCaT cells were exposed to etoposide as genotoxic stress, we found a predominant transcriptional expression of MICA, and not of MICB which remained with no expression changes. In conclusion, our results show that MICA and MICB are differentially expressed at a protein level; also suggest that both molecules could show different cell surface-shedding behavior. Moreover, MICA and MICB responded differentially to stress stimuli, which may contribute to the triggering of different immune activation pathways in NK cells.

### PA18/4 HETEROGENEITY OF CIRCULATING HLA-G IN VIVO: COMPLEXES AND NITRATED PROTEIN FORMATION

A. Díaz-Lagares<sup>1</sup>, E. Alegre<sup>1</sup>, M. Galarza<sup>1</sup>, A. Arroyo<sup>1</sup>, Á. González<sup>1</sup>

<sup>1</sup>Clinica Universitaria de Navarra, UNAV, Biochemistry, Pamplona, Spain

**Introduction:** HLA-G is a membrane suppressive molecule that can also be released to the medium. HLA-G presents several isoforms and can also undergo post-translational modifications, which are glycosylation, multimers formation through disulfide-bridges, and tyrosine nitration. As the presence of circulating multimers and nitrated HLA-G have not been demonstrated in vivo, the aim of this work was to investigate if these modified HLA-G molecules can occur in vivo.

**Methods:** Exudates and blood samples were obtained anonymously from patients during analytical routine. The protocol was approved by the Local Ethic Committee. HLA-G was analyzed by ELISA, and nitrites by colorimetry using commercial kits. Four exudates and four plasma samples were selected based on HLA-G production and nitrite concentration. Samples were separated by reducing or non-reducing SDS-PAGE, and HLA-G was detected by western blot using the anti-HLA-G antibodies 4H84 or MEM-G/1. Cell lysates from U-937-HLA-G1 transfected cells were used as controls.

**Results:** Under non-reducing conditions a band of HLA-G at 45 kDa was observed that also appeared in the lysate controls, but there were other intense bands at higher molecular weight: 75-100 kDa and at 150-200 kDa. HLA-G concentration in samples measured by ELISA was not related to the intensity of the bands observed in the western blots. Under reducing conditions we observed a band at 25 kDa, and a ladder of bands at 50-100 kDa. However under these reducing conditions the band at 45 kDa was not visible in some samples, which implies this band can also correspond to a dimer. These results suggest that HLA-G is present



in biological fluids mainly as truncated protein and as multimers linked by disulfide-binding, but other forms of aggregates could also be present. Immunoprecipitation with anti nitrotyrosine antibody and non-reducing western blot with anti-HLA-G antibody showed a band of nitrated HLA-G at 45 kDa, and at 150–200 kDa. These bands at high molecular weight disappeared in the western blot under reducing conditions, which indicates that nitrated HLA-G is also present in multimers. **Conclusion:** HLA-G circulates *in vivo* forming complexes and as nitrated protein. This heterogeneity in the molecule could affect HLA-G quantification by ELISA.

#### PA18/5 HLA-B27 HEAVY CHAIN HOMODIMER FORMATION AND NATURAL KILLER CELL FAMILY INTERACTIONS – A COMPARISON OF TWO HLA-B27 SUBTYPES IN VITRO

J.L. Giles<sup>1</sup>, J. Shaw<sup>1</sup>, S. Kollnberger<sup>1</sup>, K. McHugh<sup>1</sup>, A. Ridley<sup>1</sup>, K. Maenaka<sup>2</sup>, K. Kuroki<sup>2</sup>, P. Bowness<sup>1</sup>

<sup>1</sup>Weatherall Institute for Molecular Medicine, Oxford University, Human Immunology Unit, Oxford, United Kingdom, <sup>2</sup>Kyushu University, Medical Institute of Bio-regulation, Fukuoka, Japan

**Background:** Possession of the Human Leukocyte Antigen (HLA) B27 strongly predisposes to the development of a group of inflammatory arthritides – such as Ankylosing Spondylitis. A pathogenic role for HLA B27 homodimers has previously been suggested. HLA-B27 exists as polymorphic variants, with different subtypes being more strongly associated with disease than other variants. HLA-B\*2705 is strongly associated with disease and HLA-B\*2709 is less strongly associated with disease.

Here we investigate whether B\*2705 forms more heavy chain homodimers *in vitro* compared to B\*2709, and whether the two subtypes demonstrate different binding properties to the natural killer cell family of receptors KIR3DL1, KIR3DL2, LILRB1 and LILRB2.

##### Methods:

- 1) HLA-B27 was expressed in *E. coli* as inclusion bodies. These were purified and then refolded by limiting dilution in the presence of different peptide ( $\pm$   $\beta$ 2m).
- 2) Isolation and quantification of refolded homodimer and heterotrimeric protein complexes was conducted by gel exclusion chromatography.
- 3) Fluorescent-labelled tetrameric complexes of HLA-B27 heavy chains were used to stain cell lines transfected with KIR (killer cell immunoglobulin-like receptors) and LILR (leukocyte immunoglobulin like receptors).
- 4) Surface Plasmon Resonance (SPR) studies were performed to compare the binding of HLA-B27 homodimers and heterotrimers to the LILR proteins.

**Results:** HLA-B\*2705 forms more homodimer than HLA-B\*2709 *in vitro*. Preliminary evidence suggests that HLA-B\*2705 and HLA-B\*2709 homodimers and heterotrimers demonstrate similar binding properties to different natural killer cell family of receptors.

**Conclusions:** HLA-B27 homodimer formation and natural killer cell family interactions may play a role in Ankylosing Spondylitis pathogenesis. Subtype-specific *in vitro* differences observed in our study could explain differing disease associations.

#### PA18/6 CHARACTERIZATION OF A NOVEL MUTATION IN BETA 2-MICROGLOBULIN GENE AND RESTORATION OF MHC CLASS I ANTIGENS USING ADENOVIRAL VECTOR ADCMBV2M IN ONE MELANOMA CELL LINE

A.B. del Campo<sup>1</sup>, N. Aptsiauri<sup>1</sup>, R.M. Mendez<sup>1</sup>, S. Zinchenko<sup>1</sup>, J. Carretero<sup>1</sup>, G. Gaudernak<sup>2</sup>, G. Gonzalez-Aseguinolaza<sup>3</sup>, F. Ruiz-Cabello<sup>1</sup>, F. Garrido<sup>1</sup>

<sup>1</sup>Hospital Universitario Virgen de las Nieves, Granada, Spain, <sup>2</sup>The Norwegian Radium Hospital, Oslo, Norway, <sup>3</sup>Center for Applied Medical Research, Laboratory of Gene Therapy of Viral Hepatitis, Pamplona, Spain

**Introduction:** Many cancer cells evade immune surveillance via low expression or a total loss of HLA class I antigen, which often are caused by LOH or mutations in the beta-2-microglobulin (b2m) gene. Molecular defects underlying the total loss of HLA class I expression in cancer can be regulatory and treatable with cytokines, or it can be a structural genetic defects often caused by mutations in the b2m gene which can be corrected only with gene therapy. Cancer immunotherapy frequently leads to immunoselection of HLA-negative tumor cell lines with irreversible structural defects which give rise to progressing metastases.

**Objectives:** The main objective of this work was to study molecular mechanisms responsible for the total loss of HLA class I expression (both in basal conditions and after incubation with IFN- $\gamma$ ) in a human melanoma cell line and to recover normal expression of HLA class I using adenoviral vectors.

**Results:** Although we detected normal transcript of the beta2m gene using RT-PCR, the sequencing of the b2m gene revealed the presence of a point mutation (G>T) in exon 2 that produces a stop codon. The second copy of the gene in chromosome 15 was found to have loss of heterozygosity (LOH). These two defects cause a lack of a functional beta2m protein leading to a total loss of HLA class I. We have used adenoviral vector generated by cre-lox recombination system that carries wild type human b2m gene to recover normal expression of HLA class I. Infection of the studied melanoma cell line with this vector led to a 90–100% recovery of surface expression of HLA class I molecules which was stable for up to 15 days *in vitro*.

**Conclusions:** We propose that restoration of beta 2-microglobulin expression using adenoviral vectors is an attractive option to recover normal HLA class I expression in patients with metastatic cancer and irreversible total loss of class I molecules due to mutational events.

#### PA18/7 ANTHROPOLOGICAL VIEW OF IRANIAN ETHNIC GROUPS BASED ON HLA CLASS II GENE VARIATIONS IN COMPARISON TO MITOCHONDRIAL DNA HAPLOGROUPS

S. Farjadian<sup>1</sup>, D. Pettener<sup>2</sup>, L. Castri<sup>2</sup>, M. Sazzini<sup>2</sup>, G. Romeo<sup>2</sup>, A. Ghaderi<sup>1</sup>, D. Luiselli<sup>2</sup>

<sup>1</sup>Shiraz University of Medical Sciences, Shiraz, Iran, Islamic Republic of, <sup>2</sup>University of Bologna, Bologna, Italy

However the results of HLA class II gene diversity revealed a close genetic relation among eleven Iranian ethnic groups with some genetic similarities to Macedonians, Greeks, and Italians; since HLA genes are under strong selection by local infectious agents, mtDNA control region was considered in this study to trace maternal lineage. The variation of the first hypervariable segment (HVS-I) of the mitochondrial DNA was analyzed among 718 Iranians belonged to fourteen different ethnic groups: Pars, Azeri, Kurd, Lur, Baloch, Gilak, Mazandarani, Qashqaei, Turkmen, Armeni, Zoroastrian, Arab, Jew, and the people of Qeshm Island. The results of this study showed that, haplogroups H and U were the most common among Iranians while haplogroupe W (24.6%) was exclusively detected within Baloch people. Iranians are maternally more related to Caucasians, Near Eastern, and Mediterraneans than other Asians, Europeans, or Africans. The results of AMOVA revealed no differentiation among the 14 ethnic groups and about 97.31% of the total variance was attributed to variation within populations. The results of SAMOVA also showed no geographical structure among these ethnic groups. According to the results of MDS; Zoroastrians, Jews, Balochis, and the people of Qeshm can be considered as outliers. These populations also revealed a remarkable degree of long term evolution in phylogenetic analysis. Although the results of this study cleared some obscure points about the genetic relation among Iranian ethnic groups, further data based on complete mtDNA sequencing as well as analysis of paternally transmitted Y chromosome in combination with autosomal gene variations are required to solve the genetic history of the Iranians.

#### PA18/8 IL-10 MODULATES DIFFERENTIALLY MICA AND MICB EXPRESSION ON MELANOMA CELL LINES

A.E. Serrano<sup>1</sup>, E. Menares-Castillo<sup>1</sup>, M. Gatica-Andrades<sup>1</sup>, M. Garrido-Tapia<sup>1</sup>, C.J. Hernández<sup>1</sup>, M.N. López<sup>1</sup>, F. Salazar-Onfray<sup>1</sup>, J.C. Aguillón<sup>1</sup>, M.C. Molina<sup>1</sup>

<sup>1</sup>Universidad de Chile, Programa Disciplinario de Inmunología. ICBM., Santiago, Chile

NKG2D is an important activating receptor involved in the induction of cytotoxicity by NK and CD8(+) human lymphocytes. The NKG2D ligands are major histocompatibility complex class I chain-related protein A and B (MICA and MICB) and UL16-binding proteins (ULBP). These molecules are mainly expressed on the surface of viral infected, tumours and stressed cells. We suggest that the regulation of NKG2D ligands expression allows tumour immune escape, possibly regulated by soluble factors. IL-10 is an important immunoregulatory cytokine, present in several tumours. In our study, we analyzed MICA and MICB expression and their functional relevance in human melanoma cell lines in response to IL-10 stimulus. Malignant melanoma cell lines treated with IL-10 were used to analyze MICA/B expression by qRT-PCR and flow cytometry. Our results demonstrate that IL-10 decrease melanoma cell-surface expression of MICA but not MICB. Also we showed that the down regulation of MICA occurs at the mRNA level, and this effect is dependent to the recombinant, viral or human IL-10 dose.

**Conclusion:** IL-10 would have a role in the regulation of MICA expression on the melanoma cell surface. Interestingly, both NKG2D ligands did not show de same expression pattern despite the fact that MICA and MICB bind the same receptor. These findings could be related to different roles of MICA and MICB in the effector functions of cytotoxic cells.

Financed by: FONDECYT 11060452 FONDEF DO6I1005.

#### PA18/9 MHC CLASS I CHAIN-RELATED GENE A TRANSMEMBRANE POLYMORPHISM IN SPANISH WOMEN WITH BREAST CANCER

R. Lavado-Valenzuela<sup>1</sup>, M.J. Bravo<sup>1</sup>, M. Benavides<sup>2</sup>, M. Cobo<sup>2</sup>, A. Alonso<sup>1</sup>, A. Caballero<sup>1</sup>

<sup>1</sup>Carlos Haya Hospital, Immunology Service, Málaga, Spain, <sup>2</sup>Carlos Haya Hospital, Medical Oncology Section, Málaga, Spain

**Background:** The immune response is mainly undertaken by the innate response. The immune system recognizes the tumoral cells through receptors like NKG2D. MIC-A is a ligand of this receptor and is expressed on transformed cells. We studied 5 alleles of a microsatellite in the MICA transmembrane region, one of which (MICA-A5.1) gives rise to a truncated protein (a soluble form).

**Aim:** To determine whether MIC-A transmembrane polymorphism affects genetic predisposition to human breast cancer in Spanish patients.

**Material and methods:** The study included 110 breast cancer patients from Málaga, Spain and 121 healthy volunteers, all from the same geographical area. MICA TM microsatellite polymorphism was analyzed using a polymerase chain reaction (PCR)-based method. PCR products were electrophoresed in an ABI Prism 3100 Genetic Analyzer and their sizes were determined using the Genescan 672 software.

**Results:** No significant differences were seen in the A4, A5.1, A6 or A9 alleles between patients and controls. The only significant difference was found in the frequency of the A5 allele, which was significantly reduced in patients compared with the controls (P=0.04). Given that an association between the HLA-B7 allele and susceptibility to breast cancer has been described in our area, we also analyzed the distribution of the frequency of the MICA TM alleles in HLA-B7 patients compared to HLA-B7 controls. The frequency of the A5.1 allele was higher in patients than in controls (P=0.001). Again, we found that the frequency of the A5 allele was reduced in HLA-B7 patients compared with HLA-B7 controls, though the significance disappeared after Bonferroni correction. Comparison of the frequency

of the A5.1 allele in HLA-B7 patients with patients with other HLA alleles showed that it was even more strongly increased in HLA-B7 patients ( $\chi^2$  (1 d.f.) = 20.837;  $P=0.00005$ ; after Bonferroni correction,  $P=0.00003$ ) This difference was not found in healthy controls. In addition, we found that 100% of the patients with the HLA-B7 allele also had the A5.1 allele.

**Conclusions:** MICA-A5 allele appears to confer protection against human breast cancer. HLA-B7/MICA-A5.1 combination appears to confer susceptibility to breast cancer in our geographical area.

#### PA18/10 INTRACELLULAR DETECTION AND LOCALIZATION OF HLA-DR, DM, DO AND CD74 IN K562 LEUKEMIA CELL LINE AND POSSIBLE IMPLICATIONS OF HLA-DO IN MAINTENANCE OF THEIR SURFACE CLASS II NEGATIVE STATE

L. Papadimitriou<sup>1</sup>, I. Athanassakis<sup>1</sup>

<sup>1</sup>University of Crete, Biology, Heraklion, Greece

**Aim:** Loss of expression of human leukocyte antigen (HLA) on tumor cells alters the onset and modulation of immune response through lack of activation on CD4<sup>+</sup> lymphocytes. Most leukemia cells use such mechanism to escape immune surveillance. Moreover in patients with leukemia or lymphoma the expression of HLA-class II molecules correlate with better survival. Here, K562 leukemia cells were examined as to intracellular HLA-DR, DM, DO and CD74 expression, if any.

**Methods:** Cytoplasmic immunofluorescence of K562 was performed and the cells were analyzed by flow cytometry and confocal microscopy. The protein profile was also confirmed by RT-PCR analysis.

**Results:** The results presented here showed that despite the negative HLA-DR surface expression, K562 cells contain intracellular HLA-DR and DM molecules, while constitutively expressing the CD74 antigen and the DO $\beta$  chain. Double immunofluorescence experiments revealed the co-localization of all these molecules in endosomal compartments which were identified as late endosomes by the presence of Rab7 protein. To answer the question on how DO $\beta$  chain alone could leave ER and enter the endosomal compartments stable transfectants of K562 with a DO $\beta$ -eGFP fusion construct were isolated. Confocal microscope analysis of the transfectants verified the endosomal localization of the DO $\alpha$  chain indicating that indeed this molecule can leave ER without the need of DO $\alpha$  chain. In addition transfectants of K562 with a DO $\alpha$ -dsRED and double transfectants of K562 with DO $\alpha\beta$  were constructed. These manipulations allowed the following up of DO  $\alpha$  and  $\beta$  chains without the use of any antibody. We also analyze the antigens profile in K562 treated with IFN $\gamma$  for 6 hours as well as in K562 cells transfected with a CIITA transcription factor construct. The results showed that both factors can lead HLA-DR to the cell surface.

**Conclusions:** We conclude that K562 cells despite the absence of any surface class II antigen they contain intracellular HLA-DR molecules which can be transferred to the membrane upon stimulation with IFN $\gamma$ . The presence of DO $\beta$  chain in the same endosomal compartments with class II molecules provide new insights in the role of HLA-DO in antigen presentation showing that this is a more complicated issue than originally thought.

#### PA18/11 ANALYSIS OF CELL SURFACE EXPRESSION OF THE HLA CLASS I AND II ANTIGENS IN SEVERAL HUMAN PROSTATE CELL LINES

A.B. del Campo<sup>1</sup>, J. Carretero<sup>1</sup>, N. Aptsiauri<sup>1</sup>, R. Mendez<sup>1</sup>, S. Zinchenko<sup>1</sup>, S. Ward<sup>2</sup>, F. Ruiz-Cabello<sup>1</sup>, F. Garrido<sup>1</sup>

<sup>1</sup>Hospital Universitario Virgen de las Nieves, Granada, Spain, <sup>2</sup>Onyx Ltd, St George's University of London, London, United Kingdom

**Objectives:** HLA class I abnormalities in tumor cells, with a frequency up to 90% in some types of tumor, have been well documented and are caused by distinct molecular mechanisms. Some of them represent abnormalities in the regulatory mechanisms controlling expression of the components of the antigen processing machinery (APM) or of the class I heavy chain molecules leading to selective downregulation of HLA-A or -B loci. On the other hand, a total loss of HLA class I expression is frequently associated with structural defects in one copy of the beta 2-microglobulin gene associated with the loss of another copy in chromosome 15 due to LOH. We have studied tumor cell lines obtained from patients with prostate cancer (6 tumor cell lines and 7 normal prostate cell lines) kindly provided by Onyx, St George's University of London as a part of ENACT project, European Network for the identification and validation of antigens and biomarkers in Cancer and their application in clinical Tumor Immunology. The main objective was to characterize the tumor cell surface expression of the HLA class I and II antigens in these cell lines.

**Method and Materials:** Five tumor cell lines came with cell lines established from a corresponding autologous normal prostate tissue. All the studied cell lines were immortalized by the transfer of HPV16-E6/E7 genes. HLA- Class I genomic typing was performed by PCR-SSO; FACS analysis with a panel of monoclonal antibodies was used to assess HLA class I-II expression. Quantitative real-time PCR was used to analyze the expression level of the APM.

**Results:** In one cell line we detected a total loss of HLA class I expression due to a beta 2 microglobulin gene deletion. We have characterized the beta 2 microglobulin gene defect and restored its expression by an adenoviral vector carrying the wild type human b2m gene. In several cell lines we encountered a downregulation of HLA locus A and locus B. The molecular mechanism underlying the observed HLA class I alterations and their implication in tumor immune escape are discussed.

#### PA18/12 IN VIVO BIOLOGICAL BEHAVIOR OF TUMOUR CLONES WITH DIFFERENT MHC CLASS I EXPRESSION AFTER INJECTED DIFFERENT CELL DOSE

I. Romero<sup>1</sup>, E. Berrugilla<sup>2</sup>, C. Garrido<sup>3</sup>, I. Linares<sup>1</sup>, G. Federico<sup>1</sup>, G.-L. Angel Miguel<sup>1</sup>

<sup>1</sup>Hospital Universitario Virgen de las Nieves, Granada, Spain, <sup>2</sup>Hospices Universitario Virgen de las Nieves, Granada, Spain, <sup>3</sup>Hospital Universitario Central de Asturias/ Virgen de las Nieves, Granada, Spain

In our laboratory, we have generated a murine tumour system GR9, methylcholanthrene-induced fibrosarcoma. This system is composed of the primary tumour clones and metastatic cell lines obtained after injection of these clones in immunocompetent BALB/c mice. The tumour clones showed significant differences in the expression of MHC class I molecules. We have compared five clones and analyzed local growth and spontaneous metastatic process. Different cell dose were injected subcutaneously in immunocompetent BALB/c mice and primary tumour and metastatic nodes were isolated and adapted tissue culture. MHC class I expression was measured in these cell lines by flow cytometry and compared with the injected cell.

The five selected clones have different expression of class I molecules, thus A7 and G2 clones show high levels of expression, the B7 clone shows intermediate levels and finally, B11 and C5 show lower levels of expression.

We found no differences in the local, neither in used cell dose, of four clones included in this study. Only G2 clone, which present high expression of class I molecules, didn't grow locally and the primary tumour was rejected. However, A7 clone which has lower expression of the molecule L<sup>d</sup> on the surface did grow locally.

In spontaneous metastasis assays, we found significant differences between four clones and also with the cell dose. The number of spontaneous metastasis obtained was significantly lower in clones with low expression of class I molecules. In the assays with the clone A7, which shows high expression of MHC class I molecules, was obtained a high number of metastatic nodules, unlike the assays with B11, which expresses very low levels of MHC class I molecules, metastatic nodules did not generated any dose. We have studied the MHC molecules expression on surface of all metastatic nodules. Our results show a downregulation of the three class I molecules and total loss of the L<sup>d</sup> molecule expression in some cases. This could be consequence of an important role played by the molecule L<sup>d</sup> in the local growth tumour and its subsequent evolution to metastasis.

#### PA18/13 CHARACTERIZATION OF GENETIC POLYMORPHISM OF BOVINE LYMPHOCYTE ANTIGEN DRB3.2 LOCUS IN SARABI CATTLE

S.M. Emam<sup>1</sup>, G. Nikbakht Brujeni<sup>1</sup>, M. Mousavi<sup>2</sup>, N. Barjesteh<sup>1</sup>

<sup>1</sup>University of Tehran, Faculty of Veterinary Medicine, Microbiology & Immunology, Tehran, Iran, Islamic Republic of, <sup>2</sup>University of Tabriz, Tabriz, Iran, Islamic Republic of

Major Histocompatibility Complex molecules have a key role in adaptive Immune responses. Moreover, genetic polymorphism in the loci which code MHC molecules within a population provides a great ability for APCs to present all possible antigens with different chemical binding affinity. Second exon of BoLA-DRB3 is the most polymorphic locus at the MHC region in cattle. In addition, an association between possession of certain BoLA-DRB3 locus (MHC class II) and resistance or susceptibility to infectious diseases and some production traits have been reported. In this study, 46 samples belong to Sarabi cattle ecotype were genotyped for second exon of BoLA-DRB3 alleles by polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP) by 3 restriction Enzymes. Among 54 previously described PCR-RFLP defined alleles, 26 alleles were identified as well as 4 more new alleles that were observed in this population. To confirm PCR-RFLP genotyping, 22 samples were cloned and sequenced and other samples were sequenced directly. DRB3.2\*46 was the most frequent allele (~9.8%) and DRB3.2\*2, \*3, \*9, \*10, \*13, \*23, \*25, \*33, \*39, \*43, \*45, \*48, \*New3, \*New4 were the least frequent alleles (~1% for each one). On the whole, 16 alleles consist of BoLA-DRB3.2\*46, \*1, \*15, \*12, \*22, \*8, \*35, \*New2, \*27, \*29, \*6, \*7, \*18, \*11, \*24, \*New1 had about 85% of overall frequency. Among this population, 45 haplotype were observed and 43 haplotype were heterozygote (~95% heterozygosity). Some of these alleles have been reported in Iranian Holstein population, but the frequencies of identified alleles are completely different. The obtained result indicates that exon 2 of BoLA-DRB3 is highly polymorphic in Sarabi cattle.

#### PA18/14 POPULATION SPECIFIC DISTRIBUTION OF MICA ALLELES AND HAPLOTYPES. POSSIBLE RELEVANCE IN CANCERS

T. Lukanov<sup>1</sup>, M. Ivanova<sup>1</sup>, A. Nedialkova<sup>1</sup>, G. Gaudernack<sup>2</sup>, E.-M.I. Suso<sup>2</sup>, A. Mihaylova<sup>1</sup>, E. Naumova<sup>1</sup>

<sup>1</sup>University Hospital 'Alexandrovska', Central Laboratory of Clinical Immunology, Sofia, Bulgaria, <sup>2</sup>The Norwegian Radium Hospital, Department of Immunology, Oslo, Norway

In the present study we analyze for the first time MICA polymorphism and associations with other HLA genes in the healthy Bulgarian population. In order to assess their possible role as biomarkers in cancer, MICA polymorphism was also performed in Norwegian patients with advanced malignant melanoma (MM) and prostate cancer (PCA).

MICA allele frequencies were assessed in 60 unrelated healthy individuals from the Bulgarian population and 37 individuals with MM and 25 with PCa from the Norwegian population by using PCR-SBT. MICA allele (af) and haplotype frequencies were estimated by maximum-likelihood analysis and standardized linkage disequilibrium (LD) values were calculated. Phylogenetic and correspondence analyses were performed by using neighbor-joining method. Comparative analysis between the two different cancer groups and 7 Caucasian populations were performed by  $\chi^2$  method.

Significant MICA heterogeneity was observed in the Bulgarian population. Of 65 previously known MICA, 16 MICA were observed. The most frequent alleles were MICA\*00901 (af=0.172), 00801 (af=0.145) and 00201 (af=0.144), while the most rare alleles were MICA\*019 (af=0.013) and 038 (af=0.013). Association analysis suggested strong LD between MICA and HLA class I and class II genes. The most frequent haplotypes were MIC A\*004 – HLA-A\*0201, MIC A\*00801 – HLA-A\*1101; MIC A\*00201 – HLA-B\*3801 and MIC A\*00201 – DRB1\*0402.

Compared to healthy controls, alleles MICA\*002, 005, 024, 038, 042, 044 (MM) and 007 (PC) showed statistically significant higher frequency ( $p < 0.05$ ). Interestingly, the MICA\*042 was found only in MM group ( $p < 0.05$ ).

Comparisons with other ethnic groups showed close relation between Caucasians suggesting possible evolutionary selection of MICA polymorphic variants. Strong association between MICA and HLA-A, -B and -DRB1 was observed. PCa and MM samples showed different allele frequencies when compared with each other or with healthy Caucasians. Our results showed that MICA might be associated with malignant melanoma and prostate cancer. Therefore, a study of MICA variations is important to establish new biomarkers, which can be assessed for clinical relevance and ability to predict quality of life.

#### PA18/15 SOLUBLE HLA-G IN THE PERITONEAL FLUID

V. Urbonas<sup>1</sup>, A. Eidukaite<sup>1</sup>

<sup>1</sup>Institute of Immunology, Vilnius University, Vilnius, Lithuania

HLA-G belongs to the HLA nonclassical class I. HLA-G mainly is expressed on trophoblast and some tumours. The heavy chain of soluble HLA-G interacts with inhibitory receptors such as ILT-2, ILT-4, p49 and KIR2DL4 expressed on natural killer cells, T cells, monocytes, and/or dendritic B cells. An increased amount of soluble HLA-G (sHLA-G) molecules are found in the peripheral blood in various malignant diseases. HLA-G expression in cancer cells has been shown to be important for the escape of immunosurveillance by host T-lymphocytes and natural killer cells.

**Objective:** Presence (determination and evaluation) of sHLA-G in patients with non-malignant diseases in peritoneal fluid.

We examined 58 women from 24 to 39 years of age who complained of pain in the abdomen area: 31 of them were laparoscopically diagnosed to have endometriosis (group I). Other 27 women were without any pathological alterations (group II).

ELISA technique was used to measure the soluble HLA-G level in the samples. The amount of sHLA-G was determined in the peritoneal fluid. The presence of sHLA-G was detected in the peritoneal fluid of more than a half (56%) of the investigated women. The sHLA-G amount varied from 0 to 64 U/ml and these results have demonstrated that sHLA-G levels in investigated groups statistically were not significant.

**Conclusions:** The results have demonstrated that sHLA-G can be accumulated in the peritoneal fluid in non-malignant conditions. The source of these molecules could be peritoneal fluid macrophages or cells of other origin. There is no evidence in the literature on the sHLA-G in the peritoneal fluid in endometriosis. sHLA-G can be important in regulation of activity of cytotoxic cells in the peritoneal fluid.

#### PA18/16 HLG EXPRESSION CAN BE CHANGE BY ABSENCE OF CD2+ CELL

O. Onal<sup>1</sup>, B. Basturk<sup>1</sup>, R. Karakus<sup>1</sup>, C. Aybay<sup>1</sup>

<sup>1</sup>Gazi University, Faculty of Medicine, Immunology, Ankara, Turkey

**Introduction:** Denoting less polymorphism when compared to classical HLA class I molecules, the HLA-G molecule has 7 different isoforms. Initially, HLA-G was considered to be expressed in trophoblastic cells but it was later on proved to have permanent expression in immune system privaled and to have determinable soluble forms. HLA-G is known as a molecule that causes immune suppression.

EBV is associated with several malignant tumors besides being a persistent and common agent of infection. Either immune expression or immune response incompetence of the immune system against EBV has a major role in EBV's persistence.

**Aim:** The aim of the study was to search expression variance of the HLA-G molecule on the surface of B cells dependent on its transfection with EBV, time and presence/absence of CD2+ cells and to search variance of soluble HLA-G level.

**Material and methods:** To this end, B95-8 cell source existent in cell bank of Gazi University Head Department of Immunology which produces EBV virion has been used in the transfection of B lymphocytes. In the presence and absence of CD2+ cells and in the presence and absence of EBV transfection; 24, 48 and 72-hour cultures have been prepared and HLA-G expression on the surface of cells have been measured by flow cytometry by using monoclonal antibodies and sHLA-G levels, IL-10 and TGF-beta levels have been measured by ELISA method.

**Results:** In respect of the study results, HLA-G expression on the surface of CD19+ cells was determined as independent from EBV transfection yet showing a time related increase in the absence of CD2+ cell.

**Discussion:** The cells of the cellular immunity-NK cells and T lymphocytes- have been shown to control the expression of HLA-G. Which of the two types of cells control this mechanism; NK cells or T lymphocytes? The answer to this question is important for the explanation of the factors which controls the expression of HLA-G.

The relationship between the expression of HLA-G and the immune system is thought to be reciprocal. Not only HLA-G inhibits the immune system but the immune system might control HLA-G expression.

### PB03 – T CELL SUBSETS

#### PB03/1 PKB/AKT SIGNALS SUPPORT TREG DIFFERENTIATION AND FUNCTION BUT OPPOSE TH17 DIFFERENTIATION

M. Pierau<sup>1</sup>, S. Engelmänn<sup>1</sup>, D. Reinhold<sup>1</sup>, B. Schraven<sup>1</sup>, U. Bommhardt<sup>1</sup>

<sup>1</sup>Otto von Guericke University Magdeburg, Institute of Molecular and Clinical Immunology, Magdeburg, Germany

CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells (nTreg) are essential regulators of immune suppression and peripheral tolerance. Transforming growth factor  $\beta$  (TGF $\beta$ ) is essential to maintain Foxp3 expression and peripheral nTreg function. In concert with TCR/CD28 signals, TGF $\beta$  is a potent inducer of suppressive function in naïve T cells and, therefore, of inducible Tregs (iTregs). TGF $\beta$  signals also play a key role in the generation of IL17 producing Th17 cells, which promote inflammatory immune responses and the development of autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE).

The signaling events involved in Treg and Th17 differentiation are not yet well understood. One important signaling cascade downstream of TCR/CD28 ligation is the PI3K/PKB/mTOR pathway, which regulates survival, growth and differentiation processes in conventional T cells. However, the role of PKB/Akt in Treg development and function is unclear. Here, we show that elevated PKB (myrPKB) signals antagonize the immunosuppressive effect of TGF $\beta$ 1 on proliferation of naïve CD4<sup>+</sup> T cells from wild type and CD28-deficient mice. PKB signals alter the susceptibility of conventional CD4<sup>+</sup> T cells to Treg mediated suppression and enhance nTreg suppressor function. Upon TCR and TGF $\beta$ 1 co-stimulation, CD4<sup>+</sup> T cells from PKB transgenic mice readily express Foxp3, thereby acquiring suppressor capacity. These effects of elevated PKB signals on TCR/TGF $\beta$  regulated T cell differentiation correlate with marked and sustained activation of the transcription factors STAT5 and Foxp3 and reduced NFATc1 levels. Moreover, elevated PKB signals impair TGF $\beta$ /IL6 mediated differentiation of naïve CD4<sup>+</sup> T cells into the Th17 lineage and alter the severity of EAE disease in wild type and CD28-deficient mice. Thus, active PKB has differential impact on TGF $\beta$  mediated T cell effector functions indicating an important role of PKB in balancing immunosuppressive versus inflammatory immune responses.

#### PB03/2 INVARIANT NKT CELLS INHIBIT DEVELOPMENT OF THE TH<sub>17</sub> LINEAGE

L.T. Mars<sup>1</sup>, L. Araujo<sup>2</sup>, P. Kerschen<sup>1</sup>, S. Diem<sup>2</sup>, E. Bourgeois<sup>2</sup>, L.P. Van<sup>2</sup>, N. Carrié<sup>1</sup>, M. Dy<sup>2</sup>, R.S. Liblau<sup>1</sup>, A. Herbelin<sup>2</sup>

<sup>1</sup>INSERM U563, Autoimmunity and Immune Regulation, Toulouse, France, <sup>2</sup>CNRS UMR 8147 – Hôpital Necker, Paris, France

T cells differentiate into functionally distinct effector subsets in response to pathogen encounter. Cells of the innate immune system direct this process; CD1d-restricted iNKT cells, for example, can either promote or inhibit Th<sub>1</sub> and Th<sub>2</sub> responses. Recently, a new subset of CD4<sup>+</sup> T helper cells, called Th<sub>17</sub>, was identified that is implicated in mucosal immunity and autoimmune disorders. To investigate the influence of iNKT cells on the differentiation of naïve T cells we used an adoptive transfer model of traceable antigen-specific CD4<sup>+</sup> T cells. Transferred naïve CD25<sup>+</sup>CD62L<sup>+</sup> CD4<sup>+</sup> T cells were primed by antigen-immunization of the recipient mice permitting their expansion and Th<sub>17</sub> differentiation. This study establishes that *in vivo* activation of iNKT cells during T cell priming impedes the commitment of naïve T cells to the Th<sub>17</sub> lineage. *In vivo* cytokine neutralization experiments revealed a role for IL-4, IL-10 and IFN- $\gamma$  in the iNKT cell-mediated regulation of T cell lineage commitment. Moreover, by comparing IL-17 production by antigen-experienced T cells from unmanipulated wild-type mice and iNKT cell-deficient mice, we demonstrate an enhanced Th<sub>17</sub> response in mice lacking iNKT cells. This invigorated Th<sub>17</sub> response reverts to physiological levels when iNKT cells are introduced into Ja18<sup>-/-</sup> mice by adoptive transfer, indicating that iNKT cells control the Th<sub>17</sub> compartment at steady state. We conclude that iNKT cells play an important role in limiting development of the Th<sub>17</sub> lineage and suggest that iNKT cells provide a natural barrier against Th<sub>17</sub> responses.



**PB03/3 HUMAN RHINOVIRUSES INDUCE IL-35 PRODUCING REGULATORY T CELLS VIA INDUCTION OF B7-H1 (CD274) AND SIALOADHESIN (CD169) ON DENDRITIC CELLS**M. Seyerl<sup>1</sup>, S. Kirchberger<sup>1,2</sup>, O. Majdic<sup>3</sup>, J. Seipelt<sup>3</sup>, C. Jindra<sup>4</sup>, C. Schrauf<sup>1</sup>, J. Stöckl<sup>1</sup><sup>1</sup>Institute of Immunology, Medical University of Vienna, Vienna, Austria, <sup>2</sup>William Dunn School of Pathology, University of Oxford, Oxford, United Kingdom, <sup>3</sup>Max F. Perutz Laboratories, Department of Medical Biochemistry, Medical University of Vienna, Vienna, Austria, <sup>4</sup>Laboratory of Viral Oncology, DIAID, Department of Dermatology, Medical University Vienna, Vienna, Austria

IL-35, a heterodimer of EBV-induced gene 3 (EBI3) and the p35 subunit of IL-12, has been recently identified as an inhibitory cytokine. Here we demonstrate that human dendritic cells (DC) activated by human rhinoviruses (R-DC) induce suppressor function in co-cultured human T cells. The inhibitory effect was found in the culture supernatant of CD4<sup>+</sup> and CD8<sup>+</sup> peripheral blood T-cells activated with R-DC but not with naïve T cells from cord blood. Neutralizing mAbs against IL-10 and TGF- $\beta$  failed to revert the effect. We found however that the R-DC induced regulatory T cells produced and released IL-35 and antibodies against EBI3 or depletion of IL-35 removed the inhibitory effect of the supernatant of the regulatory T cells. Most importantly, blocking of B7-H1 (CD274) and Sn (CD169) on R-DC with specific mAbs against both receptors prevented the induction of IL-35. Thus, inhibitory signals delivered from R-DC to T cells via B7-H1 and sialoadhesin (Sn) were critical for the induction of IL-35 producing regulatory T cells. These observations suggest that an altered accessory molecule repertoire on DC upon interaction with HRV down-modulates adaptive immune responses during the viral infection.

**PB03/4 DECTIN-1 ACTIVATION PATHWAY AS A NEW TARGET TO INDUCE NEONATAL TH1/TH17 POLARIZATION**B. Jaron<sup>1</sup>, C. Leclerc<sup>1</sup>, R. Lo-Man<sup>1</sup><sup>1</sup>Institut Pasteur, Paris, France

Neonatal immune responses are generally characterized by low antibody titres, poor cell-mediated cytotoxicity and Th2 biased T lymphocyte responses to intracellular pathogens and vaccines. Thus, identifying adjuvants capable of inducing pro-inflammatory responses such as Th1 and Th17 during neonatal immune responses, represents an important goal for vaccine development against infectious diseases.

We first evaluated the capacity of neonatal CD4 T cells to differentiate into Th1 or Th17 pathway *in vitro*. Neonatal T cells were able to differentiate into Th1 cells under appropriate conditions. As for adult T cells, neonatal Th17 polarization can also be achieved following anti-CD3/CD28 stimulation by adding the critical cytokines, TGF- $\beta$  and IL-6, driving the Th17 differentiation. This shows that there is no intrinsic defect in neonatal CD4 T cells for T helper differentiation. We then investigated the *in vivo* conditions allowing neonatal Th1/Th17 polarization, through triggering of different innate activation pathways. In the context of ovalbumin (OVA) immunization, adjuvants triggering Toll-like receptors, alone or together with Nod-like receptors, failed to induce OVA-specific Th1 and Th17 responses. This impairment was due to the activation of neonatal regulatory B cells (Bregs) following TLR triggering, which down-regulate dendritic cell functions. A recently described alternative innate activation pathway involves Syk dependent C-type lectin receptors. As Bregs do not express Dectin-1, a C-type lectin receptor implicated in the recognition of  $\beta$ -glucans, we tested the *in vivo* adjuvant capacity of Dectin-1 agonist. Under these conditions both OVA-specific Th1 and Th17 responses could be induced in neonatal mice.

In conclusion, Dectin-1, but neither TLR nor NOD signalling efficiently promote Th1/Th17 responses in the neonatal context.

**PB03/5 IL-7-DRIVEN HOMEOSTATIC PROLIFERATION INDUCES THE GENERATION OF SHORT-LIVED FUNCTIONALLY DISTINCT MEMORY CD4<sup>+</sup> T CELLS EXPRESSING CD45RA**V. Libri<sup>1</sup>, S.E. Jackson<sup>1</sup>, R.I. Azevedo<sup>1,2</sup>, J.E. Cook<sup>1</sup>, J. Curnow<sup>3</sup>, M. Salmon<sup>3</sup>, P.C.L. Beverley<sup>4</sup>, A.N. Akbar<sup>1</sup><sup>1</sup>University College London, Department of Immunology and Molecular Pathology, London, United Kingdom, <sup>2</sup>Instituto de Medicina Molecular, Unidade de Imunologia Clínica, Lisbon, Portugal, <sup>3</sup>University of Birmingham, Department of Rheumatology, MRC Centre for Immunoregulation, Birmingham, United Kingdom, <sup>4</sup>Edward Jenner Institute for Vaccine Research, Compton, United Kingdom

Human naïve T cells express CD45RA and lose it upon antigen priming whereas CD45RO represents a marker for memory and effector T cells. Reversion to CD45RA expression is observed in a distinct population of resting CD8<sup>+</sup> memory T cells. Initially, due to the low percentage, this revertant population was not identified in CD4 T cells and to date memory CD4<sup>+</sup> T cells expressing CD45RA have been poorly characterised. Using a combination of CD45RA and CD27 markers to define the memory populations in CD4<sup>+</sup> T cells, we performed a phenotypical and functional study of the CD4<sup>+</sup> CD27<sup>+</sup>CD45RA<sup>+</sup> subset (revertants). Here we demonstrate that, despite their phenotypic profile (CCR7 CD28 CD57<sup>+</sup> IL7R<sub>low</sub>), revertants are not at an end-stage of differentiation and they have longer telomeres than effector memory (CD27<sup>+</sup>CD45RA<sup>+</sup>) cells. We provide evidence that CD27<sup>+</sup>CD45RA<sup>+</sup> T cells are fully functional and show a characteristic cytokine profile represented by high levels of IFN- $\gamma$ , IL-13 and GM-CSF production. This CD27<sup>+</sup>CD45RA<sup>+</sup> subset is short-lived as this population is unable to activate telomerase activity, is defective in Akt (Ser473) phosphorylation and expresses low level of Bcl-2. Interestingly we show that CD27<sup>+</sup>CD45RA<sup>+</sup> T cells can revert to CD45RA<sup>+</sup> when cultured *in vitro* in the presence of IL-7. In conclusion CD4<sup>+</sup> memory T cells expressing CD45RA represent a functional population which is able to exert effector functions in response to TCR stimulation but is prone to die shortly after activation. We hypothesise that *in vivo* this population might be continuously replaced by revertants originating from the CD4<sup>+</sup>CD27<sup>+</sup>CD45RA<sup>+</sup> reservoir through a homeostatic process driven by IL-7.

**PB03/6 ONE-POT, MIX-AND-READ PEPTIDE-MHC CLASS I TETRAMERS**A. Stryhn<sup>1</sup>, C. Leisner<sup>2</sup>, N. Loeth<sup>1</sup>, K. Lambert<sup>1</sup>, S. Justesen<sup>1</sup>, E. Smidt<sup>1</sup>, M. Claesson<sup>1</sup>, S. Buus<sup>1</sup><sup>1</sup>University of Copenhagen, Institute for International Health, Immunology and Microbiology, Copenhagen N, Denmark, <sup>2</sup>Swiss Federal Institute of Technology, Zurich, Switzerland

**Objectives:** Cytotoxic T Lymphocytes (CTL) recognize complexes of peptide ligands and Major Histocompatibility Complex (MHC) class I molecules presented at the surface of Antigen Presenting Cells (APC). Detection and isolation of CTLs are of importance for research on CTL immunity, and development of vaccines and adoptive immune therapy. Peptide-MHC tetramers have become important reagents for detection and enumeration of specific CTLs.

**Methods:** Conventional peptide-MHC-tetramer production involves recombinant MHC production, *in vitro* refolding, biotinylation and tetramerization; each step followed by various biochemical steps such as chromatographic purification, concentration etc. Such cumbersome production protocols have limited dissemination and restricted availability of peptide-MHC tetramers effectively precluding large-scale screening strategies involving many different peptide-MHC tetramers.

**Results:** We have developed an approach whereby any given tetramer specificity can be produced within 2 days with very limited effort and hands-on time. The strategy is based on the isolation of correctly oxidized, *in vivo* biotinylated recombinant MHC I heavy chain (HC). Such biotinylated MHC I HC molecules can be refolded *in vitro*, tetramerized with streptavidin, and used for specific T cell staining; all in a one-pot reaction without any intervening purification steps.

**Conclusions:** We have developed an efficient "one-pot, mix-and-read" strategy for peptide-MHC tetramer generation, and demonstrated specific T cell straining comparable to a commercially available MHC-tetramer. We have generated and validated 55 peptide-MHC tetramers representing 24 different human MHC (HLA) class I proteins. The technique should be readily extendable to any binding peptide and pre-biotinylated MHC (at this time we have over 60 different pre-biotinylated HLA proteins). It is simple, robust, and versatile technique with a very broad application potential as it can be adapted both to small- and large-scale production of one or many different peptide-MHC tetramers for T cell isolation, or epitope screening.

**PB03/7 IDENTIFYING THE CORRELATIONS BETWEEN THE MRNA EXPRESSION OF THREE TRANSCRIPTION FACTORS GATA-3, C-MAF AND HELIOS AND THE EXPRESSION OF THE CYTOKINE IL-4 WITHIN BY CD4 T CELLS RESPONDING TO TH2 ANTIGEN *IN VIVO***K. Serre<sup>1</sup>, E. Mohr<sup>1</sup>, K.M. Toellner<sup>1</sup>, R. Bird<sup>1</sup>, A.F. Cunningham<sup>1</sup>, I.C.M. MacLennan<sup>1</sup><sup>1</sup>MRC Centre for Immune Regulation, IBR, University of Birmingham, Birmingham, United Kingdom

**Objectives:** To decipher how Th2-cytokine-producing CD4 T cells are induced *in vivo* in response to a Th2-inducer – alum-precipitated OVA.

**Methods:** Mouse T cell differentiation was studied using OVA-specific CD4 T (OTII) cells transferred into congenic WT mice. The kinetics of cytokine mRNAs up-regulation was compared with transcription factor expression in OTII cells responding in the node draining the alumOVA sc immunization.

**Results:** All OTII cells in the responding node upregulated CD69 12h after immunization. Real time RT-PCR shows the up-regulation of IL-4, IL-13, GM-CSF, IL-3, IL-21, IL-2 and IL-10 mRNA in the responding OTII population. Cytokine production was confirmed at the protein level and reveals that by 3 days some 2-4% of OTII cells produced IL-4, GM-CSF and IL-3.

Specific Th2 and novel transcription factors – GATA-3, c-Maf and Helios – were also selectively induced. We propose that Helios, an Ikaros family member, is a new candidate regulator of Th2 features in T cells. This is consistent with findings indicating that Ikaros regulates the IL-4 locus.

Given the low number of IL-4-producing cells, information at the population level gives poor insight into the specific mechanisms regulating IL-4 induction. The kinetics of cytokine and transcription factor expression was evaluated in single OTII cells isolated after different rounds of division visualized by CFSE dilution. This shows that IL-2, IL-10 and IL-4/IL-13-production are acquired sequentially during the differentiation program. By contrast, GATA-3 and Helios are up-regulated before the OVA-responding cells start to proliferate, and well before IL-4/IL-13 expression that starts after about four-six divisions. Although the vast majority of the OTII cells upregulate c-Maf and/or Helios and/or GATA-3 only about 5-10% express IL-4 mRNA. Finally, some 70% of IL-4-positive OTII cells are positive for GATA-3 and/or Helios while 100% express c-Maf mRNA.

**Conclusions:**

- 1) the definition of Th2-polarization solely as induction of IL-4-production, does not reflect the diversity of effector T helper subsets generated during a Th2-response.
- 2) GATA-3, c-Maf and Helios expressed individually, if necessary are not sufficient for IL-4 induction. Specific stoichiometric studies of various transcription factors may be necessary to devise ways to modulate IL-4 gene expression *in vivo*.

PB03/8

**CD69 REGULATES T<sub>H</sub>-17 CELL DIFFERENTIATION AND CONTROLS INFLAMMATION**P. Martín<sup>1</sup>, M. Gómez<sup>2</sup>, A. Lamana<sup>2</sup>, M. Ramírez-Huesca<sup>1</sup>, A. Cruz-Adalia<sup>1</sup>, C. Gutiérrez<sup>1</sup>, F. Sánchez-Madrid<sup>1,2</sup><sup>1</sup>National Centre for Cardiovascular Research, Vascular Biology and Inflammation, Madrid, Spain, <sup>2</sup>Hospital Universitario de la Princesa, Servicio de Inmunología, Madrid, Spain

**Objectives:** The early leukocyte activation antigen CD69 is highly expressed at sites of inflammation but its physiological function in such processes remains unclear. In order to further address the role of CD69 in inflammatory processes, we have explored the role of CD69 in the differentiation of T helper lineages and its effect on a murine model of OVA-induced allergic asthma and contact hypersensitivity in CD69 deficient mice.

**Methods:** The molecular mechanisms involved in the development of T helper subsets and the role of CD69 in the signalling pathways controlling Th differentiation, have been analyzed in an antigen-specific model by crossing Knock-out CD69 mice (CD69KO) of C57BL/6 strain with OTII transgenic mice expressing a TCR specific for OVA peptide (OVAp<sub>323-339</sub>). For the study of the role of CD69 in the development of inflammatory responses *in vivo*, we have analyzed two animal models of autoimmune and inflammatory diseases; allergic asthma and cutaneous contact hypersensitivity reactions.

**Results:** Here we show that the leukocyte activation antigen CD69 negatively regulates differentiation of pro-inflammatory helper T cells (TH-17). Upon antigen stimulation *in vivo* or *in vitro*, CD4<sup>+</sup> T cells from CD69<sup>-/-</sup> mice secrete higher amounts of interleukin 17 than WT cells, and *in vitro* stimulation induces greater mRNA expression of IL-17, IL-23R and the orphan nuclear receptor RORγt. Enhanced TH-17 differentiation observed in CD69<sup>-/-</sup> cells requires elevated expression of sphingosine-1-phosphate type 1 receptor. CD69 deficiency exacerbates inflammation in mouse models of antigen-induced cutaneous contact hypersensitivity and airway allergy, and IL-17 production is increased in CD69<sup>-/-</sup> animals in both models. Furthermore, neutralizing anti-IL-17 antibodies reduce skin inflammation. These results provide important new insights into the mechanism of CD69-mediated modulation of immune inflammatory processes.

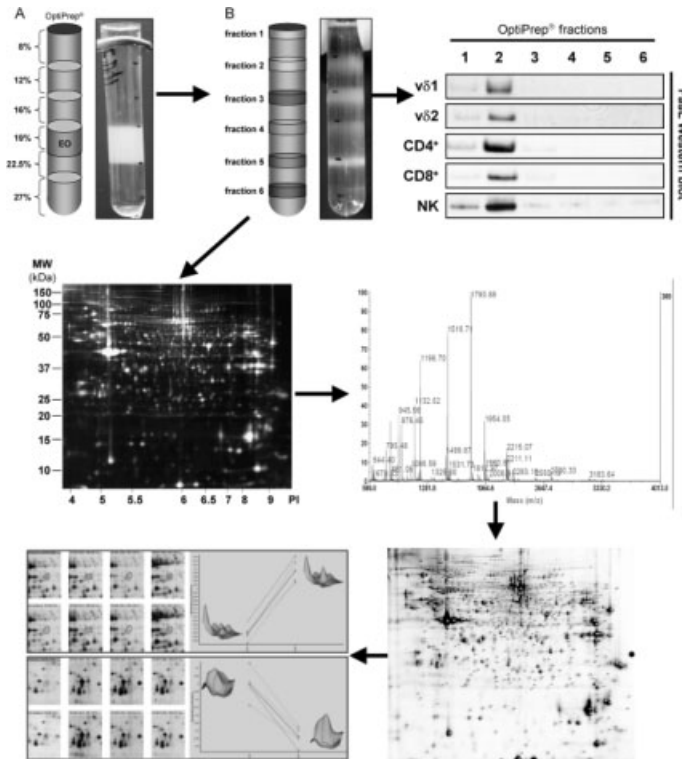
**Conclusion:** The results presented here identify a previously unknown function of CD69 as a regulator of T lymphocyte differentiation toward the TH-17 lineage. Our study reveals an important regulatory role for this molecule in T cell differentiation; links its expression to both TH-17 differentiation and S1P<sub>1</sub> modulation for the first time; and increases our knowledge of the origin and progression of important chronic immune inflammatory diseases.

PB03/9

**THE PROTEOME OF SECRETORY LYSOSOMES FROM T AND NK CELLS**H. Schmidt<sup>1</sup>, C. Gelhaus<sup>2</sup>, M. Nebendahl<sup>1</sup>, M. Lettau<sup>1</sup>, D. Wesch<sup>1</sup>, R. Lucius<sup>3</sup>, M. Leippe<sup>2</sup>, O. Janssen<sup>1</sup><sup>1</sup>Medical Center Schleswig-Holstein Campus Kiel, Institute of Immunology, Kiel, Germany, <sup>2</sup>Zoological Institute, CAU Kiel, Department of Zoophysiology, Kiel, Germany, <sup>3</sup>Institute of Anatomy, CAU Kiel, Kiel, Germany

**Objectives:** T lymphocytes and Natural Killer (NK) cells are main cytotoxic effector cells of the immune system. They use effector molecules including granzymes, perforin, granulysin and FasL to kill tumor and virally infected cells. Most importantly, the cytotoxic effector molecules are stored in so-called secretory lysosomes (SL), which represent dual-function organelles that combine properties of conventional lysosomes and exocytotic vesicles.

**Methods:** We established a method to enrich SL from human T and NK cell populations. Individual fractions were characterized by Western blotting and electron microscopy. Enriched SL of NK, γδ, CD4 and CD8 cells were compared using 2D Difference Gel Electrophoresis (2D-DIGE). The protein profile was analyzed by peptide mass fingerprinting and cell specific protein distribution was analyzed using the DeCyder Software package.



[Workflow]

**Results:** We identified over 700 individual spots in fractions corresponding to SL and potentially a second form of secretory granule. Although the overall protein content was very similar for all T cell populations, we detected a cell line specific pattern of functionally relevant proteins. Selected differentially expressed proteins were verified by Western blotting and intracellular FACS staining.

**Conclusion:** We provide the first comprehensive and comparative proteome map for enriched secretory lysosomes of individual lymphocyte subpopulations. Individual differences in SL components point to different ways to convey effector function for example in αβ vs. γδ T cells.

**PB03/10 HUMAN INTERLEUKIN-17-PRODUCING CELLS ORIGINATE FROM A CD161+CD4+ T-CELL PRECURSOR**L. Maggi<sup>1</sup>, L. Cosmi<sup>1</sup>, R. De Palma<sup>2</sup>, V. Santarlasci<sup>1</sup>, M. Capone<sup>1</sup>, F. Frosali<sup>1</sup>, G. Rodolico<sup>2</sup>, V. Querci<sup>1</sup>, G. Abbate<sup>2</sup>, R. Angelini<sup>1</sup>, L. Berrino<sup>2</sup>, E. Lazzeri<sup>1</sup>, P. Parronchi<sup>1</sup>, F. Liotta<sup>1</sup>, E. Maggi<sup>1</sup>, S. Romagnani<sup>1</sup>, F. Annunziato<sup>1</sup><sup>1</sup>University of Florence, Florence, Italy, <sup>2</sup>Second University of Naples, Naples, Italy

Classically, naive CD4<sup>+</sup> T cells have been thought to differentiate into two main lineages, Th1 and Th2 cells. More recently, a third subset of CD4<sup>+</sup> effector T cells which produce IL-17 has been described in mice, which was named as Th17 afterwards. Recently, we showed that, in addition to human memory T cells producing IL-17 alone (Th17), there is a number of T cells in both blood and tissues that co-produce IL-17 and IFN- $\gamma$  (Th17/Th1). We also found that these two cell types express both ROR $\gamma$ t and T-bet and that Th17 cells could be shifted to Th1 by the addition of IL-12, an effect that was partially antagonized by IL-23, suggesting a flexibility of human Th1 cells and their possible common developmental origin with Th1 cells. Furthermore, we and others identified IL-23 receptor (IL-23R) and CCR6 as surface molecules expressed by human Th17 cells. In this study, by using the microarray assay we found that CD161 was one of the most up-regulated genes in human Th17, in comparison with Th1 or Th2, clones. Accordingly, T-blasts from all Th17 clones expressed CD161 on their surface, whereas all Th1 or Th2 clones examined were CD161<sup>-</sup>. All IL-17-producing cells were found to be included within the CD161<sup>+</sup> fraction of adult circulating CD4<sup>+</sup> T cells, but they were not CD1-restricted natural killer T (NKT), but NKT-like, cells. When CD161<sup>+</sup> or CD161<sup>-</sup> cells were sorted from umbilical cord blood (UCB) naive CD4<sup>+</sup> T cells and activated in presence of IL-1b plus IL-23, Th17, Th17/Th1 or Th1 cells developed from the CD161<sup>+</sup> fraction, whereas CD161<sup>-</sup> cells could never be induced to differentiate into IL-17-producing cells. On the opposite, in presence of IL-12, both CD161<sup>+</sup> and CD161<sup>-</sup> cells only differentiated into Th1 cells. These findings indicate that CD161 is a novel surface marker for human IL-17-producing cells and demonstrate that human Th17 and Th17/Th1 cells exclusively originate from a NKT-like CD4<sup>+</sup> T-cell precursor in presence of IL-1b and IL-23 as polarizing cytokines.

**PB03/11 COMBINED ACTIVATION SIGNALLING BREAKS ESTABLISHED CD8+ T CELL TOLERANCE AND RESULTS IN EFFECTOR CTL GENERATION**J.P. Böttcher<sup>1</sup>, D. Stabenow<sup>1</sup>, P.A. Knolle<sup>1</sup>, L. Diehl<sup>1</sup><sup>1</sup>University of Bonn, Institute of Molecular Medicine and Experimental Immunology, Bonn, Germany

In the liver, soluble and gut-derived antigens are efficiently taken up by the resident antigen presenting cells, the liver sinusoidal endothelial cells (LSEC). Importantly, LSEC are capable of cross-presenting these antigens on MHC class I molecules to naive CD8<sup>+</sup> T cells. This cross-presentation results in the induction of antigen-specific T cell tolerance and the generation of a unique, tolerant CD8<sup>+</sup> T cell subset. Notably, LSEC-mediated T cell tolerance is not deletional. Rather, tolerant CD8<sup>+</sup> T cells survive in an inactive state, incapable of cytotoxic activity or production of effector cytokines.

As LSEC-tolerised T cells redistribute to secondary lymphoid organs, we wondered whether their initially established tolerant phenotype can be overruled and converted into an effector T cell type, e.g. by matured professional APCs. To investigate this, we tested the impact of the three CD8<sup>+</sup> T cell activation signals – TCR triggering (signal 1), costimulation (signal 2) and Interleukin-12 (signal 3) – on tolerant CD8<sup>+</sup> T cells. In order to determine the effect of the different activation signals on tolerant T cells and to analyse the differences and similarities to naive CD8<sup>+</sup> T cell activation, we chose an easily manipulable in vitro system stimulating cells with antibody-coated beads. Stimulation of LSEC-tolerised CD8<sup>+</sup> T cells with the combination of signals 1 and 2 elicited rapid proliferation and clonal expansion, but these T cells did not produce IFN- $\gamma$  nor exerted cytolytic activity. However, the deficiency in these key effector functions was completely overcome by additional IL-12 leading to development of fully competent effector cytotoxic T lymphocytes. Strikingly, the early cytokine response of tolerised CD8<sup>+</sup> T cells to full activation signals was superior to naive T cells, a feature also shared by memory T cells. Our data shows that the tolerant fate of LSEC-primed CD8<sup>+</sup> T cells is not ultimate, but can be reverted by appropriate stimulation usually provided by fully matured APCs. This could be of importance in the combat of infections by environmental pathogens which previously, due to their gastro-intestinal origin, led to LSEC-mediated tolerance induction.

**PB03/12 IL-21 MEDIATED TH17 DIFFERENTIATION IS DEPENDENT ON IRF4**E. Bothur<sup>1</sup>, A. Brüstle<sup>1</sup>, K. Reinhard<sup>1</sup>, A. Guralnik<sup>1</sup>, G. Walter<sup>1</sup>, A. Mahiny<sup>1</sup>, E. von Löw<sup>1</sup>, M. Lohoff<sup>1</sup>, M. Huber<sup>1</sup><sup>1</sup>Institut für Medizinische Mikrobiologie und Hygiene, Philipps-Universität Marburg, Marburg, Germany

Antigenic stimulation of murine naive T helper (Th) cells in the presence of TGF $\beta$  and IL-6 induces the differentiation of the Th17 subset. During the course of Th17 differentiation, the cells secrete IL-21 which amplifies its own production in a STAT3 dependent manner and induces the expression of the IL-23 receptor thereby promoting stabilization of the Th17 phenotype. Interferon regulatory factor 4 (IRF4) has been shown to be an indispensable factor for Th17 differentiation. Here we demonstrate that IRF4 is absolutely necessary not only for IL-21 induction but also for responsiveness of Th17 cells to IL-21. IRF4 deficient (*Irfa*<sup>-/-</sup>) Th cells cultured in the presence of TGF $\beta$  and IL-21 showed a severe defect in both IL-17 and IL-21 production. While STAT3 phosphorylation was normal under these conditions, levels of IL-23 receptor and the lineage-specific orphan nuclear receptors ROR $\alpha$  and ROR $\gamma$ t were strongly diminished. This lack of Th17 specific factors seems to be one reason for the IL-17 defect as we were able to regain IL-17 production in *Irfa*<sup>-/-</sup> cells to some extent by retroviral reintroduction of ROR $\alpha$  and ROR $\gamma$ t. In contrast, we found the expression of Forkhead box P3 (Foxp3), a key factor of the opponent regulatory T cell subtype, highly up-regulated. This elevated Foxp3 level presumably makes a further contribution to the *Irfa*<sup>-/-</sup> phenotype as overexpression of Foxp3 counteracted IL-21 mediated IL-17 production in WT Th cells. Together these data highlight IRF4 as a central factor of the IL-21 mediated Th17 program at several levels.

**PB03/13 THE THYROID SELF-ANTIGEN, THYROGLOBULIN, INDUCES CD4+ T CELLS FROM HEALTHY DONORS TO PROLIFERATE WITH MEMORY CELL KINETICS AND LEADS PRIMARILY TO PRODUCTION OF THE REGULATORY CYTOKINE, IL-10**C.H. Nielsen<sup>1</sup>, M.P. Galdiers<sup>2</sup>, C.J. Hedegaard<sup>1</sup>, R.G.Q. Leslie<sup>2</sup><sup>1</sup>Copenhagen University Hospital, Rigshospitalet, Institute for Inflammation Research, Copenhagen, Denmark, <sup>2</sup>University of Southern Denmark, Institute of Medical Biology, Odense, Denmark

**Objective:** Thyroglobulin (TG), as auto-antigen, induces in vitro proliferation of T cells from normal individuals, with a cytokine production differing from that of patients with autoimmune thyroid disease. We aimed to investigate whether normal TG-responsive CD4<sup>+</sup> T cells show the reaction pattern of naive or memory cells. For comparison, keyhole limpet haemocyanin (KLH) and tetanus toxoid (TT) were employed as primary and secondary foreign antigens, respectively.

**Results:** Upon incubation with CFSE-stained normal peripheral blood mononuclear cells (PBMCs), TG elicited rapid T cell proliferation, characteristic of a secondary response. However, the TG-specific subpopulation responded with a cytokine profile quite distinct from that observed with T cells responding to TT. While TT induced pro-inflammatory cytokines (i.e. IL-2/IFN- $\gamma$  followed by IL-4/IL-5), TG stimulated persistent release of the regulatory cytokine, IL-10. Some donors, though, also responded with late IFN- $\gamma$  production, suggesting that the regulatory action of IL-10 could be over-ridden. Coating of the PBMCs with bi-specific anti-CD45/anti-IL-10 beads revealed that monocytes were the primary source of IL-10 production, as indicated by the consistent right-ward shift of the cell profile on flow-cytometry. However, counterstaining with anti-CD45RO and CD45RA revealed that TG induced IL-10 production in 3.1 $\pm$ 1.7 memory cells per 10,000 CD4<sup>+</sup> T cells, whereas the numbers of memory cells producing IL-10 in response to TT and KLH were non-significant (0.38  $\pm$  0.52 and 0.52  $\pm$  0.43 cells per 10,000 CD4<sup>+</sup> T cells, respectively), as were the number of naive CD4<sup>+</sup> T cells stimulated for IL-10 production by either antigen. Concordantly, depletion of CD14<sup>+</sup> monocytes abrogated the production of IL-10. Notably, the same was true upon depletion of CD3<sup>+</sup> T cells (p < 0.002), indicating that IL-10 production by monocytes was regulated by T cells.

**Conclusions:** Our findings suggest that active peripheral tolerance towards TG may prevail in the normal population, and that IL-10 is produced by monocytes under direct control of TG-specific memory T cells. However, the balance between pro- and anti-inflammatory cytokine responses to this auto-antigen is altered in a sub-group of healthy individuals. The implication of these findings, in terms of predisposition of certain individuals to contract autoimmune diseases, remains to be elucidated.

**PB03/14 DIFFERENT MECHANISMS OF CD4+ AND CD8+ T CELL PROLIFERATION?**H. Rabenstein<sup>1</sup>, R. Obst<sup>1</sup><sup>1</sup>Ludwig Maximilians-University Munich, Institute for Immunology, Munich, Germany

T cell clonal expansion is regulated by antigen, but exactly how this occurs is unclear. It has been realized that the extent of proliferation and pathways of differentiation differ between CD4<sup>+</sup> and CD8<sup>+</sup> T cells, suggesting an intrinsic difference between the two subsets which has not been shown directly. Previous data suggest that CD8<sup>+</sup> T cell proliferation is programmed early without further antigenic requirement. By contrast, it has been observed for CD4<sup>+</sup> T cells that antigen persistence is necessary throughout the expansion phase for effector and memory differentiation. Here, we compared CD4<sup>+</sup> and CD8<sup>+</sup> T cell proliferation side-by-side using three different approaches for terminating antigen presentation.

T cells of AND and OT-I T cell receptor transgenic mice were stimulated with anti-CD3/CD28 in vitro for 48h and then transferred either into antigen expressing or antigen-free hosts. CD4<sup>+</sup> T cells proliferated only in the presence of antigen whereas CD8<sup>+</sup> T cells kept dividing regardless of antigen.

Upon adoptive transfer of naive T cells into wildtype mice, dendritic cells expressing both antigen and diphtheria toxin receptor were transferred as well. By diphtheria toxin injection the dendritic cells were depleted to stop antigen presentation in vivo. CD4<sup>+</sup> T cell proliferation ceased as soon as antigen presentation was terminated.

A novel transgenic mouse line was generated for tet-inducible expression of the K<sup>b</sup>-restricted epitope Ova<sub>257-264</sub> in dendritic cells. Initial experiments showed that antigen expression could not be completely turned off in vivo. However, in chimeras where only bone marrow derived cells present the epitope, antigen presentation could be terminated swiftly. Here, we found CD8<sup>+</sup> T cell division to be more variable than that of CD4<sup>+</sup> T cells. Interestingly, dendritic cell activation via CD40 has more of an impact on CD8<sup>+</sup> than on CD4<sup>+</sup> T cells. This was found not to be due to pMHC stabilization.

In summary, our data suggest that antigen-independent proliferation is not an intrinsic feature of CD8<sup>+</sup> T cells, but an inducible mechanism that cannot be triggered in CD4<sup>+</sup> T cells. To identify the genes involved microarray analysis will be employed.



**PB03/15 DIFFERENTIAL MIRNA EXPRESSION ALONG CD8<sup>+</sup> T CELL DIFFERENTIATION IN HUMANS**T. Yamamoto<sup>1</sup>, Y. Tsunetsugu-Yokota<sup>1</sup>, A. Roux<sup>2</sup>, B. Autran<sup>2</sup>, V. Appay<sup>2</sup><sup>1</sup>National Institute of Infectious Diseases, Tokyo, Japan, <sup>2</sup>INSERM U945, UPMC Paris6, Paris, France

**Objectives:** The differentiation of CD8<sup>+</sup> T cells following priming of naïve cells is central in the establishment of the immune response against pathogens. Yet, the factors that govern this process and the generation of distinct memory CD8<sup>+</sup> T cell subsets remain poorly understood in humans. The recent discovery of miRNAs as critical regulators of gene expression in the mammalian immune system has represented a revolution in the field of immunology. Here, we aim at exploring the potential relationship between miRNA expression and the differentiation of memory CD8<sup>+</sup> T cells in humans.

**Methods:** We measured the expression of miRNAs using TaqMan Low Density Arrays and single primer qPCR in five distinct subsets of primary T cells. These subsets were sorted using 5 color flow cytometry (based on the expression of CD8, CD27, CD28, CD45RA and CCR7) and define the major steps of the CD8<sup>+</sup> T cell differentiation pathway in humans (from naïve to highly differentiated cells).

**Results:** We show that there are significant associations between the expression of certain miRNAs and specific subsets of CD8<sup>+</sup> T cell differentiation. For instance, we found a preferential expression of the miR-17-92 cluster during early differentiation. In contrast, miRNAs like miR-155, miR-24 and miR-142-3p presented significantly higher expression levels in late effector memory cells compared to the other CD8<sup>+</sup> T cell subsets. These miRNAs are likely to regulate the functional properties of these different CD8<sup>+</sup> T cell subsets.

**Conclusion:** Distinct miRNA expression patterns within primary CD8<sup>+</sup> T cell subsets implies differential miRNA mediated regulation at different stages of CD8<sup>+</sup> T cell post thymic differentiation. The present work provides the first evidence of a link between miRNAs and the establishment of CD8<sup>+</sup> T cell memory in humans.

**PB03/16 ROLE OF IL-9 PRODUCING 'TH9' CELLS IN VIVO**C. Wilhelm<sup>1</sup>, M. Veldhoen<sup>1</sup>, J. van Snick<sup>2</sup>, H. Helmbj<sup>3</sup>, B. Stockinger<sup>1</sup><sup>1</sup>National Institute for Medical Research, London, United Kingdom, <sup>2</sup>Ludwig Institute for Cancer Research, Brussels Branch, Brussels, Belgium, <sup>3</sup>London School of Hygiene & Tropical Medicine, London, United Kingdom

CD4<sup>+</sup> helper T cells differentiate into functionally distinct subsets after encountering antigen. For a long period it was believed that T helper 1 (Th1) and Th2 cells comprise the main angle in T cell mediated responses. It was only recently that the discovery of a new subset of interleukin-17 (IL-17) producing T helper cells (Th17) helped to complete this view. In a recent paper we could show that T helper cells producing IL-9, therefore termed 'Th9' cells, could further complement the T helper cell compartment. IL-9 producing cells can be induced from naïve T cells in the presence of IL-4 and TGF- $\beta$  or develop from Th2 cells in the presence of TGF- $\beta$  (Veldhoen et al., 2008, Nature Immunology). Additionally, T cells expressing a dominant negative form of the TGF- $\beta$  receptor II under the CD4 promoter (CD4dnTGF $\beta$ RII) do not produce IL-9 but are still able to produce IL-4. T cells cultured with IL-4 and TGF- $\beta$  show a distinct transcription factor and cytokine profile, which clearly separates them from Th1, Th2, Tregs and Th17 cells. To address the mechanism of IL-9 production in vivo and the question if these cells arise from naïve T cells in vivo or rather divert from Th2 cells in the presence of TGF- $\beta$  we are establishing IL-9 reporter mice. This enables us to address the interplay of IL-4 and IL-9 function in infectious models.

**PB03/17 CD20<sup>+</sup> T CELLS: FUNCTIONAL AND PHENOTYPICAL CHARACTERISTICS OF A HIGHLY DIFFERENTIATED EFFECTOR LYMPHOCYTE SUBSET**E. Wilk<sup>1</sup>, T. Witte<sup>1</sup>, N. Marquardt<sup>1</sup>, T. Horvath<sup>1</sup>, K. Kalippke<sup>1</sup>, K. Scholz<sup>1</sup>, T. Germerott<sup>2</sup>, R.E. Schmidt<sup>1</sup>, R. Jacobs<sup>1</sup><sup>1</sup>Hannover Medical School, Clinic for Immunology and Rheumatology, Hannover, Germany, <sup>2</sup>Hannover Medical School, Institute of Legal Medicine, Hannover, Germany

**Objectives:** About 1.6% of human peripheral blood T cells coexpress CD20, which is known as a common marker of mature B cells. Rituximab is a therapeutic anti-CD20 antibody used for in vivo depletion of B cells in proliferative and autoimmune diseases. However, the mechanisms of action are not fully understood, as not all of the therapy-mediated effects can be explained by the depletion of antibody secreting cells. Therefore we analyzed CD3<sup>+</sup>CD20<sup>+</sup> T cells in RA patients and healthy controls in order to address the question if the small population of CD20<sup>+</sup> T cells is functionally active and could explain some effects observed under rituximab treatment, which have so far not been clarified.

**Methods:** Phenotype and apoptosis of PBMC from healthy donors and RA patients were examined by four color FACS analyses. Cytokine production was determined by intracellular staining and in supernatants. Proliferation of sorted T cell populations was analyzed in H3-thymidine uptake assays.

**Results:** In healthy individuals 0.1-6.8% (mean 1.6%, n=142) of peripheral blood T cells coexpress CD20. There was no significant difference to RA patients as 0.4-2.6% (mean 1.2%, n=27) of their T cells were CD20<sup>+</sup>. Under rituximab therapy, the patients' CD20<sup>+</sup> T cells are eliminated along with the B cells. Amongst the CD20<sup>+</sup> T cells 55% coexpress CD8 and 45% coexpress CD4. Polyclonal CD3<sup>+</sup>CD20<sup>+</sup> cells are functionally characterized by constitutive cytokine production (i.e. IL-1 $\beta$ , TNF), low proliferative capacity, a high activation state, and enhanced susceptibility to apoptosis.

**Conclusion:** Our findings suggest that CD20<sup>+</sup> T cells represent a terminally differentiated cell type with immune regulatory and proinflammatory capacities. We hypothesize that depletion of CD20<sup>+</sup> T cells is an additional mechanism by which anti-CD20 therapy functions in RA patients.

supported by DFG Priority Programme 1110/JA1058, Graduate Program of Lower Saxony, TUI-Foundation

**PB03/18 SUBOPTIMAL TCR STIMULATION IS SUFFICIENT FOR IL-10 PRODUCTION IN AUTO-REACTIVE HUMAN CCR6<sup>+</sup> MEMORY T CELLS**L. Rivino<sup>1,2</sup>, P. Gruarin<sup>3</sup>, S. Steinfelder<sup>1</sup>, B. Haeringer<sup>1</sup>, C. Loddenkemper<sup>4</sup>, S. Abrignani<sup>3</sup>, F. Sallusto<sup>2</sup>, A. Lanzavecchia<sup>2</sup>, J. Geginat<sup>1,2</sup><sup>1</sup>Charité Medical School and DRFZ, RCIS, Berlin, Germany, <sup>2</sup>Institute for Research in Biomedicine, Bellinzona, Switzerland, <sup>3</sup>Istituto Nazionale di Genetica Molcolare (INGM), Milan, Italy, <sup>4</sup>Charité Medical School, RCIS, Berlin, Germany

IL-10 produced by T cells is important for the prevention of autoimmunity and immunopathology, but the phenotype and function of IL-10 producing human memory T cells is unclear. CCR6 was expressed on a fraction of human CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> memory T cells with high IL-10 producing capacities, and CCR6 could be induced by tolerogenic T cell priming with TGF- $\beta$  and IL-10. CCR6<sup>+</sup> T cells were localized close to myeloid DC (mDC) in human spleens, and they produced suppressive IL-10 upon sub-optimal TCR stimulation by autologous AIRE<sup>+</sup> mDC ex vivo. However, CCR6<sup>+</sup> memory T cells could express IL-2, IFN- $\gamma$  and CD40L following optimal TCR stimulation. Auto-reactive CCR6<sup>+</sup> T cell lines responded to various recall antigens and we isolated auto-reactive CCR6<sup>+</sup> T cell clones that produced IL-10 upon suboptimal stimulation with autologous mDC, but that proliferated and secreted IL-2 upon strong activation with tetanus toxoid. We propose that CCR6 is expressed on context-dependent memory T cells that secrete suppressive IL-10 upon encounter of autologous DC under steady-state conditions, but that contribute to secondary immune responses upon full activation by their specific recall antigen.

**PB03/19 IL-17 MAINTAINS PROTECTIVE IMMUNITY DURING MYCOBACTERIUM TUBERCULOSIS INFECTION**J. Behrendts<sup>1</sup>, D. Rueckerl<sup>1</sup>, M. Heßmann<sup>1</sup>, U. Mueller<sup>2</sup>, G. Alber<sup>2</sup>, Y. Iwakura<sup>3</sup>, S. Ehlers<sup>4</sup>, C. Hoelscher<sup>1</sup><sup>1</sup>Research Center Borstel, Infection Immunology, Borstel, Germany, <sup>2</sup>University of Leipzig, Leipzig, Germany, <sup>3</sup>University of Tokyo, Tokyo, Japan, <sup>4</sup>Research Center Borstel and Christian-Albrechts-University, Microbial Inflammation Research, Borstel and Molecular Inflammation Medicine, Kiel, Germany

Because a variety of autoimmune disorders have now been shown to depend on interleukin (IL)-17-producing T helper (TH)17 cells, therapeutic blockade of TH17 development may provide a novel approach to avoid adverse consequences of anti-inflammatory strategies such as reactivation of latent tuberculosis (TB). To evaluate the potential risk of interfering with IL-17-dependent inflammation, we analyzed the outcome of experimental TB in IL-17-deficient ( $\gamma\gamma$ ) mice after infection with *Mycobacterium tuberculosis* (*Mtb*). IL-17 was important for the induction of neutrophil chemokines after *Mtb* infection, but was not involved in granuloma formation and protection during the first three months of *Mtb* infection. *Mtb*-infected IL-17 $\gamma\gamma$  mice efficiently generated interferon-gamma (IFN $\gamma$ )-producing T cells and IFN $\gamma$ -dependent effector responses. However, IL-17 $\gamma\gamma$  mice were not able to control mycobacterial replication during the chronic phase of experimental TB and died significantly earlier than corresponding wildtype mice. This breakdown of immune protection in IL-17 $\gamma\gamma$  mice was associated with a drop in the frequency of IFN $\gamma$ -producing CD4<sup>+</sup> T cells. Our findings reveal that IL-17 is essential for maintaining CD4<sup>+</sup> T cell-dependent protection during chronic stages of TB. Hence, interfering with IL-17-dependent pathways as an anti-inflammatory therapeutic approach will possibly incur the danger of reactivating latent TB. (Supported by the Inflammation Research Excellence Cluster).

**PB03/20 JAKMIP1 IS EXPRESSED UPON T CELL DIFFERENTIATION AND HAS AN INHIBITORY FUNCTION IN CYTOTOXIC T LYMPHOCYTES**V. Libri<sup>1,2</sup>, D. Schulte<sup>1</sup>, A. van Stijn<sup>3</sup>, J. Ragimbeau<sup>1</sup>, L. Rogge<sup>1</sup>, S. Pellegrini<sup>1</sup><sup>1</sup>Institut Pasteur, Department of Immunology, Paris, France, <sup>2</sup>University College London, Department of Immunology and Molecular Pathology, London, United Kingdom, <sup>3</sup>Academic Medical Center, Department of Experimental Immunology and Department of Internal Medicine, Amsterdam, Netherlands

Jakmip1 (Jak and microtubule interacting protein) belongs to a family of three related genes encoding proteins rich in coiled-coils. Jakmip1 is expressed predominantly in neuronal and lymphoid cells and co-localizes with microtubules. We have studied the expression of Jakmip1 mRNA and protein in distinct subsets of human primary lymphocytes. Jakmip1 is absent in naïve CD8<sup>+</sup> and CD4<sup>+</sup> T lymphocytes from peripheral blood, but is highly expressed in antigen-experienced T cells. In cord blood T lymphocytes, induction of Jakmip1 occurs upon TCR/CD28 stimulation and parallels induction of effector proteins, such as granzyme B and perforin. Further analysis of CD8<sup>+</sup> and CD4<sup>+</sup> T cell subsets showed a higher expression of Jakmip1 in the effector CCR7<sup>+</sup> and CD27<sup>+</sup> T cell subpopulations. In a gene expression follow-up of the development of CMV-specific CD8<sup>+</sup> response, Jakmip1 emerged as one of the most highly upregulated genes from primary infection to latent stage. To investigate the relationship between Jakmip1 and effector function, we monitored cytotoxicity of primary CD8<sup>+</sup> T cells silenced for Jakmip1 or transduced with the full length protein or the N-ter region. Our findings point to Jakmip1 being a novel effector memory gene restraining T cell mediated cytotoxicity.

**PB03/21 THE ROLE OF NF- $\kappa$ B INHIBITOR I $\kappa$ B $\alpha$  IN T HELPER CELL HOMEOSTASIS AND DEVELOPMENT**M. P. Schuster<sup>1</sup>, L. K. Clayton<sup>2</sup>, I. Schmitz<sup>1</sup><sup>1</sup>Heinrich-Heine-University, Medical Microbiology and Hospital Hygiene, Düsseldorf, Germany, <sup>2</sup>Dana-Farber Cancer Institute and Harvard Medical School, Laboratory of Immunobiology and Dept. of Medical Oncology, Boston, United States

NF- $\kappa$ B was originally described as an eukaryotic transcription factor controlling the expression of the immunoglobulin  $\kappa$ -chain in murine B cells. Subsequently, NF- $\kappa$ B was found to be crucial in important cellular processes like cell death, proliferation, immune response and hematopoiesis. NF- $\kappa$ B is regulated by inhibitory proteins, the I $\kappa$ Bs, which can be divided into two subfamilies. Prototypical I $\kappa$ Bs, like I $\kappa$ B $\alpha$ , inactivate NF- $\kappa$ B by sequestering it to the cytoplasm. On the other hand, I $\kappa$ Bs of the BCL-3 subfamily are nuclear proteins, which can have negative as well as positive effects on NF- $\kappa$ B-dependent transcription. I $\kappa$ B $\alpha$  is a member of the BCL-3 subgroup of NF- $\kappa$ B inhibitors as it is highly homologous to I $\kappa$ B $\alpha$  (43%) and BCL-3 (30%). It was originally identified as an inhibitor of NF- $\kappa$ B rapidly induced in immature CD4<sup>+</sup>CD8<sup>+</sup> double positive T cells upon negative selection. We addressed the question whether I $\kappa$ B $\alpha$  has a role in differentiation of T helper cell subsets and whether it affects the expression of their characteristic cytokines. First, we analysed I $\kappa$ B $\alpha$  expression of *in vitro* differentiated Th1, Th2, Th17 and Treg cells of C57/BL6 mice. We observed strong induction of I $\kappa$ B $\alpha$  in PMA/Ionomycin restimulated Th1 and Th17 cells, but low induction in iTreg and Th2 subsets. Further, we analysed the proliferation rates of the indicated subsets, comparing T cells from I $\kappa$ B $\alpha$ -deficient and wild type mice using CFSE. FACS analysis revealed an important role for I $\kappa$ B $\alpha$  in Th1 and Th17 cell proliferation, as cell numbers are significantly decreased in differentiated T helper cells derived from I $\kappa$ B $\alpha$ -deficient mice. Although I $\kappa$ B $\alpha$  is not expressed in Treg cells, it was unexpectedly found to be important for Treg development *in vivo*. Hence, intracellular Foxp3 staining in thymus, spleen and lymph node revealed decreased expression of intracellular Foxp3 and cell surface GITR in CD4<sup>+</sup>CD25<sup>+</sup> cells from I $\kappa$ B $\alpha$ -deficient mice compared to wild type mice. We conclude that I $\kappa$ B $\alpha$  plays a crucial role in T helper cell development and homeostasis.

**PB03/22 DELAYING BCG VACCINATION FROM BIRTH TO 10 WEEKS OF AGE RESULTS IN AN ENHANCED MEMORY CD4 T CELL RESPONSE**B. M. N. Kagina<sup>1</sup>, B. Abel<sup>1</sup>, M. Bowmaker<sup>1</sup>, T. J. Scriba<sup>1</sup>, S. Gelderbloem<sup>1</sup>, E. Smit<sup>1</sup>, M. Erasmus<sup>1</sup>, N. Nene<sup>2</sup>, G. Black<sup>2</sup>, G. D. Hussey<sup>1</sup>, A. C. Hesselings<sup>3</sup>, W. A. Hanekom<sup>1</sup><sup>1</sup>University of Cape Town, South African Tuberculosis Vaccine Initiative (SATVI), Institute of Infectious Diseases and Molecular Medicine and School of Child and Adolescent Health, Cape Town, South Africa, <sup>2</sup>Stellenbosch University, Molecular Biology and Human Genetics, Department of Biomedical Sciences, Cape Town, South Africa, <sup>3</sup>Stellenbosch University, Desmond Tutu TB Centre, Department of Paediatrics and Child Health, Faculty of Health Sciences, Tygerberg, Cape Town, South Africa

**Background:** In most tuberculosis (TB) endemic countries, bacillus Calmette Guérin (BCG) is usually given around birth to prevent severe TB in infants. The neonatal immune system is immature. Our hypothesis was that delaying BCG vaccination from birth to 10 weeks of age would enhance the vaccine-induced immune response.

**Methods:** In a randomized clinical trial, BCG was administered intradermally either at birth (n=25) or at 10 weeks of age (n=21). Whole blood was collected at 10 weeks, 20 weeks, and at 1 year of age, to measure vaccine-specific T cell responses. BCG-specific CD4 and CD8 T cell responses subsets were assessed for their intracellular cytokine expression profiles and their associated memory phenotypes using the markers, CD45RA and CCR7. Infants infected with *M. tuberculosis* over the first year of life were excluded from analysis.

**Results:** Both groups of infants had a robust BCG-induced CD4 T cell response 10 weeks after vaccination, with those in the delayed vaccination group demonstrating higher frequencies of most CD4 T cell subsets, compared with the birth vaccination group. Strikingly, at 1 year of age, infants who received delayed vaccination had higher frequencies of BCG-specific CD4 T cell subsets, particularly polyfunctional T cells co-expressing IFN- $\gamma$ , TNF- $\alpha$  and IL-2.

**Conclusions:** Delaying BCG vaccination from birth to 10 weeks of age enhances the quantitative and qualitative BCG-specific T cell response, when measured at one year of age. Our results suggest that the age at which BCG is administered may be a critical variable influencing the vaccine induced immune responses in infants.

**PB03/23 PHENOTYPIC AND FUNCTIONAL ANALYSIS OF EFFECTOR CD4 T-CELLS DURING *M. TUBERCULOSIS* INFECTION**I. Lyadova<sup>1</sup>, M. Kapina<sup>1</sup>, N. Shmitova<sup>1</sup>, I. Vasilyeva<sup>1</sup><sup>1</sup>Central Institute for Tuberculosis, Immunology, Moscow, Russian Federation

Effector Th1 cells play pivotal role in protective immune response during bacterial infections, but may also cause severe immunopathology, especially during chronic infections. One of the factors that determine functional activity of effector Th1 lymphocytes and may affect their protective/pathological potential is the degree of their differentiation. Therefore we have analyzed whether and how the degree of effector CD4 T-cell differentiation is associated with their functional activity and severity of *M. tuberculosis* infection, disease which is caused by bacteria and which pathogenesis largely depends on host Th1 reactivity. We show that during tuberculosis (TB), a population of effector CD45RO<sup>+</sup>CD62L<sup>-</sup> CD4 T cells is composed of several subsets that differ by the expression of CD27, CD28, and CD57 receptors. Differentiation of effector CD4 T cells goes from CD27<sup>+</sup>CD28<sup>+</sup>CD57<sup>-</sup> "early" effector cells through mature CD27<sup>+</sup>CD28<sup>+</sup>CD57<sup>+</sup> effector cells to terminally differentiated CD27<sup>+</sup>CD28<sup>+</sup>CD57<sup>+</sup> and CD27<sup>+</sup>CD28<sup>+</sup>CD57<sup>+</sup> effector cells. Differentiation of CD27<sup>+</sup>CD28<sup>+</sup>CD57<sup>+</sup> effector cells into CD27<sup>+</sup>CD28<sup>+</sup>CD57<sup>+</sup> lymphocytes is accompanied by an increase in cell activation, production of IFN- $\gamma$  and TNF- $\alpha$ , and enhanced stimulation of macrophage antibacterial activity. This stage of effector cell differentiation occurs in the lungs, is promoted by cell stimulation with the antigen, and is controlled by host genetic factors. In contrast to CD27<sup>+</sup>  $\rightarrow$  CD27<sup>+</sup> differentiation stage, further stages of effector differentiation (CD27<sup>+</sup>CD28<sup>+</sup>CD57<sup>+</sup> to CD27<sup>+</sup>CD28<sup>+</sup>CD57<sup>+</sup> and CD27<sup>+</sup>CD28<sup>+</sup>CD57<sup>+</sup>) result in almost complete loss of cell capacity to produce IFN- $\gamma$ . In contrast, intracellular content of granzymes increases, which may be considered as a sign of terminal and "loss of function" differentiation of effector CD4 T cells. Accumulation of highly and terminally differentiated effector CD4 T cells is a characteristic feature of severe TB infection. During mild forms of TB and following successful TB treatment, the differentiation of effector CD4 T cells is less profound suggesting that terminal differentiation may represent part of the mechanism through which during severe TB infection immune response exhausts. In conclusion, the degree of effector T-cell differentiation is an important factor that affects host protective immunity and may represent a valuable target for TB immunomodulation.

**PB03/24 MEMBRANE POTENTIAL OF CD4<sup>+</sup> T CELLS IS A SUBSET SPECIFIC FEATURE THAT DEPENDS ON THE DIRECT CELL-TO-CELL CONTACTS WITH MONOCYTES**N. M. Marek<sup>1,2</sup>, P. Trzonkowski<sup>1</sup>, M. Myśliwiec<sup>3</sup>, K. Zorena<sup>2</sup>, K. Raczyska<sup>4</sup>, J. Myśliwska<sup>2</sup><sup>1</sup>Medical University of Gdańsk, Department of Clinical Immunology and Transplantation, Gdańsk, Poland, <sup>2</sup>Medical University of Gdańsk, Department of Immunology, Gdańsk, Poland, <sup>3</sup>Medical University of Gdańsk, Clinic of Pediatrics, Hematology, Oncology and Endocrinology, Gdańsk, Poland, <sup>4</sup>Medical University of Gdańsk, Department and Clinic of Ophthalmology, Gdańsk, Poland

**Introduction:** CD4<sup>+</sup> T cells play crucial role in the regulation of immune response. Mature CD4<sup>+</sup> T cells, depending on the antigenic experience can be divided into three subsets: naive (Tn), central memory (Tcm) and effector memory (Tem) T cells. These subsets exhibit various thresholds of activation, migration pattern, cytokine synthesis efficiency and immunophenotype. In addition, they differ in the expression of potassium channels upon stimulation.

**Aims:** We aimed to check the impact of direct cell-to-cell contacts on membrane potential of Tn, Tcm and Tem subsets and to investigate if membrane potential and its changes upon stimulation are altered in diabetes.

**Methods:** Twenty six healthy adolescents and 56 age-matched type 1 diabetic (DM1) patients were enrolled into the study. Membrane potential was measured with flow cytometry in Tn (CD4<sup>+</sup>CD27<sup>+</sup>CD45RO<sup>-</sup>), Tcm (CD4<sup>+</sup>CD27<sup>+</sup>CD45RO<sup>+</sup>) and Tem (CD4<sup>+</sup>CD27<sup>-</sup>CD45RO<sup>+</sup>) subsets stained with monoclonal antibodies and fluorescent dye DiBAC<sub>4</sub>(3). Cells were analyzed before and after stimulation with margatoxin, beta-endorphin and ionomycin in heterogeneous mixture of PBMC. Buffy coats obtained from volunteer blood donors were used for isolation of monocyte enriched PBMC (mePBMC) and pure subsets of Tn, Tcm and Tem cells. Sorted CD4<sup>+</sup> T subpopulations were seeded on 24-well plates in triplicates and incubated for 15h. During the last 3h of the incubation T cells were cocultured in direct and indirect contact with mePBMC. Subsequently, cells were analyzed as it was described for non-isolated subsets.

**Results:** We found that subpopulations of CD4<sup>+</sup> T cells differed in their membrane potential when analyzed in suspension of PBMC. In addition, non-stimulated and stimulated CD4<sup>+</sup> T cells from diabetic individuals, notably Tem subset, tended to be more depolarized than respective cells from healthy individuals. Sorted subpopulations of CD4<sup>+</sup> T cells exhibited different membrane potentials as compared with non-isolated lymphocytes. Addition of autologous mePBMC partially or completely restored potential values of resting and stimulated CD4<sup>+</sup> cells found in PBMC.

**Conclusions:** Membrane potential of CD4<sup>+</sup> T cells is a subset specific feature that, at least partially, depends on the direct contacts with monocytes. Higher depolarization of Tem cells from DM1 patients might reflect persistent activation of the adaptive immune system in diabetes.

**PB03/25 IDENTIFICATION OF T CELL SUBSETS IMPLICATED IN THE RESISTANCE TO *BRUCELLA MELITENSIS* INFECTION IN MOUSE EXPERIMENTAL MODEL**M.-A. Vitry<sup>1</sup>, C. De Trez<sup>2</sup>, J.-J. Letesson<sup>3</sup>, E. Muraile<sup>2</sup><sup>1</sup>Facultés Universitaires Notre-Dame de la Paix, Unité de Recherche en Biologie Moléculaire, Namur, Belgium, <sup>2</sup>Université Libre de Bruxelles, Bruxelles, Belgium,<sup>3</sup>Facultés Universitaires Notre-Dame de la Paix, Namur, Belgium

**Objectives:** *Brucella* organisms are facultative intracellular Gram-negative coccobacilli that cause brucellosis in humans and animals. Acute human brucellosis is characterized by undulating fever, which may result in chronic disease with serious clinical manifestations. Despite progress in mouse models of brucellosis, much remains unknown regarding cellular components of the innate and adaptive immune responses induced by *Brucella* infection.

The focus of this study was to determine which lymphocyte subsets are implicated in the immune response against *B. melitensis* infection.

**Methods:** C57BL/6 wild type and deficient mice were injected intra-peritoneally with  $4 \times 10^4$  CFU of *B. melitensis* strain 16M in 500  $\mu$ l of PBS. Bacterial growth in vivo was evaluated by plating serial dilutions of spleen homogenates on 2YT.

**Results:** Using a battery of genetically deficient C57BL/6 mice, we showed that CD4<sup>+</sup>, beta2microglobulin<sup>-/-</sup> and MuMT<sup>-/-</sup> mice displayed enhanced bacterial count in spleen 5 days post infection when compared to wild type mice. In contrast, at 28 days post infection only CD4<sup>+</sup> mice presented a reduced control of infection. These results demonstrate that CD4<sup>+</sup> T cells are the major lymphocyte population implicated in the control of chronic phase of *B. melitensis* infection. Resistance and susceptibility to *Brucella* infection has been previously associated to T helper 1 (Th1) and Th2 response, respectively. However, this paradigm is mainly derived from experiments using neutralising antibodies. In addition, the importance of the recently described Th17 subset has never been analysed during *Brucella* infection. In order to gain insight about the role of Th subsets in *Brucella* control, we compared the susceptibility of IL-4<sup>-/-</sup>, IL-12p35<sup>-/-</sup> and IL-17R<sup>-/-</sup> C57BL/6 mice. Results show that IL-12p35<sup>-/-</sup> mice displayed an enhanced bacterial count at early (5 days) and later time (28 days) post infection when compared to wild type mice. Surprisingly, we did not find any negative role of IL-4 in *Brucella* control. Moreover, the absence of IL-4 led to an increased bacterial count 5 days post infection. IL-17R<sup>-/-</sup> and wild type mice presented similar bacterial growth.

**Conclusion:** Taken together, our results confirm the predominant role of IL-12-mediated CD4<sup>+</sup> Th1 cells in the control of *Brucella* infection.

#### PB03/26 INTERLEUKIN-6/STAT3 SIGNALLING REGULATES THE ABILITY OF NAÏVE T CELLS TO ACQUIRE B CELL HELP CAPACITIES

F. Eddahri<sup>1</sup>, S. Denanglaire<sup>1</sup>, O. Leo<sup>1</sup>, F. Andris<sup>1</sup>

<sup>1</sup>Université Libre de Bruxelles, Laboratoire de Physiologie Animale, Gosselies, Belgium

The conditions leading to the activation/differentiation of T helper (Th) cells dedicated for B cell antibody production are still poorly characterized. We demonstrate that interleukin (IL)-6 promotes the differentiation of naïve T lymphocytes into helper cells able to promote B cell activation and antibody secretion. IL-6 driven- acquisition of B cell help capacity requires expression of the STAT3, but not STAT4 or STAT6 transcription factors, suggesting that the ability to provide help to B cells is not restricted to a well-defined Th1 or Th2 effector population. T-cell specific STAT3-deficient mice displayed reduced humoral responses in vivo that could not be related to an altered expansion of CXCR5-expressing helper T cells. IL-6 was shown to promote IL-21 secretion, a cytokine that was similarly found to promote the differentiation of naïve T cells into potent B cell helper cells. Collectively these data indicate that the ability to provide B cell help is regulated by IL-6/IL-21 through STAT3 activation, independently of Th1, Th2, Th17 or T<sub>H</sub> differentiation.

#### PB03/27 ROLE OF HISTONE DEACETYLASES IN THE ACTIVATION OF T HELPER CELLS

R. Glauen<sup>1</sup>, E. Sonnenberg<sup>1</sup>, T. Strohm<sup>1</sup>, A. Batra<sup>1</sup>, P. Mascagni<sup>2</sup>, M. Zeitz<sup>3</sup>, B. Siegmund<sup>1</sup>

<sup>1</sup>Charité – Universitätsmedizin Berlin, Campus Benjamin Franklin, Research Center Immunoscience, Berlin, Germany, <sup>2</sup>Italfarmaco, Cinisello, Italy, <sup>3</sup>Charité – Universitätsmedizin Berlin, Campus Benjamin Franklin, Medizinische Klinik I, Berlin, Germany

**Objectives:** Several histone deacetylase (HDAC) inhibitors are currently in clinical studies for a variety of solid and hematological cancers. Recently, an anti-inflammatory potency was demonstrated by our group for HDAC inhibitors in vitro for lamina propria lymphocytes and CD4<sup>+</sup> cells and in vivo by using different models of experimental colitis in mice.

**Methods:** To further evaluate the relevance of acetylation/deacetylation during the process of T cell development and activation, naïve T helper cells were polarized to Th1, Th2, Th17 or T reg cells in the presence or absence of ITF2357. The expression pattern of the HDAC 1-11 was analyzed via real-time PCR. To characterize the significance of single HDAC, cells were transfected with small interfering RNA.

**Results:** While the in vitro generation of FoxP3<sup>+</sup> cells could be enhanced in the presence of ITF2357, the differentiation to Th17 cells was clearly suppressed. To define the role of single HDAC, expression of the HDAC 1-11 in naïve or activated CD4<sup>+</sup> T cells, compared to in vitro generated T helper cell subsets was analyzed. The expression pattern of the T cell subsets varied between Th1 and Th2 cells on the one hand and Th17 and FoxP3<sup>+</sup> T cells on the other hand. Namely, HDAC 5 and 9 expression in Th17 and FoxP3<sup>+</sup> cells was significantly increased compared to the other cell types. Additionally, we analyzed the expression pattern of CD4<sup>+</sup> effector cells sorted from the colon, spleen or peripheral lymph nodes from healthy or colitis mice. Again, HDAC 5 and 9 were significantly increased at the site of inflammation. When CD4<sup>+</sup> T cells were transfected with small interfering RNA against the HDAC5, 7, and 9 before activation, this specific gene knock-down mimicked the effect of HDAC inhibitors regarding their anti-inflammatory potency, identifying this HDAC as crucial factors in T helper cell activation.

**Conclusion:** The present study indicates, that (un)specific HDAC inhibition at the site of inflammation exerts an anti-inflammatory potency and modulates T cell differentiation and development. Furthermore, we are now able to target single HDAC, thus allowing for a further specification of the anti-inflammatory effects observed.

#### PB03/28 IL-21 IS A POTENT INDUCER OF IL-10 AND IFNG IN HUMAN NEWBORNS

A. Doganci<sup>1</sup>, C. U. Meyer<sup>1</sup>, F. Zepp<sup>1</sup>

<sup>1</sup>University Children's Hospital Mainz, Pediatric Immunology, Mainz, Germany

**Objective:** IL-21, member of the IL-2 cytokine family, is a pleiotropic cytokine that mediates its functions through a heterodimeric receptor, comprising the subunits IL-21R and the common  $\gamma$ -chain. IL-21 is mostly produced by CD4<sup>+</sup> T cells, but molecular mechanisms which regulate IL-21 synthesis remain to be investigated.

In order to assess the effect of IL-21 in the specific context of the neonatal immune response, we analyzed its role in CD4<sup>+</sup> T cells in both human cord and adult blood.

**Methods:** Umbilical cord blood samples were collected from healthy full-term infants born by cesarean sections. Leukocytes from adults were enriched from leucapheresis products. After density centrifugation, the mononuclear cell fraction (PBMC) was washed and cells were magnetically labelled for CD4<sup>+</sup> T cells and enriched in a magnetic field, following stimulation with IL-21 for ascertainment of T cell response in vitro.

**Results:** Our data indicate that addition of IL-21 significantly induced the production of IFN $\gamma$  ( $p=0,011$ ;  $n=15-18$ ) and IL-10 ( $p=0,013$ ;  $n=11$ ) in aCD3/CD28-stimulated, neonatal CD4<sup>+</sup> T cells. In contrast adult CD4<sup>+</sup> T cells showed only minor differences with or without the addition of IL-21 [during stimulation]. Furthermore we noticed significantly enhanced phosphorylation of Stat-1 ( $n=6-9$ ;  $p=0,008$ ) in cord blood compared to adult blood derived CD4<sup>+</sup> T cells.

**Conclusions:** Taken together, our results indicate a new function for IL-21 that was unanticipated given that IL-21 is required for TH17 production in humans. Here we could demonstrate that IL-21 is a potent inducer of IL-10 and IFN $\gamma$ , indicating that IL-21 is possibly involved in the generation of IL-10-producing Tr1-like cells. Otherwise IL-21 seems to be implicated in the Th1 immune response and could potentially compensate the restricted Th1 immune response in human newborns.

#### PB03/29 REGULATION OF T CELL DIFFERENTIATION BY THE INTRACELLULAR TGF- $\beta$ INHIBITOR SMAD7

M. Hasan<sup>1</sup>, B. Neumann<sup>1</sup>, E. Santos<sup>1</sup>, D. Lukas<sup>2</sup>, A. Croxford<sup>2</sup>, J. Song<sup>2</sup>, C. Becker<sup>3</sup>, A. Waisman<sup>2</sup>, I. Kleiter<sup>1</sup>

<sup>1</sup>University of Regensburg, Neurology Department, Regensburg, Germany, <sup>2</sup>Johannes Gutenberg University, I. Medical Department, Mainz, Germany

**Background:** The transforming growth factor (TGF)- $\beta$  has a pivotal role in T cell differentiation, blocking T helper (Th)1 and 2 differentiation under certain conditions and driving T regulatory (Treg) and Th17 polarization in others.

**Objectives:** To investigate the role of Smad7, the intracellular inhibitor of TGF- $\beta$  signalling, in T cell homeostasis and T cell differentiation.

**Methods:** We used conditional gene targeting to generate mice with a specific deletion of Smad7 in T cells under the control of a CD4 promoter. The distribution of thymic and peripheral T cell subsets was investigated in 6-week old mice by flow cytometry. For in vitro T cell differentiation, naïve CD4<sup>+</sup>CD62L<sup>+</sup> T cells were obtained from 8-week old mice, incubated under Th1, Th17, and Treg polarization conditions, and analyzed by flow cytometry for differentiation markers, Realtime-PCR for transcription factors, and ELISA for cytokine profiles. Wild type mice and mice with a T cell-specific overexpression of Smad7 served as controls. Differentiations were done in the presence of increasing concentrations of TGF- $\beta$  to enhance and neutralizing anti-TGF- $\beta$  antibody to block downstream effects of TGF- $\beta$  in Smad7 deficient and overexpressing cells.

**Results:** Apart from a decrease of activated CD69<sup>+</sup> T cells in the periphery, the distribution of T cell subsets was unaltered in mice with a T cell-specific deletion of Smad7, in particular no increase in the frequency of Tregs was found. When stimulated in vitro under differentiation conditions, Smad7 deficient T cells showed an increase in Treg and Th17 cells, whereas Th1 differentiation was inhibited. Furthermore, T cells lacking Smad7 produced increased concentrations of TGF- $\beta$ . Conversely, T cells with a T cell-specific overexpression showed less Treg and Th17, and more Th1 differentiation.

**Conclusion:** Downstream effects of TGF- $\beta$  on T cell differentiation are increased in Smad7 deficient and inhibited in Smad7 overexpressing T cells. Thus, Smad7 is a major regulator of T cell differentiation.

#### PB03/30 WHY DO HIV-SPECIFIC CD8+ T CELLS FAIL TO CONTAIN VIRAL REPLICATION MORE COMPLETELY?

P.R. Santos<sup>1</sup>, E. Turnbull<sup>1</sup>, M. Monteiro<sup>1</sup>, A. Legrand<sup>1</sup>, K. Conrod<sup>2</sup>, A. Worth<sup>2</sup>, I. Williams<sup>3</sup>, H. Pircher<sup>4</sup>, P. Borrow<sup>2</sup>, B. Rocha<sup>1</sup>

<sup>1</sup>INSERM U591 – Medical Faculty René Descartes Paris 5, Paris, France, <sup>2</sup>The Jenner Institute – University of Oxford, Viral Immunology Group, Compton, United Kingdom, <sup>3</sup>Mortimer Market Centre for Sexual Health and HIV Research, London, United Kingdom, <sup>4</sup>Institute of Medical Microbiology and Hygiene – University of Freiburg, Department of Immunology, Freiburg, Germany

**Objectives:** Although CD8<sup>+</sup> T cells have been shown to be important in controlling HIV-1 replication, why these cells are not more efficacious in containing viraemia remains poorly understood. In contrast to T cells specific for virus infections that are well controlled (e.g. CMV), HIV-specific T cells may undergo a skewed maturation that impairs their functional capacity. Therefore, we studied the functional properties of distinct subsets of HIV- and CMV-specific CD8<sup>+</sup> T cells.

**Methods:** We used flow cytometric-based analysis and single-cell multiplex RT-PCR to study persistent virus specific CD8<sup>+</sup> T cells.



**Results:** In any given individual, HIV- and CMV-specific CD8<sup>+</sup> T cells exhibited considerable phenotypic diversity. The majority of HIV- and CMV-specific CD8<sup>+</sup> cells were CCR7<sup>+</sup>. However, these cells showed heterogeneity in terms of CD27 and CD28 expression. CD27<sup>+</sup>CD28<sup>+</sup> (DP), CD27<sup>+</sup>CD28<sup>+</sup> (27SP) and CD27<sup>+</sup>CD28<sup>+</sup> (DN) subsets were found in all donors. Nevertheless, CMV-specific cells were predominantly 27SP and DN whereas all three subsets were represented within HIV-specific cells. Interestingly, the subset composition of HIV-specific CD8 T cells differed in acute and chronic infection. DN cells were largely absent during the acute phase, suggesting that they may emerge later in infection.

Within each subset, the proportion of HIV-specific T cells expressing IFN $\gamma$  mRNA was higher than that of both healthy donors and CMV-specific cells. The proportion of cells expressing perforin and granzymeA was significantly lower in HIV- and CMV-specific cells when compared to healthy donors but similar in both infections. Conversely, we found an impaired cytolytic potential *via* the granzymeB pathway (as assessed by co-expression of perforin and granzymeB mRNAs) in HIV-specific cells from chronically infected individuals. Our preliminary results indicate, however, that HIV-specific cells may have a strong cytotoxic capacity in acute infection.

Finally, in the chronic phase, the majority of HIV-specific cells were KLRG1<sup>+</sup>IL7R<sup>+</sup>, irrespective of surface phenotype. Notably, in the acute-phase HIV samples we studied, a substantial fraction of HIV-specific cells were KLRG1<sup>+</sup>, indicating that this marker may be expressed later in infection.

**Conclusion:** Our results emphasise the phenotypic and functional complexity of CD8 T cell responses to persistent virus infections. Understanding this complexity may inform more rational prophylactic vaccine design.

#### PB03/31 LEISHMANIA MAJOR INFECTION IN RESISTANT C57BL/6 AND SUSCEPTIBLE BALB/C MICE: THE EARLY EXPRESSION OF TH1/TH2 CYTOKINES DOES NOT REFLECT THE NATURE OF THE SUBSEQUENT ADAPTIVE RESPONSE

J. Barthelmann<sup>1</sup>, J. Westermann<sup>1</sup>, K. Kalies<sup>1</sup>

<sup>1</sup>University of Luebeck, Anatomy, Luebeck, Germany

**Objective:** The *Leishmania major* infection model in mice provides the prototypic example of the Th1/Th2 polarization that determines the outcome of infections. The resistance in C57BL/6 mice is associated with a Th1, and susceptibility in BALB/c with a Th2 response. It has been shown that the addition of Th1 or Th2 cytokines early after infection influences the development to either a Th1 or Th2 phenotype. On closer examination it becomes apparent that making a wild-type BALB/c more resistant does not necessarily mean that a Th1 response comparable to IFN $\gamma$  production, parasite clearance and healing as achieved in wild-type C57BL/6 mice develops. Vice versa, it is not trivial to render a Th1 phenotype of resistant mouse strains into a susceptible Th2 phenotype as observed in BALB/c mice. In our study we reexamine the course of leishmaniasis closely from 8 h until 6 weeks in resistant and susceptible mice and compare the basic relationship between cytokine expression, parasite dissemination, antibody production and disease progression *in vivo*.

**Methods:** The foot pads of resistant C57BL/6 and susceptible BALB/c mice were infected with 2x10<sup>6</sup> *Leishmania major* parasites. The T and B cell zones of the draining lymph node were isolated using laser-microdissection. Cytokine expression and parasitic load were analysed using real-time PCR.

**Results:** We demonstrate that the Th1/Th2 polarization is not induced before the third week of infection and coincides with increasing numbers of parasites in the draining lymph nodes of both mice strains. 10 times more parasites are detected within the lymph node of susceptible BALB/c mice after 3 weeks. At the same time point a differential increase of IgG isotypes was found. IgG1 is elevated in BALB/c whereas IgG2b prevails in C57BL/6.

**Conclusion:** We conclude that the number of parasites determines the Th1/Th2 polarization in draining lymph nodes 3 weeks after infection. The parasite numbers are regulated by the type of antigen presenting cells that carry the parasites from the skin into the lymph node. Since the ratio of the IgG isotypes influences the uptake of leishmania amastigotes by antigen presenting cells, we suggest that IgG isotypes contributes to Th1/Th2 polarization.

#### PB03/32 INTERLEUKIN-17-PRODUCING T HELPER CELLS AS NEW POTENTIAL PLAYER MEDIATING GRAFT VERSUS HOST DISEASE IN PATIENTS UNDERGOING ALLOGENEIC STEM CELL TRANSPLANTATION

E. Dander<sup>1</sup>, A. Balduzzi<sup>2</sup>, G. Zappa<sup>1</sup>, G. Lucchini<sup>3</sup>, P. Perseghin<sup>3</sup>, V. André<sup>1</sup>, M. Migliavacca<sup>2</sup>, E. Biagi<sup>2</sup>, G. Solinas<sup>4</sup>, A. Villa<sup>5</sup>, E. Berti<sup>5</sup>, P. Allavena<sup>4</sup>, A. Rovelli<sup>2</sup>, A. Biondi<sup>1,2</sup>, G. D'Amico<sup>1</sup>

<sup>1</sup>Research Center M Tettamanti, Clinica Pediatrica, Università Milano-Bicocca, Monza (Mi), Italy, <sup>2</sup>Università di Milano-Bicocca, Clinica Pediatrica, Ospedale San Gerardo, Monza (Mi), Italy, <sup>3</sup>Ospedale San Gerardo, Servizio Trasfusionale, Monza, Italy, <sup>4</sup>Istituto Clinico Humanitas, Dipartimento di Immunologia e Infiammazione, Rozzano (Mi), Italy, <sup>5</sup>Università di Milano-Bicocca, Consorzio 'M.I.A.', Monza (Mi), Italy

**Introduction:** Graft-vs-host disease (GVHD) is a major obstacle to safe allogeneic hematopoietic stem cell transplantation (HSCT), leading to significant morbidity and mortality. Recently, a subset of T helper lymphocytes named TH-17, has been shown to play a central role in mediating several autoimmune diseases.

**Objectives:** The aim of our study was to investigate the relationship between TH-17 cells and GVHD occurring in transplanted patients.

**Methods:** Having obtained an informed consent, we collected blood samples from 46 patients who received unmanipulated HSCT as well as samples from 15 healthy donors (HD) volunteers. 27 patients developed GVHD, while 19 never experienced it. All GVHD cases were monitored at multiple time-points during treatment.

**Results:** An increased TH-17 population (up to 2.1% of peripheral blood CD4<sup>+</sup> T lymphocytes) was observed in patients with active GVHD, as detected by elispot assays and flow cytometry. This increase correlated with the ongoing inflammatory process, characterized by high TNF- $\alpha$  (mean=29.8 pg/ml, range=3.7-97.1 pg/ml), IL-6 (mean 52.5 pg/ml, range=1-407 pg/ml) and IL-8 (mean 22.2 pg/ml, range 3.9-125.0 pg/ml) plasma levels. Moreover, patients with active GVHD showed increased IL-17 plasma levels. On the other hand, the percentage of TH-17 cells drastically decreased in patients with inactive GVHD. The proportion of detected TH-17 was inversely correlated with CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Regulatory T cells. TH-17 cells, present in the PB of patients with GVHD, consisted of both IL-17<sup>+</sup>/IFN- $\gamma$ <sup>+</sup> and IL-17<sup>+</sup>/IFN- $\gamma$ <sup>-</sup> subsets and expressed the IL-23R. Interestingly, IFN- $\gamma$ +TH-17 cells were able to infiltrate GVHD lesions, as observed in paraffin-embedded liver and skin sections. Finally, we demonstrated a strong correlation between the levels of TH-17 cells and the GVHD clinical status.

**Conclusions:** These findings support the hypothesis that TH-17 are involved in the active phases of GVHD and may represent a novel cellular target for developing new strategies for GVHD treatment.

#### PB03/33 MICRORNAS PROFILING DEFINES FUNCTION OF HUMAN LYMPHOCYTE SUBSETS

M. Pagani<sup>1</sup>, G. Rossetti<sup>1</sup>, R. Rossi<sup>1</sup>, L. Wenandy<sup>1</sup>, P. Gruarin<sup>1</sup>, M. Moro<sup>1</sup>, R. De Francesco<sup>1</sup>, J. Geginat<sup>2</sup>, S. Abrignani<sup>1</sup>

<sup>1</sup>National Institute of Molecular Genetics (INGM), Milano, Italy, <sup>2</sup>Charité Research Centre for Immunosciences (RCIS), Berlin, Germany

**Introduction:** MicroRNAs play a major role in the post-translational regulation of gene expression. They are non-coding RNAs ~22 nucleotides long that bind mostly to the 3' untranslated region of cognate mRNAs affecting their stability and/or translation. Hundreds of human microRNAs have been identified so far, many of which show cell- or developmental stage- specific expression profile. MicroRNAs play a pivotal role in various biological processes, including cell differentiation, apoptosis, proliferation and different diseases like cancer.

**Objectives:** Most of the distinct cell populations and subsets of the immune system circulate in peripheral blood and can therefore be separated by flow-cytometry, making it an interesting model system to study the role of microRNAs in cell differentiation.

**Methods:** Few microRNAs (i.e., miR181, miR150 and miR155) have been so far reported to have a specific cell-lineage expression during lymphocyte development and to play a role in immune response modulation. As little is known on the microRNAs expression in functional subsets of human T and B cells, we performed a microRNAs genome-wide analysis of purified human B (naïve and memory) and T (Th1, Th2, Th17, Treg, naïve, central and effector memory) lymphocytes by Real-Time quantitative PCR. RT-qPCR has sensitivity superior to other methods used for microRNAs analysis (microarray or bead-based flow cytometry), enabling microRNA profiling of poorly represented cell subsets. To have a more comprehensive overview of microRNAs and mRNAs expression changes in the same cell subsets, we analyzed gene expression with Illumina bead array technology.

**Results:** We report that a few microRNAs are preferentially expressed and that their expression patterns change during the functional differentiation of lymphocyte subsets. Experiments are in progress to validate these findings with the corresponding mimics and/or antagonists and assessment of the expected lymphocytes' function *in vitro*.

#### PB03/34 THE CHEMOKINE I-TAC (CXCL11) IS AN EARLY COMPONENT OF TH2 TYPE IMMUNITY

K. Roebrock<sup>1</sup>, J. Ehrchen<sup>1,2</sup>, G. Varga<sup>1</sup>, T. Vogl<sup>1</sup>, C. Sunderkötter<sup>2</sup>, J. Roth<sup>1</sup>

<sup>1</sup>Institute of Immunology, Medical Faculty, Münster, Germany, <sup>2</sup>Medical Faculty Münster, Department of Dermatology, Münster, Germany

**Objectives:** Differentiation of T-helper (Th) cells into different subsets has a major impact on the course of diverse inflammatory diseases. Experimental *leishmaniasis* is an excellent model system to investigate the mechanisms underlying Th-cell differentiation *in vivo*. A Th1 response is critical for a protective immune response in genetically resistant C57BL/6 mice. Susceptible BALB/c mice on the other hand develop a Th2 response and succumb to progressive disease. The decisive events for the development of a Th1 or Th2 response take place during the first two days after infection. There is growing evidence that the microenvironment of the infected tissue delivers the initial triggers that affect Th-cell differentiation. To identify early components of Th2-immunity we analyzed differential gene expression 16h after infection with *Leishmania (L.) major* in the skin of susceptible BALB/c compared to resistant C57BL/6 mice.

**Methods:** We used microarray technology (Affymetrix) and bioinformatical analysis (GenMAPP, Genedata Expressionist) to detect *L. major* induced gene expression patterns in the skin of Balb/c and C57BL/6 mice 16h after infection. Results were confirmed by RT-PCR, immunohistochemistry, laser-microdissection and *in situ*-hybridization. The effect of I-TAC on Th2 differentiation was analyzed by measuring footpad swelling, determination of parasite loads and determination of cytokine production from DCs and T-cells.

**Results:** In susceptible, but not in resistant mice, we found a marked up-regulation of the chemokine I-TAC (CXCL11) in the epidermis during the first two days of infection. Treatment of resistant mice with I-TAC at the first day of infection resulted in subsequently decreased levels of the important Th1-instructing cytokine IL-12 in draining lymph nodes and a Th2 shift. I-TAC also inhibited *L. major* induced IL-12 production *in vitro*. Most important, in infection experiments the I-TAC induced Th2 shift resulted in a significant and sustained deterioration of disease.

**Conclusion:** This is the first identification of an early signal produced by specialized tissue cells which instructs systemic Th2-differentiation in experimental *leishmaniasis*. The Th2 instructing capacity of I-TAC is integrated by DCs via suppression of IL-12 which in turn promote Th2-polarization.

**PB03/35 KERATINOCYTES DETERMINE TH1/TH2 DICHOTOMY DURING EARLY EXPERIMENTAL LEISHMANIASIS**J. Ehrchen<sup>1,2</sup>, K. Roebrock<sup>1</sup>, D. Foell<sup>1</sup>, E. von Stebut<sup>1</sup>, J. M. Weiss<sup>1</sup>, D. Viemann<sup>1</sup>, G. Varga<sup>1</sup>, H.-J. Schubert<sup>3</sup>, J. Roth<sup>1</sup>, C. Sunderkötter<sup>2</sup><sup>1</sup>Institute of Immunology, Medical Faculty, Münster, Germany, <sup>2</sup>Medical Faculty Münster, Department of Dermatology, Münster, Germany, <sup>3</sup>Medical Faculty, Department of Dermatology, Mainz, Germany, <sup>4</sup>Medical Faculty, Department of Dermatology, Ulm, Germany, <sup>5</sup>Institute of Immunology, University of Veterinary Medicine, Hannover, Germany**Objectives:** Resistance in experimental *leishmaniasis* depends on the development of a *Leishmania* (*L.*) *major* specific Th1 response, while Th2 differentiation in BALB/c mice results in susceptibility. It has been shown, that the decisive events for the development of a Th1 or Th2 response take place during the first two days after infection. There is growing evidence that the microenvironment of the infected tissue delivers the initial triggers that affect Th-cell differentiation. We therefore analyzed differential gene expression 16h after infection with *L. major* in the skin of resistant C57BL/6 and susceptible BALB/c mice.**Methods:** We used microarray technology (Affymetrix) and bioinformatical analysis (GenMAPP, Genedata Expressionist) to detect *L. major* induced gene expression patterns in the skin of Balb/c and C57BL/6 mice 16h after infection. Results were confirmed by RT-PCR, ELISA, laser-microdissection and *in-situ*-hybridization. To confirm the relevance of cytokine production by resident tissue cells IL-6 gene deficient C57BL/6 mice were reconstituted with wildtype bone marrow and infected with *L. major*.**Results:** We detected a strong up-regulation of immunomodulatory mediators in the infected skin in both mouse strains. Employing bioinformatical analysis, laser-microdissection and *in-situ*-hybridization we found that the epidermis is a major source of these early signals. This epidermal gene induction was significantly stronger in resistant mice and encompasses several genes known to affect Th1/2 differentiation such as IL-12, IL-1b, osteopontin, IL4 and IL-6. Their expression was temporally restricted to the crucial time of Th1/2 differentiation. In experimental *leishmaniasis* IL-12, IL-1b and also IL4 (later on the classical Th2 cytokine) have been demonstrated to induce a Th1 response when present in an initial and temporally restricted phase of infection. Moreover, using bone-marrow chimeric C57BL/6 mice we now demonstrate that an exclusive loss of IL-6 in non-hematopoietic cells (including keratinocytes) results in a reduced Th1 response and increased susceptibility during *L. major* infection.**Conclusion:** This is the first experimental evidence that epidermal expression of cytokines in resistant mice significantly contributes to the adaptive, Th-cell driven immune response and outcome of infection in experimental *leishmaniasis*.**PB03/36 THE EFFECT OF LOW BIRTH WEIGHT AND PREMATURITY ON THE HUMAN IMMUNE RESPONSE TO BCG VACCINATION**F. Dube<sup>1</sup>, C. Day<sup>1</sup>, S. Gelderbloem<sup>1</sup>, J. Hughes<sup>1</sup>, G. Hussey<sup>1</sup>, W. Hanekom<sup>1</sup><sup>1</sup>University of Cape Town, Cape Town, South Africa**Background:** In developing countries, >15% of infants are born preterm (< 37 weeks gestation), or have low birth weights (< 2,500g). We do not know if the current TB vaccine, BCG, protects these small infants against tuberculosis.**Objective and hypothesis:** Our aim was to determine if the magnitude and quality of the BCG-induced immune response is affected by birth weight (BW) and by gestational age (GA). We hypothesised that vaccination of preterm and low BW infants results in a suboptimal immune response, compared with vaccination of term or normal BW infants.**Methods:** Infants who received routine BCG vaccination at birth were stratified for BW and for GA. At 10 weeks of age, whole blood was collected and incubated with viable BCG. Brefeldin-A was added at 7 hours, and at 12 hour red cells were lysed, and white cells were fixed and cryopreserved. Later, cells were thawed, permeabilised and stained for CD3, CD4, CD8, IFN- $\gamma$ , TNF- $\alpha$ , IL-2, and IL-17; expression of these markers was measured by multi-parameter flow cytometry.**Results:** Infants with low BW (n=52) had a lower proportion of BCG-specific CD4 T cells co-expressing IFN- $\gamma$ , TNF- $\alpha$  and IL-2 together, compared with infants with normal BW (n=53; 16.9 $\pm$ 1.1% vs. 21.3 $\pm$ 11.2%, respectively, p=0.01), and a higher proportion of CD4 T cells expressing IFN- $\gamma$  only (33.3 $\pm$ 1.9% vs. 27.6 $\pm$ 1.5%, p=0.02). No other differences in cytokine expression were shown, according to BW and GA.**Conclusion:** Low BW infants appeared to have a lesser ability to produce CD4 T cells co-expressing IFN- $\gamma$ , TNF- $\alpha$  and IL-2, so-called polyfunctional T cells. Presence of these cells, following vaccination, has been associated with improved outcome following experimental intracellular infection. However, most cytokine expression patterns of specific cells were not affected by BW or by GA. Our study has important implications for neonatal vaccination practices worldwide.**PB03/37 CORRELATION BETWEEN THE DIVERSITY OF THE TCR REPERTOIRE AND THE EFFICIENCY OF AN IMMUNE RESPONSE AGAINST NIPPOSTRONGYLUS BRASILIENSIS**A. Seidl<sup>1</sup>, D. Voehringer<sup>1</sup><sup>1</sup>Institute for Immunology, LMU, Munich, GermanyHelminth infections cause enormous socio-economic problems in endemic regions where sanitary conditions are poor. These worm parasites are generally strong inducers of Th2-associated immune responses characterized by high numbers of Th2 cells, eosinophils, basophils and mast cells. Worm expulsion is dependent on CD4 T cells and IL-4 or IL-13. At present it is not clear whether all Th2 cells induced during infection are specific for the pathogen or whether some Th2 cells might be induced by unspecific activation of bystander T cells. Furthermore, it is unclear whether a broad repertoire of TCR specificities is required to control the pathogen. To clarify these issues, we compared the immune response of mice with broad or narrow TCR repertoires upon infection with the helminth *Nippostrongylus brasiliensis*. The TCR repertoire was reduced to an oligoclonal or monoclonal level by using DO11.10 and DO11.10/Rag2<sup>-/-</sup> mice, respectively. Since the DO11.10 receptor is not cross-reactive with *N. brasiliensis* antigens, we could study the immune response against *N. brasiliensis* in mice where parasite antigens can be recognized by only few T cells (the ones which express endogenous TCRs in DO11.10 mice) or not at all (DO11.10/Rag2<sup>-/-</sup> mice). Our results show that all Th2 cells in *N. brasiliensis* infected mice are antigen-specific cells. We found no evidence for bystander activation of Th2 cells after infection. Efficient worm expulsion, high IgE levels and the mobilization of effector cells were dependent on a TCR repertoire with broad specificities.**PB03/38 HEPATIC LIPIDS ISOLATED 30 MINUTES AFTER CUTANEOUS CONTACT SENSITIZATION ACTIVATE NAÏVE INKT CELLS IN VITRO IN A CD1D-DEPENDENT FASHION**P.W. Askenase<sup>1</sup>, M.N. Szczepanik<sup>2</sup>, N. Dey<sup>1</sup><sup>1</sup>Yale, Med, New Haven, United States, <sup>2</sup>Kracow University, Microbiology, Kracow, PolandContact sensitivity (CS) is an immune process that culminates in a localized T-cell mediated cutaneous inflammatory response. CS results from high-dose topical exposure and sensitization to a chemically reactive hapten allergen, and subsequent skin challenge with a low dose of the same chemical, with elicitation of a local immune inflammatory response. Effector T cells of CS are recruited locally and mediate a delayed hypersensitivity response at the skin site of secondary exposure to the allergen. Elicitation of T-cell dependent CS depends on events initiated early in sensitization by glycolipid-specific hepatic natural killer T cells with invariant  $\alpha$ b-TCRs (iNKT cells) that are CD1d-restricted. Hepatic iNKT cells rapidly release IL-4 as early as 7 minutes after skin sensitization. We investigated the roles of hepatic lipids and CD1d in activating hepatic iNKT cells early in CS. We found that such lipids are critical in activating iNKT cells in CS in a CD1d-dependent fashion. Lipids isolated from mouse livers just 30 minutes after cutaneous sensitization are stimulatory to naïve iNKT cells, in a CD1d manner, compared to hepatic lipids from naïve mice, indicating that stimulatory lipids rapidly accumulate in the liver following cutaneous contact sensitization. Although hepatocytes express CD1d, that is not needed for iNKT cell activation. We found evidence that hepatic iNKT cells can be activated peripherally, implying potential activation by other antigen presenting cells.**PB03/39 CHRONIC INFECTION WITH CYTOMEGALOVIRUS LEADS TO DIFFERENTIAL EXPRESSION PATTERNS OF NK CELL RECEPTORS ON PERIPHERAL BLOOD T CELLS IN HEALTHY DONORS**A. Pachnio<sup>1</sup>, O. Chagoury<sup>1</sup>, J. Harding<sup>1</sup>, M. Colonna<sup>2</sup>, P. Moss<sup>1</sup><sup>1</sup>University of Birmingham, School of Cancer Sciences, Birmingham, United Kingdom, <sup>2</sup>Washington University School of Medicine, Department of Pathology and Immunology, St Louis, United States**Objective:** Infection with cytomegalovirus (CMV) leaves a distinct imprint on the phenotypic repertoire of the cellular immune response in healthy donors. The majority of CMV-specific T cells show a highly differentiated phenotype of effector memory cells. Furthermore, studies have shown a changed profile of NK cell receptors (NKR) on T cells following acute CMV-infection, but little is known about NKR expression on CMV-specific T cells during chronic infection.

We have studied the profile of Killer immunoglobulin-like receptors (KIRs) on peripheral blood T cells in CMV-seropositive and CMV-negative donors. Furthermore, we analysed whether CMV-serostatus correlates with a changed expression pattern of the recently identified receptor FcRL6 and the inhibitory NK cell receptor CD161.

**Methods:** PBMCs of healthy CMV-seropositive and -negative donors were prepared, stained and analysed by multicolour flow cytometry. The statistical significance of the results was calculated using the Mann-Whitney U test.**Results:** Our data shows a reduced expression of KIRs stained with CD158a (KIR2DL1, KIR2DS1) on CD8+ T cells in CMV+ compared to CMV-ve donors (5% vs 2.5%). Furthermore, we find a reduced expression of the inhibitory KIR3DL1 (NKB1) on CD8+ T cells. In contrast, expression of this receptor is significantly increased on CD4+ T cells in CMV+ donors. The expression of FcRL6 on the total CD8+ T cell repertoire is 20% higher in CMV seropositive donors (57% vs 37%). MHC I-peptide tetramers were used to show that expression on CMV-specific compared to EBV-specific T cells was higher (77% compared to 51%). Levels of CD161 were found to be reduced by 8% on CD8+ T cells in CMV-positive donors, no significant changes were observed on CD4+ T cells.**Conclusion:** In summary our data show that latent CMV-infection leads to significant changes of NKR expression profiles in healthy donors. This is likely to reflect selection of clonally-distributed NKR+ T cells due to chronic infection although the determinants of this observation are unclear. Further analysis of the functional significance of these patterns of NKR expression is required.

**PB03/40 CD4<sup>+</sup> T CELL SUBSET DYNAMICS DURING CHLAMYDIA TRACHOMATIS INFECTIONS**E. Marks<sup>1</sup>, N. Lycke<sup>1</sup><sup>1</sup>Mucosal Immunobiology and Vaccine Research Center, Institute of Biomedicine, Gothenburg University, Department of Microbiology and Immunology, Gothenburg, Sweden

Infection of the genital tract by *C. trachomatis* elicits a complex immune response that confers partial-protection against reinfection but can also result in tubal infertility due to the inflammatory process induced during clearance of the bacteria. CD4<sup>+</sup> Th1 cells are thought to dominate the protective and pathological immune response, however, other CD4<sup>+</sup> T cell subsets may potentially have an important role in disease outcomes. Here we show that Th1, Th2 and Th17 populations are distinctly regulated following infection in mice. Th1 is localized primarily to the uterine tissue, while Th2 upregulation occurs later and is limited to the lower genital tract. The Th17 subset is not significantly induced following the onset of infection. Significantly, the upper and lower genital tract represent distinct segregated compartments of the immune response, with the upper genital tract responding to infection with high levels of IFN- $\gamma$ , while the lower tract is dominated by IL-10. Both the upper and lower genital tract are regulated late in the immune response by FoxP3<sup>+</sup> cells. Infection induces early Th1 differentiation which strictly infiltrates only the upper genital tract tissue, while Th2 cells infiltrate the lower genital tract during the clearance phase of the infection. FoxP3<sup>+</sup> Treg cells appear in both the upper and lower genital tissue late in infection as the presence of Th1 cells wanes, which may have important implications for immunopathogenesis of *C. trachomatis* genital tract infections.

**PB03/41 THE IL-23/IL-17 AXIS IS REQUIRED FOR PROTECTIVE IMMUNE RESPONSES AGAINST *TRYPANOSOMA CRUZI* INFECTION**H. Erdmann<sup>1</sup>, C. Rossmagel<sup>1</sup>, N. Ghilardi<sup>2</sup>, Y. Iwakura<sup>3</sup>, T. Jacobs<sup>4</sup>, C. Hoelscher<sup>1</sup><sup>1</sup>Research Center Borstel, Infection Immunology, Borstel, Germany, <sup>2</sup>Genentech, South San Francisco, United States, <sup>3</sup>University of Tokyo, Institute of Medical Science, Tokyo, Japan, <sup>4</sup>Bernhard Nocht Institute for Tropical Medicine, Immunology, Hamburg, Germany

Interleukin (IL)-12 is a potent inducer of interferon-gamma (IFN $\gamma$ )-producing T helper (TH)1 cells and promotes a protective cell-mediated immune response after infection with the protozoan parasite *Trypanosoma cruzi*, the causative agent of Chagas disease. IL-23 is structurally closely related to IL-12 and shares the IL-12/23p40 subunit and the corresponding IL-12 receptor  $\beta$ 1 subunit. However, IL-23 is not as potent as an inducer of IFN $\gamma$ -production than IL-12. Instead IL-23, but not IL-12, promotes the proliferation of IL-17-producing TH17 cells. To analyze the role of IL-23 for protective immune responses during experimental Chagas disease, IL-23p19 deficient ('-') mice were infected with *T. cruzi*. Compared to wild-type mice, IL-23p19<sup>-/-</sup> mice developed a higher parasitemia and an increased mortality. However, this susceptibility was not due to an impaired TH1 immune response. Because IL-23 supports the development of IL-17-secreting TH17 cells, we infected IL-17<sup>-/-</sup> mice with *T. cruzi* to study the relevance of IL-17 for protective immune responses. Like IL-23<sup>-/-</sup> mice, IL-17<sup>-/-</sup> mice exhibited a higher parasitemia, an elevated mortality and an altered liver pathology. Moreover, TH1 immune responses were not affected by the absence of endogenous IL-17. Together, we suggest here that in addition to TH1 cells, IL-23-dependent TH17 cells are required for a successful resolution of *T. cruzi* infection. One important effector cytokine that mediates this IFN $\gamma$ -independent arm of the protective immune response during experimental Chagas disease appears to be IL-17. Downstream effector mechanisms induced by IL-17 are currently under investigation.

**PB03/42 APOPTOTIC *LEISHMANIA MAJOR* MEDIATE A TH2 RESPONSE IN BALB/C MICE**J. Barthelmann<sup>1</sup>, J. Westermann<sup>1</sup>, K. Kalies<sup>1</sup><sup>1</sup>University of Lübeck, Centre for Structural and Cell Biology in Medicine, Institute of Anatomy, Lübeck, Germany

**Objectives:** It has recently been reported that the virulent inoculum of *Leishmania major* (*L. major*) promastigotes conventionally used for infection experiments contains about 50% of apoptotic parasites. The depletion of apoptotic parasites lead to an increased neutrophil activity *in vitro* and reduced infectivity in susceptible BALB/c mice *in vivo*. Our study aims to analyse the detrimental effect of apoptotic leishmania on the immune response in the draining lymph node.

**Methods:** We separated viable and apoptotic *L. major* by magnetic cell separation and infected susceptible BALB/c mice subcutaneously in the hind footpad with 10<sup>6</sup> viable parasites. Cytokine mRNA expression in the draining lymph node was analysed using real-time RT-PCR, leishmania-specific and total serum IgG subtypes were determined by ELISA. Statistical significance between groups was determined by the two-tailed nonparametric Mann-Whitney test for unpaired samples.

**Results:** We found that the lack of apoptotic parasites in the inoculum leads to a delay of disease progression and decreased IL-4 and IL-10 mRNA levels 6 weeks after infection, while IFN- $\gamma$  levels remain unchanged. Furthermore, Th2-associated leishmania-specific as well as total serum IgG1 levels are reduced. Disease, cytokine mRNA production and serum IgG1 after 6 weeks could be restored by adding apoptotic leishmania to the inoculum, thereby indicating that apoptotic *L. major* support the establishment of a Th2 immune response.

**Conclusion:** Overall, the removal of apoptotic parasites in the inoculum decreases the Th2 response, but does not explicitly support the establishment of a Th1 phenotype in susceptible BALB/c. It is known that the IgG subtypes facilitate the uptake of amastigote *L. major* by different antigen-presenting cells, therefore we hypothesise that the ratio of IgG subtypes contributes to the degree of susceptibility of BALB/c mice to *L. major* infections.

**PB03/43 TRANSCRIPTIONAL CONTROL OF FOLLICULAR T HELPER CELL DIFFERENTIATION**D. Stauss<sup>1</sup>, M. Lipp<sup>1</sup>, G. Mueller<sup>1</sup><sup>1</sup>Max-Delbrueck-Center for Molecular Medicine, Berlin, Germany

Follicular T helper (T<sub>FH</sub>) cells play a crucial role in plasma cell generation by providing B cell help during the germinal center (GC) reaction. T<sub>FH</sub> cells supply signals for the survival, affinity maturation, isotype switching and immunoglobulin secretion of GC B cells and are thus important players in the generation of long-lived, high-affinity antibody-secreting plasma cells as well as memory B cells. Still, the molecular mechanisms through which naïve CD4<sup>+</sup> T cells become T<sub>FH</sub> cells are largely unknown.

The project aims at the identification of critical checkpoints and master switches in the transcriptional control of T<sub>FH</sub> cells in order to better understand the development of adaptive immune responses. Large-scale gene expression analysis of human and murine T<sub>FH</sub> cells as well as other CD4<sup>+</sup> T cell populations revealed unique expression patterns for transcriptional regulators and signaling molecules belonging e.g. to the Delta Notch Signaling pathway. As cell specialization is often associated with epigenetic changes in chromatin structure we are performing genome-wide ChIP-on-Chip analyses to define the landscape of activating/repressing histone modifications during T<sub>FH</sub> cell differentiation in order to identify novel regulatory elements that may not be readily discernible through expression analysis and comparative genomics.

**PB03/44 CADMIUM INHIBITS BACTERIAL CLEARANCE VIA INDUCING T REGULATORY AND IL-17-PRODUCING CELLS**N. Hemdan<sup>1,2</sup>, U. Sack<sup>1,2</sup>, F. Emmrich<sup>1,2</sup>, J. Lehmann<sup>2</sup><sup>1</sup>University of Leipzig, Max-Bürger Research Center, Institute of Clinical Immunology and Transfusion Medicine, Leipzig, Germany, <sup>2</sup>Fraunhofer Institute for Cell Therapy and Immunology, Leipzig, Germany

**Objectives:** Cadmium (Cd) is a pervasive pollutant and discussed to exert immunomodulatory effects. Adopting an infection model of BALB/c mouse, this study aimed at investigating the effect of 2-week exposure to Cd (0, 0.07 or 0.7 mg/kg body weight) on the subsequent infection with an attenuated strain of *Salmonella enterica* (SE: 5x10<sup>6</sup> CFU).

**Methods:** Effects on animals' general health and body weight were recorded. Modification of survival rates and efficiency of bacterial clearance as well as changes in serum levels and relative mRNA expression of various cytokines in splenocytes were assessed at different post-infection (p.i.) spans.

**Results:** This study revealed that Cd had insignificant effect on body weight. In comparison to control mice, the combination of exposure to Cd and the subsequent inoculation with various SE doses significantly increased death rate up to 90%. In both liver and spleen, Cd-exposed mice revealed significantly higher bacterial burdens. Cd-exposed infected mice had increased serum and spleen mRNA expression levels of IFN- $\gamma$  on day 3 p.i. This was reverted thereafter and remained inhibited up to day 30 p.i., and coincided with decreased levels of spleen mRNA of IL-12p40 but increased levels of TNF- $\alpha$ , IL-23p19, IL-4, IL-10, IL-6, TGF- $\beta$ 1 and IL-17A up to day 30 p.i., and increased serum levels of IL-6 and IL-10 estimated on day 3 p.i.

**Conclusion:** It can be inferred that:

- 2-week exposure to Cd modulated the animal's immune response and impaired its bacterial clearance efficiency.
- The diminished production of IL-12 together with elevated IL-10 and TGF- $\beta$ 1 levels may reflect increased T<sub>reg</sub> cell activities and abolished T cell differentiation into the protective Th1 type.
- Activating T<sub>reg</sub> cells together with inducing IL-6 production might lead to lowered activities of APCs.
- The elevated levels of TGF- $\beta$ 1, IL-6 and TNF- $\alpha$  might favour the elaborate differentiation of IL-17-producing cells as depicted.
- IL-17 might lead to chronic inflammation and succumb of animals even following eradication of the pathogen.

Overall, this study provide evidence that short-term exposure to non-lethal Cd doses impaired the immune response of BALB/c mice to bacterial infection; identifying the mechanisms involved warrants further studies.



**PB03/45 ISOLATION AND EXPANSION OF ANTIGEN-SPECIFIC HUMAN T CELLS USING DYNABEADS®**

A. Kullmann<sup>1</sup>, B.M. Reed<sup>1</sup>, T. Aarvak<sup>1</sup>, K.W. Schjetne<sup>1</sup>  
<sup>1</sup>Invitrogen Dynal, Oslo, Norway

**Background:** CD137 (4-1-BB), a member of the TNFR-family, functions as a costimulatory molecule promoting proliferation and survival of activated T cells. CD137 identifies recently activated human CD8<sup>+</sup> and CD4<sup>+</sup> T cell and represent a promising approach for the isolation of viable antigen-specific T cells.

**Methods and results:** Dynabeads FlowComp Human CD137 isolates activated antigen-specific T cells by use of an agonistic anti-CD137 antibody conjugated to a modified biotin and nitrated streptavidin coated Dynabeads. The modified biotin and nitrated streptavidin facilitates a gentle release mechanism and the procedure enables isolation of bead-free antigen-specific T cells.

In a virus model, PBMC from HLA-A2<sup>+</sup> donors were stimulated with CMV-peptide for 24 h. Activated CD137 positive cells were isolated by use of FlowComp Human CD137 and the separated CD137<sup>+</sup> T cells were further expanded *ex vivo*. After 9 days expansion more than 85% of the cells expressed the antigen-specific TCR as detected with CMV-specific pentamer staining. For further expansion of the antigen-specific T cells, Dynabeads conjugated with anti-CD3, anti-CD28 and anti-CD137 antibodies were used. We found that CD137-ligation was critical to sustain expansion of viable antigen-specific T cells.

**Conclusion:** Dynabeads FlowComp Human CD137 allows for specific isolation of anti-viral CD8<sup>+</sup> and CD4<sup>+</sup> T cells directly from PBMC in an easy-to-use *in vitro* procedure. Isolated and expanded antigen-specific cells maintain their specificity and can be further expanded using Dynabeads T-Expander Human CD3/CD28/CD137. The use of Dynabeads represent an efficient way to isolate and expand highly pure and viable antigen specific T cells for further characterization or use in adoptive immunotherapy.

**PB03/46 INHIBITION OF THE TH2 SKEWED RESPONSE IN NEONATES PROMOTES THE DEVELOPMENT OF TH17 TYPE EFFECTOR T CELLS**

I. Debock<sup>1</sup>, S. Delbauve<sup>1</sup>, M. Péteín<sup>2</sup>, M. Goldman<sup>1</sup>, V. Flamand<sup>1</sup>

<sup>1</sup>Institut d'Immunologie Médicale, Université Libre de Bruxelles, Gosselies, Belgium, <sup>2</sup>Institut de Pathologie et de Génétique, Gosselies, Belgium

**Aims:** A skewing towards a Th2-type response qualifies today the immunological status of the neonates. This may account for their sensitivity to infectious agents and their propensity to develop allergic pathologies. The capacity of the neonate to mount an effector Th17-type response is not well characterized. We evaluated here the effects of the neutralization of IL-4, a key cytokine involved in the development of a Th2-type immune response and in the regulation of IL-17 dependent response, in glance with the possible appearance of a Th17 cell-mediated inflammatory immune response in murine newborn.

**Methods:** BALB/c neonates immunized at birth with (A/J × BALB/c)F1 spleen cells develop a Th2-type polarization and pathology, as well as Th2-type rejection of (A/J × BALB/c)F1 skin allografts. We perinatally injected anti-IL-4 mAb to neonates to neutralize IL-4 in this model and monitored the Th17-type response by qRT-PCR, intracellular staining and immunohistochemistry.

**Results:** We showed that hyper IgE response, lymphoid organ hyperplasia and Th2 polarization are abrogated in BALB/c mice immunized with F1 spleen cells if they are submitted to anti-IL-4 neutralizing mAb treatment. mRNA levels of IL-4, IL-5 and IL-13 were inhibited in the spleen of F1 spleen cells and anti-IL-4 mAb injected mice whereas mRNA levels of IL-17A, IL-17F, IL-22 and RORγt were enhanced. This was confirmed by the fact that intracellular IL-17A protein was detected in a significant higher percentage of CD4<sup>+</sup> T lymphocytes in animals immunized at birth and inhibited for the Th2 pathway, compared with mice immunized with F1 spleen cells and isotype control mAb. (A/J × BALB/c)F1 skin allografts were acutely rejected in mice immunized at birth with semi-allogeneic spleen cells plus anti-IL-4 neutralizing mAb with a massive neutrophils infiltrate and a significant increase of IL-17A mRNA into the graft. Neutralization of IL-17A before skin grafting leads to a delay of neutrophils infiltration into the graft.

**Conclusions:** Our findings suggest that full acquisition of function by effector Th17 cells may be reinforced when Th2 pathway is prevented early in life. This may have an impact in terms of choice of vaccine adjuvants and development of autoimmune diseases.

**PB03/47 IDENTIFYING LINEAGE RELATIONSHIPS IN HUMAN T CELL POPULATIONS**

C.L. Menckeborg<sup>1</sup>, S. Kissane<sup>1</sup>, C. Schmutz<sup>1</sup>, F. Falciani<sup>2</sup>, J. Curnow<sup>1</sup>, M. Salmon<sup>1</sup>

<sup>1</sup>University of Birmingham, School of Immunity and Infection, College of Medical and Dental Sciences, Medical School, Birmingham, United Kingdom, <sup>2</sup>University of Birmingham, School of Biosciences, College of Medical and Dental Sciences, Medical School, Birmingham, United Kingdom

**Objectives:** CD4<sup>+</sup> and CD8<sup>+</sup> T cell populations can be divided into distinct subpopulations based on the expression of the surface markers CCR7 and CD45RA. The resulting populations are referred to as naïve, central memory, effector memory and effector memory RA<sup>+</sup> (EMRA). In this study we aim to identify the potential lineage relationships between these subpopulations for both CD4<sup>+</sup> and CD8<sup>+</sup> T cells. In addition we will study the degree of similarity between the CD4<sup>+</sup> and CD8<sup>+</sup> transcriptional programmes.

**Methods:** Naïve, central memory and effector memory CD4<sup>+</sup> and CD8<sup>+</sup>, and EMRA<sup>+</sup> CD8<sup>+</sup> T cell populations were sorted from the peripheral blood of six healthy donors and their gene expression was analysed by microarray. Statistical Analysis of Microarrays (SAM) was used to identify shared genes capable of separating the four populations into distinct populations. Hierarchical clustering and principal component analysis (PCA) visualised the relationships between the different populations.

**Results:** The results from the PCA suggest that for both CD4<sup>+</sup> and CD8<sup>+</sup> T cells, naïve cells differentiate into central memory cells, which then differentiate into effector memory and subsequently, for CD8<sup>+</sup> T cells, into EMRA<sup>+</sup>. The genes identified by SAM analysis that distinguish these subpopulations include many molecules already known to be associated with T cell differentiation, including CCR7, CD45RA, granzymes, L-selectin and TNF and IFNγ receptors. Although the majority of gene differences were associated with either CD4<sup>+</sup> or CD8<sup>+</sup> differentiation, there were also genes that were associated with the differentiation of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells.

**Conclusion:** Our data suggest that both CD4<sup>+</sup> and CD8<sup>+</sup> T cells differentiate along a linear pathway of naïve to central memory to effector memory to EMRA<sup>+</sup>. The transcriptional programmes responsible for these differentiation steps were largely distinct between CD4<sup>+</sup> and CD8<sup>+</sup> T cells, although additional elements were common to both subsets.

**PB03/48 DISTINCTION OF T-CELL SUBSETS BY FLUOROSPOT ASSAY**

E. Gelius<sup>1</sup>, B. Axelsson<sup>1</sup>, P. Larsson<sup>1</sup>, T. Ernemar<sup>1</sup>, P. Andersson<sup>1</sup>, J. Fohlstedt<sup>1</sup>, N. Ahlborg<sup>1</sup>, K. Nihlmark<sup>1</sup>, S. Paulie<sup>1</sup>

<sup>1</sup>Mabtech AB, Nacka Strand, Sweden

The interest in *ex vivo* measurements of antigen-specific T cells secreting multiple cytokines has led to the development of the FluoroSpot assay. FluoroSpot is similar to ELISpot and has all the advantages of a regular ELISpot assay, including high sensitivity, robustness and ease of performance. In addition, FluoroSpot enables the simultaneous detection and enumeration of distinct cells secreting either of two cytokines as well as cells secreting both cytokines.

Cytokine-secreting PBMCs stimulated with various antigens were identified using FluoroSpot. Single cytokine-secreting cells were visualised as green or red spots. Double cytokine-secreting cells were identified by a computerised overlay and visualised as yellow spots. Antigens from CMV, EBV, Influenza, TBE, Tetanus, TB and Candida were used to study the ratios of antigen-specific single- and double-producing T cells secreting combinations of IFN-γ, IL-2, IL-4, IL-13 and/or IL-17.

The FluoroSpot assay detected frequencies of IFN-γ and IL-2 double-secreting CD8<sup>+</sup> T cells similar to what has been found by flow cytometry analysis of polyfunctional T cells. Also, distinct T-helper cell populations, such as TH1/TH2 and TH1/TH17 could be identified. The FluoroSpot assay represents a promising method for characterisation of cellular immune responses making it attractive for rapid analysis and more advanced screening of cellular immune responses.

**PB03/49 TNF-α AND CD8<sup>+</sup> T LYMPHOCYTES EXERT A PROTECTIVE EFFECT AGAINST PULMONARY PARACOCIDIOIDOMYCOSIS IN NITRIC OXIDE DEFICIENT MICE**

S. Bernardino<sup>1</sup>, V.L.G. Calich<sup>2</sup>

<sup>1</sup>Institute of Biomedical Sciences – University of Sao Paulo, Immunology, São Paulo, Brazil, <sup>2</sup>Institute of Biomedical Sciences-USP, Immunology, São Paulo, Brazil

**Introduction and objectives:** *Paracoccidioides brasiliensis* is a pathogenic fungus restricted to Latin America and its natural route of infection is the inhalation of fungal particles, which usually leads to an asymptomatic Paracoccidioidomycosis (PCM) infection. Inducible nitric oxide synthase (iNOS) is a nitric oxide (NO) generating enzyme which plays a role in the clearance of microbial infections, but its overproduction can lead to immunosuppression of cellular immunity. Our previous findings showed that early in PCM, NO deficiency results in less severe disease with lower pulmonary CFU, increased number of activated CD4 and CD8 T cells and macrophages, equivalent number of regulatory T cells in the lungs and high levels of pulmonary TNFα which seem to be important to organize lung lesions at the late period of infection. In contrast, at late period, KO mice showed higher pulmonary CFU organized in granulomas which control the fungal dissemination and resulted in similar mortality rates for both mouse strains. Increased numbers of regulatory T cells and activated CD4<sup>+</sup> T cells and macrophages in lung were seen in KO mice compared to WT group.

**Methods and results:** We investigated the role of TNFα and CD8<sup>+</sup> T cells in the immunity against of PCM using C57BL/6 iNOS KO and WT mice infected by the intratracheal route with 1x10<sup>6</sup> yeasts. Early *in vivo* TNFα (i.p.: 250 μg/0,5 mL days 0,6,12) and CD8<sup>+</sup> T depletion (i.p.: 200 μg/0,5 mL 48, 24h before infection, days 6,12) were able to abrogate the CFU differences between KO and WT mice observed at week 2. Moreover, the depletion of CD8<sup>+</sup> T cells resulted in lower number of CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes and activated macrophages recruited to the lungs. Otherwise, TNFα neutralization leads to elevated number of activated pulmonary T lymphocytes and macrophages. Furthermore, at a late period, the anti TNF-α treatment resulted in increased and precocious mortality of KO, but not WT mice, and impaired organization of lung granulomas.

**Conclusion:** The protective effect of NO absence in murine PCM appears to be mediated by TNF-α and CD8<sup>+</sup> T cells which controls fungal loads, recruitment and organization of pulmonary lesions as well as mortality rates.

**Supported by FAPESP**

**PB03/50 REGULATION OF SUPPRESSOR OF CYTOKINE SIGNALING (SOCS) MOLECULES IN HUMAN T CELLS**K. Kleinstaub<sup>1</sup>, C. Sander-Jülich<sup>1</sup>, U. Richardt<sup>1</sup>, B. Fleischer<sup>1</sup>, M. Jacobsen<sup>1</sup><sup>1</sup>Bernhard Nocht Institute for Tropical Medicine, Immunology, Hamburg, Germany

The suppressor of cytokine signaling family (SOCS) comprises crucial regulators of different cellular processes including inhibition of cytokine receptor signaling and protein degradation. T cells express a subgroup of SOCS family members including SOCS1, SOCS2, SOCS3, and the cytokine inducible SH2-containing protein (CISH), which modulate T-cell activation, cytokine response, and T helper cell polarization. Gene expression studies (in cooperation with the group of S. H. E. Kaufmann, MPIIB, Berlin, Germany) indicate a role of SOCS2, SOCS3, and CISH expression in susceptibility to human tuberculosis (unpublished data). The inherent assumption of this study is that differential SOCS family member expression patterns cause functional variations in the T cell response against *Mycobacterium tuberculosis* infection. This might be a critical factor for the development of active tuberculosis. Therefore we analyze the expression of SOCS1, SOCS2, SOCS3, and CISH in human T cells *ex vivo* and after *in vitro* restimulation in this study.

We determine expression of SOCS family members in enriched CD4<sup>+</sup> and CD8<sup>+</sup> T cells, as well as in T cell subpopulations (i.e. naïve, central memory, effector memory, and effector T cells) by quantitative PCR. Notably and in contrast to previous findings we detect no significant differences between T-cell subpopulations for SOCS3 expression. In addition, T-cell receptor specific *in vitro* stimulation leads to mRNA up-regulation for all SOCS family members already after 6 h of CD3/CD28 culture.

These findings are in contrast to previous animal studies that described predominant SOCS3 expression in naïve T cells that is down-regulated after *in vitro* T cell restimulation after 48 h.

We conclude that the regulation of SOCS3 expression seems to be different between human and mouse T cells. Taking into account that the vast majority of studies, which characterized SOCS family member functions in T cells have been performed using animal models we suggest to analyze SOCS function in human T cells where applicable. Ongoing studies in our laboratory aim at defining the influence of SOCS genes on polarization and cytokine-expression pattern of primary human T helper cells and T cell lines.

**PB03/51 HELICOBACTER PYLORI SHAPES THE CYTOKINE PROFILE OF CD4<sup>+</sup> T CELLS**M. Beigier-Bompadre<sup>1</sup>, V. Moos<sup>2</sup>, K. Allers<sup>2</sup>, T. Schneider<sup>2</sup>, T. Aebischer<sup>1</sup>, T.F. Meyer<sup>1</sup><sup>1</sup>Max Planck Institute for Infection Biology, Molecular Biology, Berlin, Germany, <sup>2</sup>Charité – Campus Benjamin Franklin, Medical Clinic I, Berlin, Germany

*Helicobacter pylori* colonizes the human gastric mucosa causing a chronic infection that could lead to peptic ulcer and some forms of gastric cancer. T cells are key effectors in the protective response to *H. pylori* challenge, and therefore a target for the bacterium in avoiding protective responses. Here, we evaluated the cytokine profile of human CD4<sup>+</sup> T cells co-incubated with *H. pylori* and mutants for the cytotoxin VacA and the enzyme gamma-glutamyl-transpeptidase (GGT). Purified CD4<sup>+</sup> T cells from healthy donors were incubated with *H. pylori* wild type or the mutants for 2h before activation of the cells with antibodies against CD3 and CD28. After 5 to 7 days of incubation, we evaluated T-cell proliferation by CFSE staining and the cytokine profile by a cytometric bead array. As previously described, *H. pylori* inhibited T-cell proliferation and the enzyme GGT was responsible for this effect (*Gastroenterology*. 2007;132:1820-33). In addition, *H. pylori* abrogated the release of IL-2, IL-10, IL-13, IL-17A, TNF and IFN- $\gamma$  in activated cells and this effect was mediated by GGT. On the contrary, the release of IL-6, IL-4 and TGF- $\beta$ 1 was not affected. The expression of the transcription factor FoxP3 evaluated by Western Blot was enhanced in activated CD4<sup>+</sup> T cells after 7 days of co-incubation with *H. pylori* compared to uninfected cells. Thus, *H. pylori* modulates the cytokine profile of CD4<sup>+</sup> T cells, which could be a mechanism used by the pathogen to shape the adaptive immune response of the host.

**PB03/52 AN IMBALANCE IN T<sub>H</sub>1/T<sub>H</sub>2 CYTOKINES WITH PREDOMINANCE OF T<sub>H</sub>2 TYPE IN UROTHELIAL CARCINOMA OF BLADDER**A. Satyam<sup>1</sup>, P. Singh<sup>2</sup>, N. Badjatia<sup>1</sup>, A. Seth<sup>2</sup>, A. Sharma<sup>1</sup><sup>1</sup>All India Institute of Medical Sciences, Department of Biochemistry, New Delhi, India, <sup>2</sup>All India Institute of Medical Sciences, Department of Urology, New Delhi, India

**Objectives:** Though studies aiming to elucidate the T<sub>H</sub>1/T<sub>H</sub>2 balance have been performed in several types of malignancies, the role of these two subsets in immunological dysfunction in bladder cancer patients is not clearly defined. The present study was aimed to evaluate the role of T<sub>H</sub>1 and T<sub>H</sub>2 derived cytokines in progression of urothelial carcinoma of bladder by determining the circulatory concentration of various cytokines and to correlate the observations with grade and severity of the disease.

**Methods:** Eighty patients with urothelial carcinoma of bladder (superficial low grade, superficial high grade & muscle invasive), and 75 controls (20 patients with renal calculus and 55 healthy individuals) were included in the study. The circulating levels of cytokines were estimated by high sensitivity ELISA kits using biotinylated antibody with streptavidin-peroxidase enzyme system.

**Results:** The mean value of T<sub>H</sub>1 cytokines; IFN- $\gamma$  and IL-2 were significantly reduced in bladder cancer patients as compared to controls. The decrease in levels of type 1 cytokines showed correlation with grades and stage of tumor. No significant change in IFN- $\gamma$  and IL-2 levels was observed between high-grade and low-grade and between muscle-invasive and high-grade tumors. Significantly enhanced values of typical T<sub>H</sub>2 cytokines IL-4, IL-5 and IL-10 were observed in patients as compared to controls (patients with renal calculus and healthy subjects). No significant increase was detected in the levels of IL-4 and IL-10 in patients with muscle-invasive vs high-grade where as significant increase was observed for IL-5.

**Conclusion:** Our study delineates that in bladder tumor patients a marked polarization exists towards the expression of T<sub>H</sub>2 type cytokines while T<sub>H</sub>1 remain suppressed. Further more the levels of these cytokines alter according to the grades of the tumor indicating that tumor cells themselves are capable of modulating the cytokine levels to a immunosuppressive state, favoring their survival, proliferation and ability to escape immune surveillance and destruction by host immune cells. This study might give significant insights about the use of T<sub>H</sub>1 type cytokines for the administration of immunotherapy to bladder cancer patients.

**PB03/53 EVALUATION OF CD8<sup>+</sup> T-CELL-MEDIATED SELECTION PRESSURE ON PROVIRAL GAG P17 AND P24 IN CHRONICALLY INFECTED HIV-1<sup>+</sup> INDIVIDUALS**S.J. Westrop<sup>1</sup>, S. Mandalia<sup>1,2</sup>, M. Nelson<sup>2</sup>, N. Imami<sup>1</sup><sup>1</sup>Imperial College London, London, United Kingdom, <sup>2</sup>Chelsea and Westminster Hospital, London, United Kingdom

**Objectives:** Functional decline of HIV-1-specific CD8<sup>+</sup> T-cell responses observed in chronic HIV-1 infection is not restored by anti-retroviral therapy, unlike CD8 T-cell responses to other viral antigens. The contribution of HIV-1 sequence variation to CD8<sup>+</sup> T-cell responses is unknown. The relationship between observed *in vitro* responses and those occurring *in vivo* is investigated by sequencing proviral HIV-1 gag and assaying the function of HIV-1-, Flu-, EBV- and CMV-specific CD8 T cells.

**Methods:** Proliferation, IFN- $\gamma$  and perforin release of HIV-1-specific CD8<sup>+</sup> T cells was assessed in 73 HIV-1<sup>+</sup> patients by <sup>3</sup>H-Thymidine incorporation and ELISpot, and compared to responses to MHC class I-restricted Flu, EBV and CMV peptides. Proviral HIV-1 gag was sequenced, translated and aligned to the peptides used in the *in vitro* assays. Sequence changes in peptides were compared between patients with relevant and non-relevant HLA-types. Amino acid changes in predicted anchor and non-anchor residues were investigated further. Non-parametric tests and MIXED models were used for statistical analysis.

**Results:** Flu-, EBV and CMV-specific responses resulted in more robust perforin (p=0.067) and greater lymphoproliferative responses (p=0.043) than Gag-specific responses, despite equal IFN- $\gamma$  release (p=0.339). Over 80% of HIV-1<sup>+</sup> patients responded with IFN- $\gamma$  production to HIV-1 Gag peptides, indicating recognition of CD8 T-cell epitopes, despite lack of proliferative response. MIXED modelling revealed that only 2 of 25 peptides exhibited significantly more amino acid sequence changes when restricted to the patients' HLA-type, relative to when restricted to an irrelevant HLA-type. One peptide had significantly more amino acid changes in predicted anchor residues, and the other in predicted non-anchor residues. However, 1 of the 25 MHC I-restricted Gag peptides studied showed significantly more amino acid changes in non-anchor residues in patients with irrelevant HLA-type compared to patients with relevant HLA-type.

**Conclusion:** CD8 T-cell proliferation specific to non-HIV-1 viral antigens is attainable in chronically infected HIV-1<sup>+</sup> individuals, despite deficient CD4<sup>+</sup> T-cell help caused by reduced numbers and HIV-1-induced anergy. Recognition of HIV-1 CD8 T-cell epitopes by chronically infected HIV-1<sup>+</sup> individuals, and modest evidence of CD8 T-cell-mediated selective pressure, implicates CD8 T-cell unresponsiveness, rather than viral escape, as a decisive cause of HIV-1 disease progression.

**PB03/54 CELLULAR VERSUS HUMORAL IMMUNE RESPONSE – HIGH AND LOW ANTIGEN DOSES DETERMINE THE DIRECTION OF THE IMMUNE RESPONSE IN THE MURINE SPLEEN**C. Stamm<sup>1</sup>, J. Westermann<sup>1</sup>, K. Kalies<sup>1</sup><sup>1</sup>University of Lübeck, Centre for Structural and Cell Biology in Medicine, Institute of Anatomy, Lübeck, Germany

**Objectives:** Immunisations with different antigen doses provoke different immune responses. Thus, injection of a low dose of sheep red blood cells (SRBC) induces a cellular immune response as judged by delayed type hypersensitivity (DTH) reaction after reexposure to the antigen, whereas the injection of a high dose induces a humoral immune response but no DTH. To figure out the underlying mechanisms, we compared the localisation of SRBC in the spleen, the expression of cytokines after primary immunisation *in vivo* and the number of proliferating T cells in the T-cell zone of the spleen.

**Methods:** To investigate the localisation of SRBC they were labelled with CFSE before injection. Spleen sections were analysed using confocal fluorescence microscopy. The expression of cytokine genes in the lymphoid compartments of the spleen after high- or low-dose immunisation was examined using laser-microdissection. To test for a DTH reaction a high dose of SRBC was injected into the footpad of mice that received a primary high- or low-dose immunisation or isolated spleen cells from high- or low-dose immunised donor mice.

**Results:** High-dose SRBC localise in the marginal zone, whereas low-dose SRBC are not detectable in the spleen but induce an increased number of B cells in the marginal zone. IFN- $\gamma$  cytokine expression in the T-cell zone of spleens from high- or low-dose immunised mice did not differ. In contrast, increased IFN- $\gamma$  expres-

sion was found in the B-cell zone after low-dose injection only. A much stronger T-cell proliferation was induced after high-dose injection of SRBC. This newly formed T cells did not respond to the second encounter with SRBC in the footpad. This unresponsiveness remained even after transferring the T cells into a naïve host and challenging them again with SRBC.

**Conclusion:** We conclude that the absent DTH reaction after high-dose injection of SRBC is not due to lower numbers of T cells. We hypothesise that the newly formed T cells are either unable to migrate into the skin or are regulatory T cells which suppress the DTH reaction in the skin.

#### PB03/55 LIVER SINUSOIDAL ENDOTHELIAL CELLS INDUCE ANTI-INFLAMMATORY CD4<sup>+</sup> T CELLS SUPPRESSING MURINE AUTOIMMUNE HEPATITIS

N. Kruse<sup>1</sup>, A. Schrage<sup>1</sup>, K. Neumann<sup>1</sup>, K. Derkow<sup>2</sup>, E. Schott<sup>2</sup>, A. Kühl<sup>3</sup>, C. Loddenkemper<sup>3</sup>, A. Hamann<sup>4</sup>, K. Klugewitz<sup>1</sup>

<sup>1</sup>Medizinische Klinik I, Charité, Universitätsmedizin Berlin, Berlin, Germany, <sup>2</sup>Medizinische Klinik m.S. Hepatologie und Gastroenterologie, Charité Campus Virchow, Berlin, Germany, <sup>3</sup>Medizinische Klinik I, Research Center ImmunoSciences (RCIS), Charité, Universitätsmedizin Berlin, Berlin, Germany, <sup>4</sup>Exp. Rheumatologie, Charité, Universitätsmedizin Berlin, Berlin, Germany

**Objectives:** Cellular mechanisms that maintain the intrahepatic immune balance are crucial in viral or autoimmune liver diseases and for allograft acceptance. For naïve CD8<sup>+</sup> T cells, liver sinusoidal endothelial cells (LSEC) have been shown to act as non-professional antigen presenting cells and thus induce tolerance by inhibition of cytotoxicity. In this study we investigated consequences of CD4<sup>+</sup> T cell priming by LSEC.

**Methods:** Priming by LSEC was investigated in bone marrow chimeric mice expressing MHC class II exclusively on non-hematopoietic cells. We studied the cytokine expression of LSEC primed CD4<sup>+</sup> T cells (T<sub>LSEC</sub>) and determined the stability of their phenotype *in vivo* by adoptive transfer into congenic mice and immunogenic antigen application. Investigating suppressive capacities of T<sub>LSEC</sub> we performed an *in vitro* suppression assay. The ability of T<sub>LSEC</sub> to influence proinflammatory reactions *in vivo* was analyzed in a model of T cell-mediated autoimmune hepatitis. Hepatic inflammation was monitored by ALT levels and histologic analyses. The migration pattern of T<sub>LSEC</sub> was investigated by an *in vivo* homing assay.

**Results:** We demonstrated that LSEC induce proliferation of naïve CD4<sup>+</sup> T cells *in vitro*. Although the expression of CD45RB was downregulated in T<sub>LSEC</sub>, these cells did not produce effector cytokines. This phenotype of T<sub>LSEC</sub> remained stable *in vivo*. The *in vivo* migration pattern of T<sub>LSEC</sub> was different from cells activated by professional antigen presenting cells isolated from the spleen, since they showed enhanced homing into lymph nodes and the intestine while they were also present in the liver. Interestingly, T<sub>LSEC</sub> negative for CD25 and Foxp3, suppressed the proliferation of naïve CD4<sup>+</sup> T cells *in vitro*. They did neither support a DTH reaction nor a hepatic inflammation and were even able to suppress hepatitis.

**Conclusion:** Priming of naïve CD4<sup>+</sup> T cells by LSEC leads to an anti-inflammatory phenotype here referred to as T<sub>LSEC</sub>. Thus liver sinusoidal endothelia may directly contribute to limiting hepatic inflammation and supporting local tolerance towards exogenous antigens or endogenous self-antigens.

#### PB03/56 SPECIFIC T-CELL RESPONSES IN LESIONS AND PERIPHERAL BLOOD FROM PATIENTS WITH BURULI ULCER

K. Becker<sup>1</sup>, M. Badusche<sup>1</sup>, G. Bretzel<sup>2</sup>, K.-H. Herbringer<sup>2</sup>, W. Nienhuis<sup>3</sup>, O. Adjei<sup>4</sup>, B. Fleischer<sup>1</sup>, M. Jacobsen<sup>1</sup>

<sup>1</sup>Bernhard-Nocht-Institute for Tropical Medicine, Hamburg, Germany, <sup>2</sup>University of Munich, Munich, Germany, <sup>3</sup>University Medical Centre Groningen, Groningen, Netherlands, <sup>4</sup>Kumasi Centre for Collaborative Research, Kumasi, Ghana

Buruli ulcer disease (BUD), caused by *Mycobacterium (M.) ulcerans*, is a neglected bacterial infection of the poor in remote rural areas. BUD is a mutilating disease leading to severe disability; it is the third most common mycobacterial infection in immunocompetent people after tuberculosis and leprosy most endemic in West Africa.

There is some evidence that a T helper type 1-mediated immune response is protective against *M. ulcerans* but the role and distribution of antigen-specific T cells in BUD lesions is hardly defined. In addition, analysis and diagnosis of specific T-cell immunity against *M. ulcerans* is hampered by concomitant infection with other atypical mycobacteria, *M. tuberculosis*, and/or *M. bovis BCG* vaccination.

Here we determine the T-cell distribution of two ulcerative lesions and peripheral blood from a BUD patient using a quantitative PCR method for analyses of T-cell receptor Vβ (TCR-BV) chains and compare the antigen-specific T-cell response in peripheral blood of children infected with *M. ulcerans* or other mycobacteria using *in vitro* restimulation with mycobacterial lysates and intracellular cytokine analyses.

TCR-BV chain analyses in two lesions from a BUD patient reveal differential expression of certain chains depending on the distance from the lesion center. Skewed TCR-BV chains are different between two distinct lesions from the same BU patients. This suggests that T cells, which infiltrate the BUD lesions, are oligoclonally expanded but a predominantly infiltrating subtype could not be identified.

Antigen-specific T-cell cytokine analyses of peripheral blood mononuclear cells from patients with BUD (n = 26) and other mycobacterial infections (n = 9) reveal IFN-γ, IL-2, and TNF-α secretion after stimulation with lysates and purified protein derivatives from a non-toxic *M. ulcerans* strain, *M. tuberculosis*, and *M. avium*. Despite crossreactivity against different mycobacterial lysates, ratios of specific T cells (percentage of *M. ulcerans*-specific T cells/*M. tuberculosis*-specific T cells in individual donors) were significantly different between the BUD patients and donors with other mycobacterial infections. Therefore concomitant measurement of T-cell cytokine expression after restimulation with different mycobacterial lysates can help to distinguish early infection with *M. ulcerans* from infection with other mycobacteria.

#### PB03/57 PLASMODIUM VIVAX ALTERS IMMUNOSUPPRESSION CAUSED BY PLASMODIUM FALCIPARUM INFECTIONS

S. Chuangchaiya<sup>1,2</sup>, K. Jangpatrapongsa<sup>2,3</sup>, J. Sirichaisinthop<sup>4</sup>, J. Sattabongkot<sup>5</sup>, K. Pattanapanyasat<sup>6,7</sup>, K. Chotivanich<sup>1</sup>, M. Troye-Blomberg<sup>8</sup>, L. Cui<sup>9</sup>, R. Udomsangpetch<sup>2,3</sup>

<sup>1</sup>Mahidol University, Department of Clinical Tropical Medicine, Faculty of Tropical Medicine, Bangkok, Thailand, <sup>2</sup>Mahidol University, Department of Pathobiology, Faculty of Science, Bangkok, Thailand, <sup>3</sup>Mahidol University, Department of Clinical Microbiology, Faculty of Medical Technology, Bangkok, Thailand, <sup>4</sup>Center of Malaria Research and Training, Ministry of Public Health, Saraburi, Thailand, <sup>5</sup>AFRIMS, Department of Entomology, Bangkok, Thailand, <sup>6</sup>Mahidol University, Department of Immunology, Faculty of Medicine, Siriraj Hospital, Bangkok, Thailand, <sup>7</sup>Mahidol University, Center of Excellence for Flow Cytometry, Office for Research and Development, Bangkok, Thailand, <sup>8</sup>Wenner-Gren Institute, Department of Immunology, Stockholm, Sweden, <sup>9</sup>Pennsylvania State University, Department of Entomology, Pennsylvania State, United States

**Objectives:** *Plasmodium falciparum* infection causes transient immunosuppression during parasitemic stage. However, immune response during simultaneous infection with both *P. vivax* and *P. falciparum* has not been investigated. In particular, it is not clear whether host immune response to malaria will be different when compare an infection with single malaria species versus mixed malaria species.

**Methods:** Human blood mononuclear cells from mixed *P. vivax-P. falciparum* infection were characterized by flow cytometry for the immunomodulatory role of T cells. In addition, antibodies to parasite-derived proteins and to PfMSP-1<sub>19</sub> and PvMSP-1<sub>19</sub> recombinant proteins were determined.

**Results:** We found that CD3<sup>+</sup>delta 2<sup>+</sup>-TCR T cells, T-killer cell phenotype, were significantly higher in the acute-mixed *P. vivax-P. falciparum* infection compared with either single *P. vivax* or *P. falciparum* infection. Interestingly, mixed malaria-infection had the highest antibodies against both *P. vivax* and *P. falciparum* compared with those antibodies obtained from the single malaria infection.

**Conclusion:** This suggests that co-infection with *P. vivax* could induce effector T-killer cells. In addition, antimalarial antibodies found in the mixed infection could have protective role against disease severity in *P. falciparum* infection as shown by the lower parasitemia in the mixed infection group. These findings imply that *P. vivax* may help resolving the severity of *P. falciparum* infection.

#### PB03/58 PHENOTYPIC CHARACTERIZATION OF PERIPHERAL BLOOD MEMORY CD8<sup>+</sup> T CELL SUBSETS, WITHIN DIFFERENT GROUPS OF PATIENTS SUFFERING AMERICAN TEGUMENTARY LEISHMANIASIS (ATL) IN THE NORTHWEST OF ARGENTINA. FIRST REGIONAL REPORT

C.M. Parodi<sup>1</sup>, A. Barrio<sup>1</sup>, M.F. García Bustos<sup>1</sup>, M.C. Mora<sup>1</sup>, F. Ramos<sup>1</sup>, J. Becker<sup>2</sup>, S. Monroig<sup>2</sup>, B. Ruibal-Ares<sup>3</sup>, M.M. E de Bracco<sup>3</sup>, M.A. Basombrío<sup>1</sup>

<sup>1</sup>Instituto de Patología Experimental (IPE) CONICET, Salta, Argentina, <sup>2</sup>Hospital San Bernardo, Dermatology, Salta, Argentina, <sup>3</sup>Instituto de Investigaciones Hematológicas (IIHEMA), Academia Nacional de Medicina, Cellular Immunology, Ciudad Autónoma de Buenos Aires, Argentina

**Aim:** Phenotypic characterization of CD8<sup>+</sup> T peripheral cells from patients suffering ATL is the purpose of this work in order to better understand their role within the pathology outcome.

**Methods:** Study groups: 1) 9 patients with diagnosis of ATL (infection duration: 5-20 years); 2) 5 ATL patients that received 2 or more complete therapy regimens but suffered frequent relapses (infection duration: 5-20); 3) 2 acute ATL patients (infection duration: < 1 month); 4) 6 healthy subjects. Isolated, cryopreserved and thawed peripheral blood mononuclear cells were stained with: Anti-CD3, CD8, CD57, CD45RO, CD27, CD127, CD45RA, CD28 -FITC, -PE or -PerCP labelled monoclonal antibodies (BD, Pharmingen). Results were evaluated by flow cytometry (FACSscan cytometer, CellQuest software).

**Results:** Lower percentages of CD127, CD27, CD28 and "early" CD8<sup>+</sup> T cells (CD27<sup>+</sup>, CD28<sup>+</sup>) were observed in the first two groups of patients compared to the control group. Likewise, increase of "late" (CD27<sup>-</sup>, CD28<sup>-</sup>) and CD57<sup>+</sup> T cells was observed, indicating the presence of highly differentiated cells. As shown in the table, these differences were more accentuated in group 1. On the other hand, no differences were found between acute ATL patients and the control group.



Cell surface receptors (%)	Group 1	Group 2	Group 3	Group 4
CD127	42.57 ± 3.23*	54.30 ± 8.27*	54.49 ± 7.53	74.62 ± 4.30
CD27	37.60 ± 2.77* ** ***	49.37 ± 4.16*	69.33 ± 1.74	80.60 ± 4.39
CD28	33.17 ± 4.24* ** ***	57.44 ± 6.50	62.24 ± 8.48	67.25 ± 7.34
CD57	48.49 ± 2.21* ***	43.76 ± 7.72*	32.31 ± 3.25	17.65 ± 5.20
CD27+, CD28+	27.50 ± 3.68*	38.22 ± 5.63*		62.92 ± 6.63
CD27-, CD28-	54.25 ± 2.41* **	31.66 ± 4.43		16.98 ± 4.60

Mean ± Standard Error. Statistical differences (p<0.05) between patient groups (1,2,3) and control group (4) \*; between group 1 and 2 \*\*; between group 1 and 3 \*\*\*.

[T cell memory receptors within CD8+ T cell subset]

**Conclusions:** The differences found between patients suffering acute and chronic ATL infection, suggest that parasite persistence could be responsible for the appearance of highly differentiated CD8+ T cells. Slightly lower percentages of these cells found in patients that present relapses after treatment could be the result of treatment that could transitorily reduce the parasitic load. Infection duration, parasitic load and therapy administration seem to be highly responsible for the appearance of distinct peripheral memory T cell subsets.

PB03/59 **DISTINCT BIFIDOBACTERIUM STRAINS ELICIT DIVERGENT T HELPER IMMUNE RESPONSES**

P. López<sup>1,2</sup>, M. Gueimonde<sup>2</sup>, A. Margolles<sup>2</sup>, A. Suárez<sup>1</sup>  
<sup>1</sup>University of Oviedo, Department of Functional Biology, Immunology Area, Oviedo, Spain, <sup>2</sup>Instituto de Productos Lácteos de Asturias (CSIC), Department of Microbiology and Biochemistry of Dairy Products, Villaviciosa, Spain

**Aims:** To evaluate the specific immune activation properties of different *Bifidobacterium* strains, some of the most relevant intestinal microorganisms.  
**Methods:** We examined the effect of 12 *Bifidobacterium* strains belonging to 4 different species, *Bifidobacterium longum*, *Bifidobacterium breve*, *Bifidobacterium bifidum* and *Bifidobacterium animalis* subsp. *lactis*, on the maturation pattern of human monocyte-derived dendritic cells (DC), as well as in their ability to induce cytokine secretion. In addition, we determined peripheral blood mononuclear cell (PBMC) proliferation and cytokine expression after exposure to bacterial strains.  
**Results:** All bifidobacteria tested were able to induce full DC maturation but showed striking differences in the levels of cytokine production, especially IL-12, IL-10, TNFα and IL-1β, suggesting that specific cytokine ratios could be used to predict the type of Th response that they may promote. In fact, analysis of cytokine production by PBMC showed that most of the tested *B. animalis* and *B. longum* strains induced the secretion of large amounts of IFNγ and TNFα, in agreement with the Th1 profile suggested by DC cytokine production. Remarkably, three of four *B. bifidum* strains induced poor secretion of these cytokines and significant amounts of IL-17, the main product of Th17 cells, in accordance with the high IL-1β/IL-12 ratio observed after DC stimulation.  
**Conclusions:** This work shows a species and strain-specific immune effects of bifidobacteria and describes a valuable method for screening possible probiotic strains with different immunomodulatory properties, which could be appropriate for clinical or biotechnological applications.

PB03/60 **HUMAN CONCEPTION DISORDERS: AN IMMUNOLOGICAL PERSPECTIVE STUDY**

H.M. Abdalla<sup>1</sup>  
<sup>1</sup>Institute of Endemic Diseases, University of Khartoum, Department of Immunology and Clinical Pathology, Khartoum, Sudan

**Background:** Pregnancy is the result of the fusion of two gametes (spermatocytes and oocytes) into a zygote which contains both maternal and paternal human leukocyte antigen. Unexplained infertility, recurrent miscarriages, premature delivery and pregnancy induced hypertension may, in some cases, be linked to immune and cytokine networks of early pregnancy. Understanding these cytokines and their actions could be the new area of unexplained conception disorders treatment for future.  
**Objective:** To compare the cytokine profiles (INF-γ and IL-4) of Peripheral Blood Mononuclear Cells from pregnant women with a normal obstetric history and women with conception disorders) non pregnant women and women with recurrent miscarriage).  
**Methods:** In this study 25 women with history of unexplained conception disorders (15 non pregnant women, 10 women with recurrent miscarriage) were compared with 75 pregnant women as controls. Blood sample and peripheral blood mononuclear cells were stimulated by phytohaemagglutinin and trophoblast antigen and secreted cytokines  
**Results:** There would be higher significant concentration of INF-γ cytokine in the women with the history of unexplained conception disorders compared with those who had normal pregnancy (p=0.00), in contrast, higher significant concentration of IL-4 cytokine in women who had normal pregnancy compared with those who had a conception disorders (p=0.04).  
**Conclusions:** we concluded that women with conception disorders show predominantly th1 pattern cytokines response in PBMCs when stimulated with trophoblast antigen compared to pregnant women.

PB03/61 **MAPPING OF T-CELL SUBSETS, TH1, TH2, TH17 AND TREG, IN RELATION TO DISEASE COURSE IN EXPERIMENTAL BORRELIA BURGDORFERI INFECTION**

L. Fryland<sup>1</sup>, S. Bergström<sup>2</sup>, P. Hultman<sup>3</sup>, C. Ekerfelt<sup>1</sup>  
<sup>1</sup>Linköping University, Department of Clinical and Experimental Medicine, Division of Clinical Immunology, Linköping, Sweden, <sup>2</sup>Umeå University, Department of Microbiology, Umeå, Sweden, <sup>3</sup>Linköping University, Department of Clinical and Experimental Medicine, Division of Molecular and Immunological Pathology, Linköping, Sweden

**Objectives:** The outcome of *Borrelia burgdorferi* infection can differ between individuals, from an asymptomatic infection to a stage with persistent symptoms despite antibiotic treatment. The mechanisms behind the different outcomes of Lyme borreliosis are unknown but we have previously shown the importance of a functional Th1-response for the optimal eradication of *Borrelia burgdorferi* in murine *Borrelia* arthritis, using a subtoxic dose of HgCl<sub>2</sub> for experimental Th2-deviation.  
The current study aimed to increase the understanding of the interplay between different T-cell subsets in relation to disease course, by studying markers for Th1, Th2, Th17, cytotoxic cells and Treg present during the course of experimental *Borrelia burgdorferi* infection.  
**Methods:** Forty-nine C3H/HeN mice were divided into three groups: *Borrelia*-infected, *Borrelia*-infected exposed to subtoxic doses of HgCl<sub>2</sub> and untreated controls. Mice were sacrificed on day 15, 28 and 43 post-*Borrelia* inoculation. Joint inflammation was evaluated by hind tibiotarsal joint swelling and histological studies on immune cell infiltration. Spirochete burden was analysed by RT-PCR of urinary bladder and culturing of ear biopsies. Ingual lymph node mRNA expression of different T-cell subset markers were analysed with real-time RT-PCR. The mRNA expression was analysed with the non-parametric tests Mann-Whitney, Friedman and Wilcoxon, the latter as a *post-hoc* test, while clinical signs of disease were analysed with Mann-Whitney.  
**Results:** Experimentally immune-deviated *Borrelia*-infected mice showed increased joint swelling (p=0.007 on day 28 p.i.) and delayed clearance of spirochetes (p=0.007 on day 43 p.i.). Unexposed *Borrelia*-infected mice showed increased mRNA expression of IL-10 (anti-inflammatory/Th2) and EBI-3 (Treg) early in infection compared to later stages (p≤0.031 for both markers). Immune-deviated *Borrelia*-infected mice showed trends for increased mRNA expression of GM-CSF (associated with cytotoxicity), IL-12p35 (Th1-deviating or associated with Treg) and Foxp3 (Treg) late in infection, compared to unexposed *Borrelia*-infected mice, whereas the latter showed a trend for increased expression of IL-12p40, which as a homodimer is anti-inflammatory.  
**Conclusion:** The results indicate that a beneficial outcome of *Borrelia* infection is associated with a rapid pro-inflammatory response and a rapid dampening of this. Increased symptoms and delayed eradication of spirochetes is associated with still ongoing pro- and anti-inflammatory responses late in infection.

PB03/62 **CHANGES IN THE LYMPHOCYTE RECEPTOR EXPRESSION AFTER PRENATAL AND POSTNATAL EXPOSITION TO POLYCHLORINATED BIPHENYLS**

E. Jahnova<sup>1</sup>, M. Horvathova<sup>1</sup>, T. Nemessanyi<sup>1</sup>, L. Palkovicova<sup>2</sup>, T. Jusko<sup>2</sup>, A. Kocan<sup>3</sup>, T. Trnovec<sup>3</sup>, I. Hertz-Picciotto<sup>4</sup>  
<sup>1</sup>Slovak Medical University, Department of Immunology and Immunotoxicology, Bratislava, Slovakia, <sup>2</sup>Slovak Medical University, Department of Environmental Medicine, Bratislava, Slovakia, <sup>3</sup>Slovak Medical University, Department of Toxic Organic Pollutants, Bratislava, Slovakia, <sup>4</sup>University of California, Department of Public Health Sciences, Davis, United States

**Objectives:** Prenatal and postnatal exposure to persistent organochlorine pollutants, such as polychlorinated biphenyls (PCBs), has been implicated as a possible cause of impaired immune function in children. Prenatal PCB exposition is connected to the incidence of infectious and allergic diseases in children and humoral immunity changes.  
**Methods:** We collected blood samples from umbilical vein after delivery (n = 350), subsequently from the children aged 6 (n = 350) and 16 months (n = 313). The cell surface receptors were analyzed by the multi-color immunophenotyping using monoclonal antibodies CD19-PC7, CD3-FITC/(CD56+16)-PE, CD4-PC7, CD8-PC5, HLADR-ECD, CD83-PC5, CD11c-PE, CD11b-FITC, CD45RO-ECD, CD45RA-PE, CD62L-PC5, CD25-FITC (Beckman Coulter).  
**Results:** The positive correlation was between prenatal PCBs exposition and frequency of B cells (CD19+), activated B cells (HLADR+CD19+), macrophage-like cells (CD19-CD11c-CD11b+) in umbilical cord, further CD19+, CD19-CD11c-CD11b+, and memory CD4 T cells (CD4+CD45RO+CD45RA-) in 6- and 16-months

old children. The negative association ( $P < 0,05$ ;  $P < 0,01$ ) has been found in naïve/resting T cells (CD4+CD45RO-CD45RA+) and terminally differentiated effector memory (TEM) T cell population (CD4+CD62L-CD45RA+). The percentage of CD19+, HLADR+CD19+, CD3+, CD8+ T-lymphocytes, and lymphoid dendritic cells (CD19-CD11c+CD11b-) was significantly raised after 6- and 16-months. CD4+ T lymphocytes, natural regulatory T cells (CD4+CD25+), suppressor inducer T cells (CD4+CD62L+) and truly naïve helper/inducer T cells (CD4+CD62L+CD45RA+), natural killer cells (CD3-CD56+CD16+) and myeloid dendritic cells (CD19-CD11c+CD11b+) were decreased ( $P < 0,05$ ;  $P < 0,0001$ ). We found significantly higher expression of memory and TEM CD4+ T cells in the both groups of children, 6- and 16-months, and CD4+CD45RO-CD45RA+ cells at 6-months. The differences in dendritic cells (CD83+CD19+) and CD19-CD11c-CD11b+ cells were not statistically significant.

**Conclusions:** In the present study we demonstrated the correlation between prenatal PCBs exposition and cell surface receptor expression in the cord blood, 6- and 16-months children. We have described a number of affected lymphocytes populations belonging to main lymphocyte subpopulations, dendritic cells, macrophage-like cells and T cells. The continual investigation of PCBs-induced immune dysfunction, cellular and molecular pathways profiling, will enhance our understanding of the individual and population health.

This work was supported by the grant MZ SR 2005/31-SZU-09

### PB03/63 IL-17 RESPONSE IS HYPERACTIVE IN BEHÇET'S DISEASE PATIENTS

N. Sayim<sup>1</sup>, E. Alpoş<sup>2</sup>, O. Yegin<sup>1</sup>

<sup>1</sup>Akdeniz University, Pediatric Immunology, Antalya, Turkey, <sup>2</sup>Akdeniz University, Dermatology, Antalya, Turkey

Behçet's Disease (BD) is a multisystemic autoimmune/inflammatory disease, vasculitic lesions attacks mucocutaneous sites eye, joint and central nervous system. Although neutrophil hyperactivity and abnormalities in immune regulation have been shown in BD, the exact pathogenesis is still unclear. Th17 cells and IL-17 have been shown to play important roles in many autoimmune diseases such as Rheumatoid arthritis, Multiple Sclerosis and Uveitis. The aims of this study were to investigate the IL-17 serum levels and IL-17 response of BD patients.

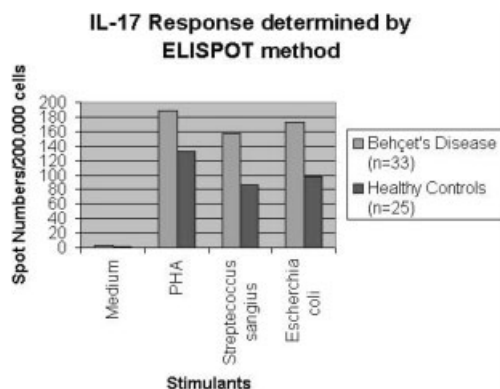
Serum IL-17 levels were determined in 45 BD patients with different clinical spectrum and active or inactive period and 33 healthy controls with ELISA method. IL-17 response of mononuclear cells to Phytohemagglutinin, Streptococcus sanguis and Escherichia coli extracts were evaluated by ELISPOT assay in 33 BD patients and compared with 25 sex and age matched healthy controls.

Serum IL-17 levels were found to be significantly increased in BD patients in active period when compared with the healthy controls, but no difference could be detected during inactive period (Table-1).

Groups	Behçet's Disease (BD)	Healthy Controls (HC)	p Values
Total BD n=45 HC n=33	20,2	11,8	<0.05
Inactive BD n=22	11,2		>0.05
Active BD n=23	28,7		<0.01
BD with Uveitis n=18	24,4		<0.05
BD with active Uveitis n=8	29,8		<0.01
BD with inactive Uveitis n=10	11,3		>0.05

[IL-17 serum levels (pg/dl) in Behçet's Di]

IL-17 response of BD patients were significantly higher than the controls when tested by ELISPOT method (Figure -1).



[IL-17 Response determined by ELISPOT]

Our results indicate that IL-17 may have a role in the pathogenesis of BD and these patients have a hyperactive IL-17 response to stimulation.

### PB03/64 PREDICTION OF BILIARY COMPLICATION AFTER LIVER TRANSPLANTATION BASED ON CYTOKINE PROFILE IN SERUM

J.H. Kim<sup>1</sup>, S.-Y. Lee<sup>2</sup>, E.-S. Kang<sup>2</sup>, J.B. Park<sup>3</sup>, C.H. Kwon<sup>3</sup>, S.-J. Kim<sup>3</sup>, J.-W. Joh<sup>3</sup>, S.-K. Lee<sup>3</sup>

<sup>1</sup>Samsung Medical Center, Department of Laboratory Medicine, Masan, Korea, Republic of, <sup>2</sup>Samsung Medical Center, Sungkyunkwan University School of Medicine, Department of Laboratory Medicine and Genetics, Seoul, Korea, Republic of, <sup>3</sup>Samsung Medical Center, Sungkyunkwan University School of Medicine, Department of Transplantation Surgery, Seoul, Korea, Republic of

**Background:** Biliary complications like as biliary strictures and bile leakages are the major concern associated with living donor liver transplantation (LDLT). Although radiologic evaluations are used for diagnostic tools, discriminative biochemical tests are insufficient. This study aims to evaluate the pattern of cytokine secretion in LDLT recipients with post-transplant biliary complication, which can explain the status of immunological activation of the patient and its clinical significance.

**Method:** Sera from 16 recipients who underwent LDLT were obtained on the pre and post transplantation day 3, 7, 15 and 30 and are subjected to multiplex bead flow cytometric cytokine analysis. Statistical analysis using Mann Whitney U test was performed to compare the mean values of cytokine level between the groups without and with biliary complications.

**Results:** Among sixteen, 5 developed biliary complications within 3 months of LTLD. Mean concentrations of IFN-gamma, TNF-alpha, IL-2, IL-12, IL-4 and IL-10 was higher on all estimated days, and IL-8 was higher in no complication group. On post operation day 7 the complication groups The patients with biliary complications had significantly increased level of Th1 and Th2 cytokine levels compared to patients without complications. Mean concentration of IFN-gamma, TNF-alpha, IL-2, IL-12, IL-4 and IL-10 The mean cytokine INF gamma mean cytokine level on post transplantation day 7 complication vs no complication group; TNF-alpha = 244.5 vs 6.3,  $p = .009$ ; IL-2 = 477.7 vs 70.8,  $p = .019$ ; IL-12 = 361.9 vs 2.1,  $p = .001$  and Th2 cytokines (mean cytokine level on post transplantation day 7; IL-4 = 504.1 vs 10.5,  $p = .019$ ) compared to patients without complications. However, there were no significant differences in the concentrations of IL-6 and IL-8 between two groups (proinflammatory cytokine IL-6 = 9.5 vs 2.3,  $p > 0.05$ ; IL-8 = 52.5 vs 81.4,  $p > 0.05$ ).

**Conclusion:** The level of serum cytokine during the first month of LDLT recipients may provide important information on the prediction and diagnosis of biliary complications which develop within 3 months after LDLT.

### PB03/65 THE ANSWER OF PERIPHERAL LYMPHOCYTES TO IONIZING IRRADIATION

L. Zárybníková<sup>1</sup>, Z. Šinkorová<sup>1</sup>, T. Iveta<sup>1</sup>

<sup>1</sup>University of Defence, Faculty of Military Sciences, Department of radiobiology, Hradec Králové, Czech Republic

**Objective:** Ionizing irradiation is external stress stimulator which causes in organism after *in vivo* irradiation reaction of the immune system. The system answers to irradiation by increasing the number of peripheral blood granulocytes, which are relative radioresistant. On the other hand, peripheral lymphocytes belong to radiosensitive cells, which due to irradiation tend toward inducing apoptosis. In our study we aimed to population of natural killers (NK cells) and T-lymphocytes,

especially to radiosensitivity of CD8<sup>+</sup> or CD8<sup>+</sup> subpopulations. We also demonstrated differences between *in vivo* and *in vitro* experimental design.

**Methods:** *In vivo*: experimental model of white large pig was whole-body irradiated by cobalt gamma emitter 0–2–4–6 and 10 Gy. Porcine peripheral blood was collected 1, 8, 24 and 48 hours after irradiation to heparinized tubes and analysed immediately after that. *In vitro*: jugular peripheral blood from non-irradiated animal was collected, transferred into 24-well polystyrene cultivation plates, irradiated by 0–0.5–1–1.5–2–4–6–8 and 10 Gy and cultivated up to the time of analyse (3, 8, 24 and 48 hours). For visualizing lymphocyte subpopulations we choose indirect double color surface immunostaining using mouse monoclonal antibodies (anti-CD3, anti-CD8) visualized by secondary anti-mouse antibodies conjugated with fluoresceins FITC and PE. The relative representation of each subpopulation was analysed by flow cytometry.

**Results:** Subpopulation of CD3<sup>+</sup>CD8<sup>+</sup> T lymphocytes did not embodied extensive changes of their representation, depending on the irradiation. Number of CD3<sup>+</sup>CD8<sup>+</sup> T cells declined 8 hours after irradiation by 4 Gy and higher, and at 48 hours they reached 20–50% of their origin values. Irradiation by 2 Gy caused at 8 hours increase to 130% which later fell down. NK cells (CD3<sup>+</sup>CD8<sup>+</sup>) increased 8 hours after irradiation to 180–300% for all tested dose of irradiation.

**Conclusions:** Only subpopulation of T lymphocytes (CD3<sup>+</sup>CD8<sup>+</sup>) is radiosensitive and decline depends on the absorbed dose and time of analyse. Documented trends of changes were similar for *in vivo* as so as *in vitro* design, nevertheless *in vitro* set in later.

#### PB03/66 EXPRESSION OF CD45 ISOFORMS IN A MULTIPLE MYELOMA PATIENT WITH A C77T SUBSTITUTION IN EXON 4 OF THE CD45 GENE

J. Gil<sup>1</sup>, A. Diaz-Alderete<sup>1</sup>, B. Jimenez<sup>2</sup>, E. Fernández-Cruz<sup>1</sup>, P. Sabín<sup>3</sup>

<sup>1</sup>Hospital General Universitario 'Gregorio Marañón', Immunology, Madrid, Spain, <sup>2</sup>Hospital General Universitario 'Gregorio Marañón', Occupational Medicine, Madrid, Spain, <sup>3</sup>Hospital General Universitario 'Gregorio Marañón', Oncology, Madrid, Spain

**Background and objectives:** Alternative splicing is very frequent in the immune system, where functional diversity is particularly important. CD45 is a transmembrane phosphatase whose activity is crucial for T-cell development and signalling. In circulating lymphocytes, the transition from naive to activated and memory T cells is marked by alternative splicing of CD45 pre-mRNA with exclusion of exons 4 (A), 5 (B), and 6 (C). A human polymorphism -the C to G transversion (C77G) in the exonic splicing silencer (ESS1) of exon 4 of CD45- disrupts this splicing, and the high-molecular-weight isoform CD45RA is not effectively silenced in memory T cells. Moreover, C77G has been associated with susceptibility to human immune-based diseases and viral infections.

A different nucleotide transition at the same position, the C77T genotype, was first described in 2003 during a study on the association between C77G and multiple sclerosis. To date, no further C77T descriptions have been reported in patients or healthy individuals, and the effect of this synonymous substitution (Pro57Pro) at the splicing and functional level remains unknown; our aim was to study the surface CD45 expression profile correlating with C77T genotype.

**Methods:** Genomic DNA was obtained and, following PCR with primers 5'-GACTACAGCAAGATGCCAGTG-3' and 5'-GGGATACTGGGTGGAAGTA-3', the PCR products were purified and sequenced. Whole peripheral blood was incubated with fluorochrome-conjugated monoclonal antibodies, lysed, washed, and analysed by flow cytometry (BD Biosciences).

**Results:** During screening for C77G in a series of patients with immune disorders, we found the heterozygous substitution C77T in a patient with multiple myeloma. This patient's circulating lymphocytes included all CD45RA+CD45RO- (34%), CD45RA+CD45RO+ (30%), and CD45RA-CD45RO+ (34%) subsets; these cells did not show the variant pattern of coexpression caused by C77G.

**Conclusion:** This is the second report of the C77T human genotype. We conclude that this change in ESS1 of exon 4 does not have the same effect on splicing regulation (i.e., inhibition of the function of the splicing silencer) and CD45 isoform expression as C77G. CD45 is an excellent model for understanding the mechanism and regulation of alternative splicing events. The functional implications of the splicing program in this and other genes deserve further study.

PB03/67 Abstract withdrawn by author

#### PB03/68 THE LYMPHOCYTES PATTERN IN HEREDITARY ANGIOEDEMA – A FAMILY STUDY

S.I. Radesi<sup>1</sup>, H. Bumbea<sup>1</sup>, M. Zamfirescu<sup>2</sup>, P. Leru<sup>3</sup>, A.M. Vladareanu<sup>1</sup>

<sup>1</sup>University Emergency Hospital Bucharest, Hematology, Bucharest, Romania, <sup>2</sup>Medlife Hyperclinic, Alergology and Clinical Immunology, Bucharest, Romania, <sup>3</sup>Colentina Clinical Hospital, Bucharest, Romania

**Objective:** Identify possible lymphocyte immunophenotyping specificity in a family with hereditary angioedema (HAE) who developed systemic lupus erythematosus (SLE) in one of its members.

**Methods:** Three generations familial case history was completed, followed by immunological screening to confirm the diagnosis. Peripheral blood immunophenotyping was performed for the following surface lymphocytic antigens: 1) CD4, CD8, CD3, CD 28, CD 45 RO, CD 25 for the T cells, 2) CD19, CD38, CD5 for the B line; 3) CD16, CD56 for the NK cells. The obtained data were compared with normal values from the literature and from 6 healthy adult controls.

**Results:** Clinical and biological investigations showed:

1. A 40 year (y) old male diagnosed with a progressive SLE with cutaneous, hematological and renal involvement 10y before has a history of abdominal pain and several episodes of facial, peripheral and laryngeal angioedema (AE) and a 33% C1 esterase activity.
2. His sons: A 10y boy with two episodes of intestinal occlusion, operated in infancy (8 and 14 months) has 26% C1 esterase activity. His 7y brother has a 4 years history of recurrent abdominal pain, peripheral and facial AE, with two episodes of spontaneously remissive laryngeal AE and a C1 esterase activity of 31%.
3. The paternal grandfather and his sister had similar history of repeated AE and died at middle age.

The immunophenotyping showed a higher number of T cells in all cases (>1800cells/dl) with an increased percent of double negative T cells (CD4-, CD8-) in children (11.04% and 17.71%) and an elevated percent of CD8+Tcells in adult (49.23%). The number of CD4+CD25+Tcells was also raised in all cases (143.5-369.6cells/dl), higher in children than in adult and in parallel progression with the worsening of their clinical status.

**Conclusion:** The HAE patients might have associated T cells dysfunctions that facilitate the clinical burst.

#### PB03/69 ORGAN-SPECIFIC HUMAN CD8+ T CELL DIFFERENTIATION IN HUMANS

B. Bengsch<sup>1</sup>, M. Kuntz<sup>1</sup>, B. Seigel<sup>1</sup>, H.E. Blum<sup>1</sup>, R. Thimme<sup>1</sup>

<sup>1</sup>Uniklinik Freiburg, Medizinische Klinik Abt. II Gastroenterologie, Freiburg, Germany

CD8+ T cells are essential antiviral effector cells of the adaptive immune system.

Recent phenotypic, functional, and gene-expression analyses have demonstrated that the differentiation stage of human peripheral CD8+ T cells can be defined by a specific combination of markers, such as CD27, CCR7 and CD45RA. In this study, we investigated whether and to what extent CD8+ T cell differentiation is influenced by organ-specific factors. CD8+ T cells were derived from blood, tonsillar tissue (lymphatic environment) and liver tissue (peripheral organ). The phenotypic analysis of CD8+ T cells in these different body compartments revealed that the distribution of CD8+ T cell differentiation subsets varies significantly. Indeed, intrahepatic CD8+ T cells display more progressed differentiation stages compared to the blood and the tonsillar tissue.

CD8+ T cells derived from tonsillar tissue display a predominantly early or naive differentiation stage.

In sum, our results indicate that organ-specific factors determine the composition of the organ-resident CD8+ T cell pool. These findings most likely reflect distinct immunological properties of the specific organs.

#### PB03/70 IMMUNE CELL PHENOTYPING AFTER SALMONELLA TYPHIMURIUM INFECTION IN MURINE EXPERIMENTAL MODELS

A.D. Iancu<sup>1</sup>, C. Dinu<sup>2</sup>, M. Neagu<sup>3</sup>, D.L. Radu<sup>1</sup>

<sup>1</sup>"Cantacuzino" National Institute of Research-Development for Microbiology and Immunology, Cellular Immunity, Bucharest, Romania, <sup>2</sup>"Cantacuzino" National Institute of Research-Development for Microbiology and Immunology, Typhoid Vaccine, Bucharest, Romania, <sup>3</sup>"Victor Babes" National Institute of Pathology and Biomedical Sciences, Immunology, Bucharest, Romania

**Objectives:** Natural or experimental infections with pathogenic bacteria are known to induce humoral and cell-mediated host immunity. *Salmonella typhimurium* is a Gram-negative bacterium which multiplies in the gastrointestinal tract of many animal species where it usually causes no disease, but in humans its growth causes gastroenteritis. *Salmonella typhimurium* infections in mice can be induced both naturally and experimentally and cause various degrees of diarrhea. The aim of this study was to determine the values of peripheral and spleen lymphocyte populations in normal conditions and after being infected by *Salmonella typhimurium*.

**Methods:** In order to establish the percentage of B, T and NK cell sets from peripheral blood, we used murine monoclonal antibody as follows: PerCP-CD45R/B220, PerCP-CD3, PE-CD4, PE-CD8 and PE-NK1.1. Cell populations were investigated on the BD FACSCanto II system built with blue (488 nm, air-cooled, 20 mW solid state) and red (633 nm, 17 mW HeNe) excitation sources and analyzed using BD FACSDiva software. Mice strains used in the experiments were BALB/c and C57/bl mice, in different experimental conditions: SPF and conventional strains. *S. typhimurium* infections were achieved by intraperitoneal inoculation with 10 germs/ 0.5 mL/ mouse.

**Results:** In peripheral blood, conventional and SPF Balb/c mice had higher percentage of CD4+ cells compared to both variants of C57/bl mice; whereas conventional and SPF C57/bl mice had higher percentage of B220+ cells in comparison to Balb/c mice. In conventional and SPF Balb/c and C57/bl strains lymphocyte populations isolated from spleen and peripheral blood showed an increasing tendency of NK1.1 cells after infection. B220 cells isolated from spleen in both SPF mice strains decreased after *Salmonella typhimurium* infection. The percentage of lymphocyte populations isolated from peripheral blood in conventional Balb/c and C57/bl mice strains showed a decreasing tendency after infection.

**Conclusion:** After infection of conventional and SPF Balb/c and C57/bl strains with *Salmonella typhimurium* lymphocyte populations isolated from spleen and peripheral blood tend to decrease.

This study was part of a project supported by NATO Project SfP 982838/2007



**PB03/71 ROLE OF TH1 AND TH2 CYTOKINES FOR TH17 CELL DEVELOPMENT IN A MONOCYTE-MEMORY T CELL CO-CULTURE SYSTEM**N. Fischer<sup>1</sup>, U. Holzer<sup>1</sup><sup>1</sup>University of Tuebingen, Children's Hospital, Tuebingen, Germany

**Objectives:** The aim of this study was to investigate the optimal induction conditions for Th17 cell development from human CD4<sup>+</sup> T cells in the presence of monocytes. Induction of IL17<sup>+</sup> cells from naive and memory CD4<sup>+</sup> T cells was compared. Additionally the influence of Th1-associated cytokines IFN- $\gamma$  and IL-2 and Th2 cytokine IL-4 on monocyte-induced Th17 cell development from memory CD4<sup>+</sup> T cells was investigated.

**Methods:** Human naive or memory CD4<sup>+</sup> T cells were co-cultured with monocytes in the presence of anti-CD3 mAb for 5 days. In a monocyte-free stimulation setup, T cells were cultured in the presence of anti-CD3 mAb or anti-CD3 and anti-CD28 mAbs. Optional IL-2 was added after 24 h. The monocyte-memory CD4<sup>+</sup> T cell co-cultures were additionally stimulated in the presence or absence of IL-4 and anti-IFN- $\gamma$  mAb. After 5 days, cells were stimulated for 4–6 hours with PMA and ionomycin in the presence of monensin. For flow cytometric analysis of Th17 cell induction, cells were harvested, stained for cell surface markers, fixed, permeabilized, and stained with anti-IL-17 antibody.

**Results:** Stimulation of naive CD4<sup>+</sup> T cells in the presence or absence of monocytes did not result in a significant fraction of IL-17-producing cells. In the monocyte-free stimulation of memory CD4<sup>+</sup> T cells, the highest percentage of IL-17 producing cells could be found by stimulation with anti-CD3 mAb and IL-2. The highest percentage of Th17 cells was found after monocyte-memory CD4<sup>+</sup> T cell co-culture in the presence of anti-CD3 mAb at a monocyte:T cell ratio of 1:4. Addition of IL-2 to monocyte-memory T cell co-cultures led to a decrease of IL-17<sup>+</sup> cells. Furthermore, neutralization of Th1 cytokine IFN- $\gamma$  resulted in an enhanced development of Th17 cells, while the Th2 cytokine IL-4 was found to potentially inhibit monocyte-induced differentiation of Th17 cells.

**Conclusion:** Our findings highlight the crucial role of monocytes in the development of human Th17 cells from TCR-activated memory CD4<sup>+</sup> T cells. Furthermore, we demonstrate that the hallmark effector molecules of Th1 and Th2 cells, IFN- $\gamma$  and IL-4, as well as IL-2 negatively regulate the generation of human Th17 cells.

**PB03/72 DISSECTION OF THE FACTORS CONTRIBUTING TO PROTECTIVE IMMUNE RESPONSE TO FLU VACCINE IN MICE**V. Ronconi<sup>1</sup>, S. Crotta<sup>1</sup>, M. Taccone<sup>1</sup>, B. Baudner<sup>1</sup>, D. O'Hagan<sup>1</sup>, A. Wack<sup>1</sup><sup>1</sup>Novartis Vaccines and Diagnostics, Siena, Italy

Influenza is still a significant cause of morbidity and mortality, with seasonal epidemics occurring worldwide. Therefore, improved vaccines that induce a broader and more potent immune response are needed to provide protection to the populations most at risk.

Formulation of vaccines with potent adjuvants is an attractive approach for enhancing the performance of vaccines composed of subunit antigens and offers the opportunity to drive the immune response into a desired Th profile, by choosing the appropriate adjuvant combinations. In the present study we investigated the different types of immune responses induced, Th1 and Th2, for their ability to confer protection against viral infection.

The MF59 adjuvant increases humoral and cellular immune responses to Flu antigens, inducing Th2-associated IgG subclasses and cytokines. The addition to MF59 of CpG oligonucleotides does not lead to a further increase of the immune response, but modifies it towards a Th1 biased profile as well as the infection with sublethal doses of influenza virus. Both Th1 and Th2 immune responses induce effective neutralizing antibody titers, as demonstrated by passive immunization experiments with sera from vaccinated or pre-exposed mice into naïve recipients. In addition, they provide protection from lethal challenge with homologous influenza virus. To investigate the role of Th1- and Th2-induced cells, we are developing an adoptive transfer model of Flu-specific CD4 T cells into naïve mice. This model will allow to assess either direct protective effector functions and whether T cell help is a limiting factor for the extent and quality of vaccine induced antibody responses.

Our investigations have significant implications for the development of new and improved Flu vaccines against pandemic and inter-pandemic influenza virus strains. They offer the opportunity to establish which type of immune response is more effective in the protection against viral infection and open the possibility to drive it into a desired direction by choosing appropriate adjuvants or combinations thereof.

**PB03/73 CELLULAR IMMUNE PARAMETERS ASSOCIATED WITH PREVENTION OF ADENOVIRUS-ASSOCIATED COMPLICATIONS IN IMMUNOCOMPETENT AND TRANSPLANTED CHILDREN**V. Guérin<sup>1</sup>, J.-H. Dalle<sup>1</sup>, B. Pédrón<sup>1</sup>, K. Yakouben<sup>1</sup>, D. Jorge Cordeiro<sup>1</sup>, L. Peltier<sup>1</sup>, M. Ouachée-Chardin<sup>1</sup>, A. Baruchel<sup>1</sup>, G. Sterkers<sup>1</sup><sup>1</sup>Robert Debré Hospital – Assistance Publique des Hôpitaux de Paris, Paris, France

**Objective:** The functions of virus-specific CD4 T-cells that are associated with protection remain to be clarified.

**Methods:** Here, we analysed adenovirus (ADV)-specific immune responses in 27 healthy children (median age: 5.30 years), 24 well-being pediatric patients sampled at 1 year after hematopoietic-stem cell transplantation (median age: 9.09 years) and 31 recipients (median age: 8.31 years) who were sampled at months 1 to 6 post-HSCT and did not evidence AdV DNAemia. Quantification of proliferative- and IFN $\gamma$ -responses to AdV-lysate were performed in all individuals and of IL2-responses in part of them.

**Results:** Results showed similar percentages of AdV-specific CD4 T-cells secreting IFN $\gamma$  in more than 2 years of age healthy children (median: 0.14%), 1 year post-HSCT recipients (0.18%) and healthy adults (0.15%). Furthermore, IFN $\gamma$ -, IL2- and proliferative-responses were highly correlated in these 3 populations. In contrast, IFN $\gamma$ -responses preceded the development of proliferative-responses and consequently were isolated at the earliest-time post-HSCT. More strikingly, a dissociation between IL2-responses and proliferative-responses could also be observed at early time post-HSCT.

**Conclusion:** This study provides new insight into quantitative and qualitative immune parameters associated with prevention of AdV-associated complications in children more than 2 years of age and indicates that CD4-memory functions are even more complex than currently appreciated.

**PB03/74 INDUCTION OF CYTOLYTIC CD4+ T CELLS TO SOLUBLE ANTIGENS AND THEIR POTENTIAL FOR ANTIGEN-SPECIFIC IMMUNOSUPPRESSION**J.-M. Saint-Remy<sup>1</sup><sup>1</sup>Catholic University of Leuven, CMVB, Leuven, Belgium

Cytolytic CD4+ T cells have been described occasionally in various settings, in particular during the course of viral infections, but their precise role and the mechanism by which they are elicited are poorly understood.

We have designed a methodology by which virtually any class II restricted T cell epitope can activate and profoundly alter the phenotype and function of CD4+ T cells, even when such cells are already polarized into Th1, Th2 or Th17 effector cells.

This methodology relies on addition to the T cell epitope of a consensus motif containing a thiooxidoreductase activity, so that the motif is located outside of the MHC class II binding cleft. This allows the reduction of disulfide bridges of T cell surface proteins with as consequence a prolongation of the synapse with the APC. This triggers a profound and stable suppression of IL-2 transcription, increased production of IFN- $\gamma$ , activation of the transcription of cytolytic proteins including granzymes and soluble FasL, and surface expression of NKG2D and its adaptor molecule DAP10. Overall, CD4+ T cells acquire the capacity to induce apoptosis of APC and of bystander effector CD4+ T cells.

Preclinical evidence of efficacy has now been established in 3 different animal models, allergic asthma, EAE as a model of multiple sclerosis and tolerance to graft. Active immunization with thiooxidoreductase motif-containing T cell epitopes in adjuvant, or passive transfer of in vitro expanded CD4+ T cells show highly significant efficacy in these 3 models.

Induction of apoptosis of APC and of bystander T cells by antigen-specific CD4+ T cells purposely transformed into potent cytolytic cells could represent a novel strategy to eliminate unwanted immune responses.

**PB03/75 SELF-LIMITATION OF HUMAN EFFECTOR TH17 CELL RESPONSES BY RECIPROCAL REGULATION OF IL-10 AND IL-17**C. Zielinski<sup>1</sup>, A. Lanzavecchia<sup>1</sup>, F. Sallusto<sup>1</sup><sup>1</sup>Institute for Research in Biomedicine, Bellinzona, Switzerland

T helper cells that produce IL-17 (Th17 cells) have recently emerged as a new T cell lineage. They can induce recruitment of neutrophils and trigger production of pro-inflammatory cytokines and chemokines by a broad range of cellular targets. Although these effector functions confer upon Th17 cells the ability to protect against certain extracellular bacteria and fungi, a dys-regulated Th17 response can induce severe tissue destruction and autoimmunity. Therefore, mechanisms must be in place to shield the host from immune-mediated damage.

The aim of this study was to investigate the stability and flexibility of the pro-inflammatory human Th17 subset and to identify factors that can modulate the cytokine profile of differentiated Th17 cells.

We are investigating how regulatory properties can be adopted by human Th17 cells. We found that Th17 cells can produce IL-10, an immuno-suppressive cytokine that confers regulatory functions. IL-10 can be induced in the CCR6+CCR4+ Th17 cell subset as well as in Th17 clones under certain stimulatory conditions, which is accompanied by down-regulation of IL-17. We also found that the Th17 phenotype is subject to a complex modulation by other cytokines. IL-23, for example, which accounts for pathogenicity in the EAE mouse model by IL-10 suppression, promotes the regulatory Th17 phenotype in the human system by up-regulation and maintenance of IL-10/IL-17 co-expression. This reciprocal regulation of pro- and anti-inflammatory cytokines might constitute a self-regulatory mechanism that allows for self-limitation of the inflammatory Th17 cell response.

The results of this study may have important implications for immune vaccination strategies and contribute to a better understanding of immuno-regulation in general.

**PB03/76 COMPARISON OF CELLULAR IMMUNE RECOVERY TOWARD CMV AND ADV BETWEEN RELATED AND UNRELATED PEDIATRIC HEMATOPOIETIC-STEM CELL TRANSPLANTATION**

D. Jorge Cordeiro<sup>1</sup>, V. Guérin<sup>1</sup>, J.-H. Dalle<sup>1</sup>, B. Pédrón<sup>1</sup>, M. Ouachée-Chardin<sup>1</sup>, K. Yakouben<sup>1</sup>, A. Baruchel<sup>1</sup>, G. Sterkers<sup>1</sup>
<sup>1</sup>Robert Debré Hospital, Paris, France

**Objective:** Adenovirus (ADV) and cytomegalovirus (CMV) are significant causes of morbidity and mortality within the first 3 months following hematopoietic-stem cell transplantation (HSCT) because of incomplete T-cell immune recovery. The risk factors that include unrelated and mismatched HSCT in adults are poorly known in children.

**Methods:** Here, we evaluated the recovery of ADV and CMV specific cellular immunity in pediatric recipients (n = 28, mean age = 7.83 years) related to the status of the transplantation (related donor (RD), n = 14; unrelated donor (UD), n = 14, with 10/10 HLA-matched (MUD), n = 10, and 9/10 HLA-matched (MMUD), n = 4). Immunological investigations included T-cell proliferation assays and the enumeration, by flow cytometry, of IFN-gamma secreting cells following in vitro stimulation with ADV and CMV lysate antigens.

**Results:** At M3, the results for RD, UD, MUD and MMUD were respectively: i) Incidence of cellular immune recovery: 71%, 71%, 60% and 100%. ii) Median of T-cell proliferation responses: 28152 cpm, 26378 cpm, 26378 cpm and 27880 cpm. iii) Median of IFN-gamma responses: 0.34%, 1.32%, 1.90% and 1.32%. At M1, lower incidence of ADV and CMV-specific immune recovery was observed in UD.

**Conclusion:** Genetic diversity appears to delay rather than decrease CMV or ADV specific cellular immune recovery in children.

**PB03/77 CELLULAR IMMUNE PARAMETERS ASSOCIATED WITH SPONTANEOUS CONTROL OF CYTOMEGALOVIRUS IN TRANSPLANTED CHILDREN**

V. Guérin<sup>1</sup>, J.-H. Dalle<sup>1</sup>, B. Pédrón<sup>1</sup>, M. Ouachée-Chardin<sup>1</sup>, K. Yakouben<sup>1</sup>, A. Baruchel<sup>1</sup>, G. Sterkers<sup>1</sup>
<sup>1</sup>Robert Debré Hospital – Assistance Publique des Hôpitaux de Paris, Paris, France

**Objective:** CD4<sup>+</sup> T-cells functions that best correlate with cytomegalovirus (CMV) control were evaluated.

**Methods:** Thirty children (mean age: 8.30 years) who received an allogeneic-hematopoietic stem-cell transplantation were included and relationship between CMV-infection and CMV-specific immune recovery as determined by proliferation assay and intracytoplasmic-IFNγ assay were studied.

**Results:** Thirteen recipients were seronegative before HSCT. None developed CMV infection or CMV-specific immunity. Seventeen recipients were seropositive: i) in 4 who spontaneously and permanently controlled CMV, the median of CMV-specific IFNγ-secreting CD4 T-cells was 9.13 per ml at M3 and 3 of 4 evidenced optimal proliferative-responses since M1, ii) in 10 patients who received anti-CMV chemotherapy because of prolonged viremia, lower (p=0.016) IFNγ responses (0.39 per ml) together with delayed and/or depressed proliferative responses were observed iii) finally, 1 patient with early CMV-associated disease had undetectable proliferative and IFNγ-responses until M3.

**Conclusion:** Both intense IFNγ-responses and early proliferative responses appear associated with optimal CMV-control.

**PB03/78 IL-17 AND IL-22 ARE ASSOCIATED WITH PROTECTION AGAINST HUMAN KALA AZAR CAUSED BY LEISHMANIA DONOVANI**

M.G.R. Pitta<sup>1</sup>, A. Romano<sup>1</sup>, S. Cabantous<sup>1</sup>, S. Henri<sup>1</sup>, A. Hammad<sup>2</sup>, B. Kouriba<sup>1</sup>, L. Argiro<sup>1</sup>, M. el Kheir<sup>2</sup>, B. Bucheton<sup>1</sup>, C. Mary<sup>1</sup>, S.H. El-Safi<sup>2</sup>, A. Dessein<sup>1</sup>
<sup>1</sup>Institut National de la Santé et de la Recherche Médicale, Unité 906, Marseille, France, <sup>2</sup>University of Khartoum, Faculty of Medicine, Department of Medical Microbiology & Parasitology, Khartoum, Sudan

IL-17 and IL-22 have been shown to increase protection against certain bacteria and fungal pathogens in experimental models. However, no human studies have demonstrated a crucial role of IL-17 and IL-22 in protection against infections. We have shown that *Leishmania donovani*, which can cause the lethal visceral disease Kala Azar (KA), stimulates the differentiation of Th17 cells, which produce IL-17, IL-22, and IFN-γ.

Comparisons of Th1, Th2 and Th17 cytokines in a cohort of subjects who developed KA or were protected against KA during a severe outbreak, showed IL-17 and IL-22 were strongly and independently associated with protection. Further examination indicated that a weak Th17 response was predictive of KA. Analysis of the cytokines that are required for the induction (IL-6, IL-1β) and for the maintenance (IL-23) of the Th17 response, suggests a defect in the Th17 differentiation pathway in KA subjects. Our results suggest that IL-17 and IL-22 play with Th1 cytokines, complementary roles in human immunosurveillance against KA, and that a defect in Th17 induction may increase the risk of KA.

**PB04 – T CELL HOMEOSTASIS, APOPTOSIS AND CELL SURVIVAL**
**PB04/1 BMI1 REGULATES MEMORY TH2 CELL SURVIVAL VIA REPRESSION OF THE NOXA GENE**

T. Nakayama<sup>1</sup>, M. Yamashita<sup>1</sup>
<sup>1</sup>Chiba University, Chiba, Japan

The maintenance of memory T cells is central to the establishment of immunological memory, although molecular details of the process are poorly understood. In the absence of the Polycomb group (PcG) gene Bmi1, the number of memory Th2 cells was reduced significantly. Enhanced cell death of Bmi1<sup>-/-</sup> memory Th2 cells was observed both in vivo and in vitro. Among various pro-apoptotic genes that are regulated by Bmi1, the expression of pro-apoptotic BH3 only protein Noxa was increased in Bmi1<sup>-/-</sup> effector CD4 T cells even in the absence of Ink4a and Arf. The generation of memory Th2 cells was restored by the deletion of Noxa but not by Ink4a and Arf. Direct binding of Bmi1 to the Noxa gene locus was accompanied by histone H3-K27 methylation. The recruitment of other PcG gene products to the Noxa gene was highly dependent on the expression of Bmi1. In addition, DNA methylation appeared to control the recruitment of PcG gene products and the expression levels of Noxa. Moreover, memory Th2-dependent airway inflammation was attenuated substantially in the absence of Bmi1. Thus, Bmi1 controls memory Th2 cell survival and function through the direct repression of the Noxa gene.

**PB04/2 ANALYSIS OF THE MOLECULAR CLOCK IN CD4+ T CELLS AND ITS RELEVANCE FOR THE FUNCTIONAL CIRCADIAN RHYTHM OF CD4+ T CELLS**

T. Bollinger<sup>1</sup>, A. Leutz<sup>1</sup>, J. Kovac<sup>2</sup>, H. Oster<sup>2</sup>, T. Lange<sup>3</sup>, C. Benedict<sup>3</sup>, W. Solbach<sup>1</sup>
<sup>1</sup>Medical Microbiology and Hygiene, University of Luebeck, Luebeck, Germany, <sup>2</sup>Circadian Rhythm Group, Max Planck Institute for Biophysical Chemistry, Goettingen, Germany, <sup>3</sup>Neuroendocrinology, University of Luebeck, Luebeck, Germany

**Background:** A number of immunological functions in CD4+ T cells are dependent on the circadian rhythm as we and others could previously demonstrate. Little is known about the underlying mechanisms. One possibility could be the circadian rhythm of the molecular clock in T cells. The molecular clock is known to control the circadian rhythm in the brain and several peripheral organs. To address this question we analyzed the expression of five clock genes (Bmal1, Per2, Cry1, Rev-erba, Dbp), the production of cytokines and the CD40L expression in CD4+ T cells from human volunteers.

**Methodology:** 15 healthy young men were examined under defined conditions over 24 h in the sleep lab. Venous blood was drawn periodically every 3 h, CD4+ T cells were isolated. T cells were split: one fraction was used for the investigation of clock gene expression and the second fraction was polyclonally stimulated and analyzed applying FACS.

**Results:** We found that on average ~32% of polyclonally stimulated highly purified CD4+ T cells express CD40L at 15:00 h whereas only ~2% of the CD4+ T cells express CD40L at 6:00 h. Additionally there is also a strong rhythm for the production of INF-γ. Furthermore, we have preliminary data demonstrating the rhythmic expression of the core clock genes Bmal1 and Cry1 in CD4+ T cells.

**Conclusions:** These findings demonstrate that highly purified CD4+ T cells have a strong functional circadian rhythm and that a possible underlying mechanism could be the molecular clock.

**PB04/3 ROLE OF GILZ AND L-GILZ, A NEW GILZ ISOFORM, IN THE REGULATION OF T CELL GROWTH**

S. Bruscoli<sup>1</sup>, E. Ayroldi<sup>1</sup>, E. Velardi<sup>1</sup>, V. Donato<sup>1</sup>, A. Bastianelli<sup>1</sup>, M. Cristina<sup>1</sup>, C. Riccardi<sup>1</sup>
<sup>1</sup>University of Perugia, Department of Clinical and Experimental Medicine, Section of Pharmacology, Perugia, Italy

Glucocorticoids (GCs) play a role in the physiologic regulation of immune system and in the pharmacological suppression of inflammatory response, through molecular mechanisms involving GR activation and modulation of gene expression. The immunosuppressive activities of GC mainly affect T lymphocytes. In fact, GCs not only modulate T lymphocyte activation/proliferation/apoptosis and contribute to thymic selection, but they also regulate cytokine production thus modulating T helper cells differentiation.

**Objectives:** We performed experiments to analyze the GILZ system role in the control of immune response. GILZ (Glucocorticoid-Induced Leucine Zipper), discovered in our laboratory in studies aimed at characterizing gene(s) targeted by dexamethasone (DEX), was shown to mediate several GC functions, such as modulation of T lymphocytes activation, IL-2 production, apoptosis and cell proliferation. Many molecular targets of GILZ have been identified, including NF-κB, AP-1, Raf-1 and Ras, all involved in GC effects.

**Results:** We have cloned a new GILZ isoform of 705 bp, coding for a protein of 234 aa, named long-GILZ (L-GILZ). Here we report that L-GILZ shares with GILZ the ability to negatively regulate several T cell functions. In fact L-GILZ, like GILZ, interacts directly with Ras leading to inhibition of T cell proliferation. Furthermore, GILZ and L-GILZ silencing resulted in an increase in concanavalin A-induced T cell proliferation suggesting a role of physiological brake in T cell activation.

Moreover, siRNA-mediated knockdown of GILZ and L-GILZ in T cells inhibited the DEX-induced anti-proliferative effect as well the DEX-induced apoptosis. Notably, both GILZ and L-GILZ inhibit the activation of NF- $\kappa$ B, an important transcription factor involved in T cell activation and survival.

**Conclusion:** These preliminary data indicate that GILZ and L-GILZ has similar features, and further support the role of GILZ system in mediating the GC-induced anti-inflammatory and immunosuppressive effects.

**PB04/4 A NEW ROLE FOR FAS IN ATTENUATING PROLIFERATION OF MEMORY/EFFECTOR TCR-RECHALLENGED T CELLS BY CONTROLLING NF- $\kappa$ B SIGNALING: IMPLICATIONS FOR LYMPHADENOPATHY AND AUTOIMMUNITY**

D. Balomenos<sup>1</sup>

<sup>1</sup>National Institute for Biotechnology, Immunology and Oncology, Madrid, Spain

Fas, the prototypic apoptosis-inducing molecule, controls T cell homeostasis and Fas-deficient lpr mice develop lymphadenopathy. Although Fas interaction with its ligand (FasL) after secondary TCR stimulation induces apoptosis, the etiology of lymphadenopathy development in lpr mice is not precisely defined. Another unexplained feature of lpr T cells is their *in vivo* hyperproliferation. We therefore sought a functional role for Fas in the regulation of activated T cell proliferation. Our data reveal a previously undescribed Fas function in regulating proliferation of T cells resistant to apoptosis after secondary TCR activation. We show that lpr T cells hyperproliferated after secondary stimulation, due to the lack of Fas/FasL-dependent regulation of division.

Although caspase-8 processing initiates the Fas apoptosis pathway, it has been recently shown that whole-molecule caspase-8 activity is essential for T cell proliferation. In fact, our studies showed that lpr T cell hyperproliferation was associated to increased whole-molecule caspase-8 activity. Furthermore, lack of Fas led to increased NF- $\kappa$ B activation in hyperproliferating effector/memory lpr T cells, which was accompanied by reduced p21 expression and increased CDK2 activation. We previously showed that p21 regulates proliferation of memory but not of naïve T cells after TCR stimulation. p21 overexpression in lpr T cells resulted in a notable reduction in T cell lymphoproliferation and lymphadenopathy development, as well as a significant decrease in lpr memory T cell accumulation and autoimmune disease manifestations. *In vitro* studies showed that p21 overexpression did not affect the lpr apoptosis defect, but reduced the hyperproliferation of lpr T cells after secondary stimulation. Overall, we show that lpr T cell hyperproliferation is critical for the deregulation of homeostasis and the development of autoimmunity in lpr mice.

The data suggest that Fas/FasL signaling controls homeostasis and lymphadenopathy by two distinct functions, by promoting apoptosis and by regulating proliferation of apoptosis-surviving T cells. This model might help to understand T cell expansion syndromes and diseases associated with Fas expression, such as the autoimmune lymphoproliferative syndrome (ALPS).

**PB04/5 TOSO IS A NOVEL REGULATOR OF DEATH RECEPTOR SIGNAL TRANSDUCTION BY FACILITATING RIP1 UBIQUITINATION**

X.-H. Nguyen<sup>1</sup>, K.-H. Lee<sup>1</sup>

<sup>1</sup>Research Center Borstel, Molecular Immunology, Borstel, Germany

Apoptosis plays fundamental roles in the development and the homeostasis of immune cells. One of the most well characterized death receptors in the immune system is CD95 (Fas/Apo-1). Although CD95 is primarily recognized as a death-inducing receptor, accumulating evidence indicates that CD95 ligation can also induce non-apoptotic signaling pathways. The regulation of CD95 mediated non-apoptotic signaling, in particular with respect to its contribution to autoimmunity, is however still unclear. The immune-specific transmembrane protein Toso was originally identified as a surface molecule with negative regulatory function on lymphocyte apoptosis. The overexpression of Toso is tightly associated with autoimmune disease, as observed by gene expression analysis of T lymphocytes from SLE and MS patients.

Employing miRNA mediated specific Toso gene knock-down, as well as Toso overexpression, we demonstrate that Toso exhibits anti-apoptotic effects on CD95-mediated signaling by a caspase independent mechanism. Thus, Toso represents the unique case of a cell surface molecule that solely by cellular expression protects from CD95-induced apoptosis. Our data further show that the anti-apoptotic function of Toso depends on RIP1 as an effector molecule. Toso is constitutively associated with RIP1 and, in response to CD95-stimulation, promotes RIP1 ubiquitination. Utilizing a specific RIP1 ubiquitination mutant (RIP1-K377R) we could demonstrate that the protective function of Toso on CD95-induced apoptosis requires site-specific ubiquitination of RIP1 at K377. Moreover, our studies reveal that in response to death receptor stimulation Toso promotes the induction of pro-survival signaling, such as the activation of MAPK and NF $\kappa$ B signaling pathways. As a result of this relative augmentation of survival signals versus apoptotic signals, Toso raises the threshold for death receptor-mediated apoptosis. Together, our studies on the role of RIP1 ubiquitination for the anti-apoptotic function of Toso provide novel insights into the complex regulatory mechanisms of death receptor-mediated signal transduction. Such knowledge on how survival and apoptotic signals are integrated into cellular fate decisions is also critical for our understanding of autoimmune disorders and may open up prospects for potential new diagnostic and therapeutic applications.

**PB04/6 IL-7 SUSTAINS CD31 EXPRESSION IN HUMAN NAÏVE CD4+ T CELLS AND PREFERENTIALLY EXPANDS THE CD31+ SUBSET IN A PI3K-DEPENDENT MANNER**

R.I. Azevedo<sup>1,2</sup>, M.V.D. Soares<sup>1</sup>, J.T. Barata<sup>3</sup>, R. Tendeiro<sup>1</sup>, A. Serra-Caetano<sup>4</sup>, R.M.M. Victorino<sup>1</sup>, A.E. Sousa<sup>1</sup>

<sup>1</sup>Instituto de Medicina Molecular, Unidade de Imunologia Clínica, Lisbon, Portugal, <sup>2</sup>University College London, Department of Immunology and Molecular Pathology, London, United Kingdom, <sup>3</sup>Instituto de Medicina Molecular, Unidade de Biologia do Cancro, Lisbon, Portugal, <sup>4</sup>Instituto de Medicina Molecular, Unidade de Citometria de Fluxo, Lisbon, Portugal

The CD31+ subset of human naïve CD4+ T cells is thought to contain the population of cells that have recently emigrated from the thymus, whilst their CD31-counterparts have been proposed to originate from CD31+ after homeostatic cell division. However, the maintenance of the CD31+ pool into old age is likely to rely on alternative mechanisms other than thymic output. One of the likely candidates is IL-7, a homeostatic cytokine known to play a key role on the survival and proliferation of naïve cells in the periphery. We sought to investigate the role of IL-7 in the homeostasis of human naïve CD4+ T cell subsets defined by CD31 expression. We provide evidence that IL-7 exerts a preferential proliferative effect on CD31+ naïve CD4+ T cells from adult peripheral blood as compared to the CD31- subset. IL-7-driven proliferation did not result in loss of CD31 expression, suggesting that CD31+ naïve CD4+ T cells can undergo cytokine-driven homeostatic proliferation whilst preserving CD31. Furthermore, IL-7 sustained or increased CD31 expression even in non-proliferating cells. Both proliferation and CD31 maintenance observed in the presence of IL-7 were dependent on the activation of phosphoinositide 3-kinase (PI3K) signalling, and likely contribute to the IL-7-mediated maintenance of CD31+ naïve CD4+ T cells during adulthood. In conclusion, our data suggest that the CD31 marker identifies a naïve CD4+ T cell subset with a unique ability to proliferate in response to IL-7, which is likely to be the main target of IL-7 during its therapeutic use.

**PB04/7 GRANZYME A AND B DEFICIENCY AFFECTS THE IMMUNE RESPONSE AGAINST A HELMINTH INFECTION IN MICE**

W. Hartmann<sup>1</sup>, B. Fleischer<sup>1</sup>, S. Kortner<sup>1</sup>

<sup>1</sup>Bernhard Nocht Institute for Tropical Medicine, Immunology, Hamburg, Germany

**Objective:** Granzyme (gzm) A and B are major cytotoxic serine proteases of NK cells and cytotoxic T cells, known to play a key role in the destruction of tumor cells or infected cells. However recent data indicate further functions of granzymes including the induction of activation induced cell death (AICD) in Th2 cells and cytokines in monocytes/macrophages. Their role in helminth infection has not been examined so far. More than 150 Mio people are infected worldwide with filarial species like *Wuchereria bancrofti* and *Onchocerca volvulus*. As CD4+ T cells, NK cells, macrophages and Treg cells affect the mixed Th1/Th2 (Th2-biased) immune defence against filariae we investigated the impact of gzm A and B deficiency on the immune response against the rodent filaria *Litomosoides sigmodontis*. Experimental infection of mice with *L. sigmodontis* is the best-characterized model to study infections with filarial nematodes.

**Methods:** Resistant wildtype C57BL/6 (wt) mice and gzm A, B and AxB knock out (ko) mice were naturally infected via mites. Worm number and worm length was analyzed at different time points. We compared cytokine and antibody responses of naïve and infected gzm ko and wt mice using standard immunological methods such as ELISA and flow cytometry. Induction of cell death was analyzed *ex vivo* and after restimulation to induce AICD.

**Results:** Gzm AxB ko and gzm B ko mice harboured lower worm numbers in the pleural cavity than wt mice. This lower worm load in gzm AxB ko mice was associated with a defence promoting Th2 cytokine and antibody shift probably partially caused by diminished AICD in CD4+ T cells. Gzm A ko mice had higher worm burdens at day 9 post infection indicating a suppressed immune response against the worms in the absence of gzm A. Further, deficiency of granzymes interfered with migration and development of macrophages.

**Conclusion:** Our data argue for a multifunctional role of granzyme A and B during a helminth infection such as gzm B-mediated cell death in CD4+ T cells and gzm A-induced production of pro-inflammatory cytokines and phagocytosis by monocytes.

**PB04/8 HEPATOCYTES AND IL-15: A FAVORABLE MICROENVIRONMENT FOR T CELL SURVIVAL AND CD8+ T CELL DIFFERENTIATION**

M.P. Correia<sup>1,2</sup>, E.M. Cardoso<sup>1,3</sup>, C.F. Pereira<sup>4</sup>, R. Neves<sup>5</sup>, M. Uhrberg<sup>5</sup>, F.A. Arosa<sup>1,2,3</sup>

<sup>1</sup>IBMC- Instituto de Biologia Molecular e Celular, Lymphocyte Biology Group, Porto, Portugal, <sup>2</sup>ICBAS, University of Porto, Porto, Portugal, <sup>3</sup>CESPU, Centro de Investigação em Ciências da Saúde (CICS), Instituto Superior de Ciências da Saúde – Norte, Gandra, Portugal, <sup>4</sup>MRC Clinical Sciences Centre, London, United Kingdom, <sup>5</sup>University Clinic of Düsseldorf, Institute for Transplantation Diagnostics and Cell Therapeutics, Düsseldorf, Germany

Human intrahepatic lymphocytes (IHL) are enriched in “non-classical” T cells co-expressing NK receptors (NKR). Although the origin of this population remains controversial, it is possible to speculate that the hepatic microenvironment, namely epithelial cells or the cytokine milieu, may play a role in its shaping. Interleukin (IL)-15 is constitutively expressed in the liver and has a key role in activation and survival of innate and tissue-associated immune cells. In this *in vitro* study, we examined whether hepatocyte cell lines and/or IL-15 could play a role in the generation of NK-like T cells. The results have shown that both HepG2 cells and a



human immortalized hepatocyte cell line are able to increase survival and to drive basal proliferation levels of T cells. Interestingly, besides driving T cell survival and proliferation, IL-15 was capable of inducing antigen (Ag)-independent upregulation of NKR, including NKG2A, Ig-like receptors (KIR), and *de novo* expression of CD56 and NKP46 in CD8+CD56- T cells. In conclusion, our study suggests that hepatocytes and IL-15 create a favorable microenvironment for T cells to grow and survive. Also, it can be hypothesized that the increased percentage of intrahepatic “non-classical” NKT cells could be in part result of a local IL-15-driven CD8+ T cell differentiation.

PB04/9

#### ROLE OF THE ORPHAN ADAPTOR PROTEIN SLY1 IN PERIPHERAL LYMPHOCYTE APOPTOSIS

D. Finkenshtadt<sup>1</sup>, B. Reis<sup>1</sup>, K. Pfeffer<sup>1</sup>, S. Beer<sup>1</sup><sup>1</sup>Institut für Med. Mikrobiologie und Krankenhaushygiene, Heinrich-Heine-Universität, Düsseldorf, Germany

SH3 protein expressed in lymphocytes 1 (SLY1) is a member of a distinct family of putative adapter and/or scaffold proteins highly conserved in mammals. SLY1 is exclusively expressed in lymphocytes and has been shown to be phosphorylated specifically upon antigen receptor engagement.

To investigate physiological functions of SLY1, *sly1*-deficient mice have been generated by our group. *Sly1*-deficient mice exhibit reduced lymphoid organ sizes, diminished marginal zone B cell numbers and severely impaired antibody responses against T cell-dependent and -independent antigens. As thymic cellularity was diminished by about 50%, we further analysed thymic development *ex vivo* and upon cultivation on the bone marrow stroma cell line OP9. These data identified SLY1 as a novel anti-apoptotic protein required for developmental progression of T cell precursors to the CD4<sup>+</sup>CD8<sup>+</sup> double-positive stage.

In the present study apoptosis in peripheral lymphocytes was analysed by measuring AnnexinV and active caspase-3 in flow cytometry. Unstimulated *sly1*-deficient lymphocytes and splenocytes showed decreased levels of active caspase-3 in comparison to wildtype cells. Stimulation with anti-CD3 or anti-CD3/CD28 further decreased active caspase-3 levels cells without altering relative differences in caspase-3 levels.

Furthermore, wildtype and *sly1*-deficient CD4<sup>+</sup> and CD8<sup>+</sup> T cells were separated by MACS and treated with either radiation, staurosporine or CD95L to trigger intrinsic or extrinsic apoptosis pathways, respectively. Compared to wildtype cells, CD8<sup>+</sup> *sly1*-deficient cells showed lower levels of apoptosis independent of the apoptotic stimulus. CD4<sup>+</sup> *sly1*-deficient cells specifically displayed decreased levels of apoptosis after CD95L stimulation compared to wildtype-cells. In contrast, apoptosis levels upon radiation or staurosporine treatment remained comparable between the two genotypes. In line with this observation, the CD4<sup>+</sup> cell to CD8<sup>+</sup> cell ratio in *sly1*-deficient mice was increased towards the CD8<sup>+</sup> lineage, indicating that SLY1 regulated apoptosis sensitivity is relevant *in vivo*.

Our data suggest a pro-apoptotic impact of SLY1 in peripheral lymphocytes and in particular a role in extrinsic apoptosis signaling pathway in CD4<sup>+</sup> T cells.

PB04/10

#### REVIVING FUNCTION IN CD4 T CELLS ADAPTED TO PERSISTENT SYSTEMIC ANTIGEN

M. Y. Braun<sup>1</sup>, K. Weatherly<sup>1</sup>, F. Gaudray-Rodriguez<sup>1</sup>, I. Salmon<sup>2</sup>, M. Noval Rivas<sup>1</sup><sup>1</sup>Université Libre de Bruxelles, Institute for Medical Immunology, Gosselies, Belgium, <sup>2</sup>Université Libre de Bruxelles, Histopathology Department, Erasme Hospital, Brussels, Belgium

**Objectives:** Chronic antigenic stimulation often leads to the intrinsic down-regulation of responsiveness that provides CD4 T cells with the capacity to tailor their activation threshold to the strength of ambient antigen presentation. This state of unresponsiveness is believed to hamper CD4 T cell responses to chronic viral infection as well as to cancer. The aim of the present study was to identify the molecular mechanisms by which CD4 T cells maintained their adaptation to persistent antigen.

**Methods:** Here we report a model where persistent stimulation by antigen led to the expansion of specific CD4 T cells in lymphopenic antigen-expressing recipients.

**Results:** Unlike naive T cells or T cells expanded in the absence of antigen, antigen-expanded T cells developed a state of long-term unresponsiveness characterized by their inability to mediate *in vivo* immune damages and to produce IL-2 and proliferate following subsequent *in vitro* antigenic challenge. Analysis of gene expression profile at the RNA level revealed that chronically-stimulated CD4 T cells expressed typical Th1 genes, such as IFN $\gamma$ , t-bet, IL-12R $\beta$  chains, CXCR3, CCR5, TWIST-1, granzyme B, perforin... Interestingly, antigen-expanded T cells co-expressed also a large series of genes known to regulate Th1 responses (IL-10, IL-21, TWIST-1, and negative costimulatory receptors ICOS, CTLA4, PD-1, PD-L1, CD80,...). Unresponsiveness in CD4 T cells chronically stimulated by antigen could be abrogated by blockade of negative costimulatory molecules (PD-1>CTLA-4) or by treatment favouring positive costimulation, such as the addition of Tol like receptor (TLR) ligands. *In vivo*, these treatments abolished CD4 T cell unresponsiveness induced by persistent antigenic exposure and converted quiescent T cell infiltration into a severe wasting disease. Combining treatments antagonising negative costimulation with those stimulating positive costimulation of T cells led to lethality in mice with T cells under persistent antigenic stimulation.

**Conclusion:** Our results demonstrate that T cell unresponsiveness maintained by chronic antigenic stimulation allow for the persistence of CD4 T cells with specificities that could be potentially useful in vaccinal strategies against pathogens and malignancies. They also emphasize the possible deleterious effect that vaccine adjuvants could have by stimulating autoreactive CD4 T cells that are maintained unresponsive through persistent self-antigen exposure.

PB04/11

#### REQUIREMENT OF IFN- $\gamma$ MEDIATED INDOLEAMINE 2,3 DIOXYGENASE EXPRESSION IN THE MODULATION OF LYMPHOCYTE PROLIFERATION BY HUMAN ADIPOSE-DERIVED STEM CELLS

O. DelaRosa<sup>1</sup>, E. Lombardo<sup>1</sup>, A. Beraza<sup>1</sup>, P. Manchego-Corvo<sup>1</sup>, C. Ramirez<sup>1</sup>, R. Menta<sup>1</sup>, L. Rico<sup>1</sup>, E. Camarillo<sup>1</sup>, L. García<sup>1</sup>, J.L. Abad<sup>2</sup>, C. Trigueros<sup>3</sup>, M. Delgado<sup>4</sup>, D. Buscher<sup>1</sup><sup>1</sup>CELLERIX S.A., R&D, Madrid (Tres Cantos), Spain, <sup>2</sup>Coretherapix SLU, R&D, Madrid, Spain, <sup>3</sup>Fundacion Inbiomed, San Sebastián, Spain, <sup>4</sup>Instituto de Parasitología y Biomedicina-CSIC, Granada, Spain

Human adipose-derived stem cells (hASCs) are mesenchymal stem cells with reduced immunogenicity and capability to modulate immune responses. Whereas the immunosuppressive activity of bone marrow-mesenchymal stem cells (BM-MSCs) has received considerable attention during the last few years, the specific mechanisms underlying hASCs-mediated immunosuppression have been poorly studied. Recent studies comparing both cell types have reported differences at transcriptional and proteomic levels, suggesting that hASCs and BM-MSCs, while having similarities, are quite different. This suggests that different mechanisms of immunosuppression may apply. Here, we report that hASCs inhibit peripheral blood mononuclear cells (PBMCs), CD4<sup>+</sup> and CD8<sup>+</sup> T cell proliferation in both cell-cell contact and transwell conditions, which is accompanied by a reduction of proinflammatory cytokines. We demonstrate that hASCs do not constitutively express immunomodulatory factors. Conditioned supernatants from hASCs stimulated by IFN- $\gamma$  PBMCs or activated PBMCs highly inhibited PBMC proliferation, indicating that inhibitory factors are released upon hASC activation. Many factors have been involved in mesenchymal stem cells-mediated immunosuppression, including IFN- $\beta$ , IL-10, hepatocyte growth factor, prostaglandin E2, transforming growth factor- $\beta$ 1, indoleamine 2,3-dioxygenase (IDO), nitric oxide and IL-10. Using pharmacological inhibitors, neutralizing antibodies and genetically modified hASCs that constitutively express or silence IDO enzyme we demonstrate that, in the case of hASCs, the IFN- $\beta$ /IDO axis is essential. Taken together, our data support the key role of IDO in the therapeutic use of hASC on immunomediated diseases.

PB04/12

#### EVIDENCE FOR INCREASED REPLICATIVE RATE OF THE NAÏVE CD4+ T-CELL POOL DURING HIV-2 INFECTION, A NATURALLY OCCURRING ATTENUATED FORM OF HIV DISEASE

R. Tendeiro<sup>1</sup>, M. Soares<sup>1</sup>, A.P. Baptista<sup>1</sup>, R. Cavaleiro<sup>1</sup>, R. Foxall<sup>1</sup>, R.S. Soares<sup>1</sup>, P. Gomes<sup>2</sup>, R.M.M. Victorino<sup>1,3</sup>, A.E. Sousa<sup>1</sup><sup>1</sup>Unidade de Imunologia Clínica, Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa, Lisboa, Portugal, <sup>2</sup>Laboratório de Virologia, Hospital Egas Moniz, Lisboa, Portugal, <sup>3</sup>Clínica Universitária de Medicina 2, Hospital de Santa Maria, Lisboa, Portugal

**Objectives:** HIV infection is characterized by a progressive loss of CD4+ T cells that has been linked to a high and persistent T-cell turnover. In agreement, evidence of a longer replicative history of CD8+ T cells estimated by telomere length measurement was documented in HIV-1-infected patients, while for CD4+ T cells only minimal or no telomere shortening has been reported as compared to seronegative subjects. HIV-2 is associated with a slow rate of CD4+ T-cell decline and consequently a prolonged length of infection. Nevertheless, for a given degree of CD4+ T-cell depletion, levels of T-cell activation and frequency of cycling cells have been shown to be comparable to those observed in HIV-1-infected individuals. Therefore, we compared CD4+ T-cell telomere length in the two AIDS associated infections.

**Methods:** Telomere length was assessed by Flow-FISH and frequency of cycling cells quantified by flow cytometry after intracellular staining for Ki67 and surface staining for CD4, CD45RO and CD31. Mann-Whitney test and Spearman correlation were used for statistical analysis.

**Results:** A reduction of total CD4+ T-cell telomere length was found in HIV-2-infected patients as compared to both healthy and HIV-1-infected individuals. Strikingly, this decrease was related to a shortening of naïve CD4+ T-cell telomeres. Furthermore, the naïve CD4+ T-cell telomere length showed a negative correlation with the frequency of cycling naïve cells, and more importantly with the proportion of the CD31+Ki67+ population within the naïve CD4+ T-cell pool, which was found to be significantly expanded in HIV-2 infected individuals. Conversely, no differences were found regarding the telomere length of the memory pool in spite of the significantly increased frequency of cycling cells in both infected cohorts as compared to healthy controls.

**Conclusion:** We found that the slow decline of CD4+ T cells that characterizes HIV-2 immunodeficiency is associated with high naïve CD4+ T-cell proliferation rate, particularly of the CD31+ subset that has been shown to be enriched in recent thymic emigrants. Our data support an important role of the impairment of naïve CD4+ T-cell homeostatic response in HIV-1/AIDS pathogenesis.

**PB04/13 FAIM REGULATES AKT ACTIVATION AND NUR77 EXPRESSION IN TCR-MEDIATED APOPTOSIS OF THYMOCYTES**J. Huo<sup>1</sup>, S. Xu<sup>1</sup>, K.-P. Lam<sup>1</sup><sup>1</sup>Bioprocessing Technology Institute, Laboratory of Immunology, Singapore, Singapore

**Objectives:** TCR-mediated apoptosis is essential for establishing central tolerance in T cells. However, the underlying mechanism of how TCR signaling culminates in an apoptotic signal in developing thymocytes is still not completely clear and remains a central question in immunology. Fas-apoptosis inhibitory molecule (FAIM) is an inducible antagonist to Fas-killing in B lymphocytes. We previously found that faim-deficient B cells and thymocytes were more sensitive to Fas-triggered apoptosis *in vitro*, faim<sup>-/-</sup> mice suffer greater mortality and exhibit exacerbated liver damage in response to Fas engagement *in vivo*, and FAIM influenced c-FLIP expression and regulated the binding of caspase-8 to death receptor during Fas-triggered apoptosis. In this report, we ask if FAIM is involved in TCR-mediated apoptosis of thymocytes.

**Methods:** We demonstrated FAIM's role in TCR-mediated apoptosis in both FAIM-deficient mouse thymocytes (*in vivo* and *in vitro*) and FAIM-over-expressing DO11.10 cells.

**Results:** We found that FAIM is up-regulated in thymocytes upon TCR engagement and that thymocytes lacking FAIM are highly sensitive to TCR-mediated apoptosis *in vitro* and exhibit enhanced activation of caspase-8 and -3. Furthermore, injection of anti-CD3 antibody leads to augmented depletion of CD4<sup>+</sup>CD8<sup>+</sup> T cells in the thymus of faim<sup>-/-</sup> mice compared to wild-type control, suggesting that FAIM may play a role in thymocyte negative selection. Mechanistically, cross-linking of the TCR on faim<sup>-/-</sup> thymocytes leads to reduced activation of Akt but results in elevated protein levels of the orphan nuclear receptor Nur77. Biochemical analyses utilizing faim<sup>-/-</sup> primary thymocytes and FAIM-overexpressing DO11.10 T-cells indicate that FAIM modulated Akt activation by influencing its lipid raft localization in TCR signaling.

**Conclusion:** Taken together, our study suggested that FAIM regulated the apoptosis of developing thymocytes.

**PB04/14 CD4<sup>+</sup> T LYMPHOCYTE RESPONSE TO PRIMARY CMV INFECTION**P. Antoine<sup>1</sup>, V. Ollislaers<sup>1</sup>, S. Lecomte<sup>1</sup>, C. Liesnard<sup>2</sup>, C. Donner<sup>3</sup>, A. Marchant<sup>1</sup>

<sup>1</sup>Université Libre de Bruxelles, Institute for Medical Immunology, Charleroi, Belgium, <sup>2</sup>Université Libre de Bruxelles, Hôpital Erasme, Department of Virology, Brussels, Belgium, <sup>3</sup>Université Libre de Bruxelles, Hôpital Erasme, Department of Obstetrics and Gynaecology, Brussels, Belgium

Acute cytomegalovirus (CMV) infection induces rapid expansion of CMV-specific cytokine-producing CD4<sup>+</sup> T cells but slow acquisition of proliferative responses. *In utero* transmission of CMV following maternal infection is associated with prolonged undetectable proliferative responses. We have observed that pregnant women with primary CMV infection have high frequencies of CD4<sup>+</sup> T cells expressing low levels of Bcl-2. The frequency of these cells normalises during the first months of infection. As Bcl-2 controls cell survival and proliferation, its regulation could play an important role in the control of proliferative responses of CMV-specific lymphocytes. The aim of the project is to characterise the phenotype, the antigen specificity and the functions of Bcl-2<sub>low</sub> CD4<sup>+</sup> T cells in pregnant women with primary CMV infection. We have observed that Bcl-2<sub>low</sub> cells are more activated and differentiated than Bcl-2<sub>high</sub> cells. In particular, the low expression of Bcl-2 is tightly associated with loss of CD28 expression, decreased CD127 (IL-7 receptor  $\alpha$  chain) expression and increased PD-1 expression. The low expression of CD127 is associated with low STAT-5 activation in response to IL-7 stimulation. These results indicate that primary CMV infection induces the modulation of Bcl-2 expression by CD4<sup>+</sup> T lymphocytes and this is associated with the modulation of other surface receptors regulating cell proliferation. Further studies will define the functional consequences of these phenotypic alterations.

**PB04/15 COMBINED INHIBITION OF DIPEPTIDYL PEPTIDASE IV (DP IV)-LIKE AND AMINOPEPTIDASE N (APN)-LIKE ENZYMATIC ACTIVITY SUPPRESSES T CELL ACTIVATION AND IL-17 PRODUCTION**A. Göhl<sup>1</sup>, U. Bank<sup>2</sup>, S. Wrenger<sup>1</sup>, A. Thielitz<sup>3</sup>, J. Faust<sup>4</sup>, K. Neubert<sup>4</sup>, M. Täger<sup>2</sup>, S. Ansorge<sup>2</sup>, D. Reinhold<sup>1</sup>

<sup>1</sup>Otto-von-Guericke University Magdeburg, Institute of Molecular and Clinical Immunology, Magdeburg, Germany, <sup>2</sup>IMTM GmbH, Department Immunopharm, Magdeburg, Germany, <sup>3</sup>Otto-von-Guericke Universität Magdeburg, University Clinic of Dermatology and Venerology, Magdeburg, Germany, <sup>4</sup>Martin-Luther-University Halle-Wittenberg, Department of Natural Sciences I, Halle, Germany

Compelling evidence has demonstrated that IL-17-producing T cells mainly contribute to the pathogenesis of autoimmune inflammation. Since synthetic inhibitors of DP IV-like and of APN-like enzymatic activity are clearly shown to suppress T cell activation *in vitro* and disease progression in models of autoimmunity, here, we tested the hypothesis whether these inhibitors target the production of IL-17. The effect of inhibitors of DP IV-like activity, Lys[Z(NO<sub>2</sub>)]-thiazolidide (LZNT) and Lys[Z(NO<sub>2</sub>)]-pyrrolidide (LZNP), as well as the APN inhibitor actinonin on IL-17 production was examined in human mitogen-stimulated T cells and in mitogen-stimulated splenocytes of C57BL/6 mice *in vitro*. Moreover, in order to investigate the mechanism by which such peptidase inhibitors limit autoimmune disease *in vivo*, we studied the effect of LZNP and actinonin activity on IL-17 production in experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis. We found that LZNT and LZNP, as well as actinonin inhibit IL-17 production in human stimulated T cells and in stimulated splenocytes of C57BL/6 mice *in vitro*. Combining DP IV and APN inhibitors increased the suppressive effect on T cell proliferation and IL-17 production in comparison to a single peptidase inhibitor. Moreover, we observed *in vivo* that the elevated IL-17 plasma levels of EAE mice are decreased by the administration of combined peptidase inhibitors. Collectively, our data suggest that the combined inhibition of DP IV-like and APN-like enzymatic activity in pathogenic T cells represents a novel and efficient therapeutic approach that targets IL-17 production in autoimmunity.

**PB04/16 TYPE I INTERFERON CONTROLS CD4<sup>+</sup>CD25<sup>+</sup> T HELPER CELL ACTIVITY BY CD4<sup>+</sup>CD25<sup>+</sup> REGULATORY T CELL- DEPENDENT AND -INDEPENDENT MECHANISMS**L. Pace<sup>1</sup>, S. Vitale<sup>1</sup>, B. Dettori<sup>1</sup>, C. Palombi<sup>1</sup>, F. Belardelli<sup>2</sup>, E. Proietti<sup>2</sup>, G. Doria<sup>1</sup>

<sup>1</sup>University of Rome Tor Vergata, Rome, Italy, <sup>2</sup>Dept of Cell Biology and Neurosciences, Istituto Superiore di Sanità, Rome, Italy

Type I IFNs are central to a vast array of immunological functions. Their early induction in innate immune responses provides one of the most important priming mechanisms for the subsequent establishment of acquired immune responses. The outcome is either promotion or inhibition of these responses, but the conditions under which one or the other prevails remain to be defined. The main objective of the present study has been to determine the involvement of IFN $\alpha$  on murine CD4<sup>+</sup> CD25<sup>+</sup> Th cell activation, as well as to define the role played by this cytokine on CD4<sup>+</sup> CD25<sup>+</sup> Treg cell proliferation and function. Although IFN $\alpha$  induces CD4<sup>+</sup> CD25<sup>+</sup> T cells co-incubated with APCs to produce large amounts of IL-2, at the same time their ability to respond to its proliferative effects is prevented. Notably, in medium supplemented with IFN $\alpha$ , IL-2 induced CD4<sup>+</sup> CD25<sup>+</sup> Treg cell proliferation is also inhibited, and lead to a decrease of their suppressive activity. Altogether, these findings indicate a dual pivotal role of IFN $\alpha$  on CD4<sup>+</sup> T cell activity, by a direct effect on CD4<sup>+</sup> CD25<sup>+</sup> Th cell activation and indirectly by affecting CD4<sup>+</sup> CD25<sup>+</sup> Treg cell-mediated suppression.

**PB04/17 ANTI-CD44-INDUCED APOPTOSIS IN T CELLS PROCEEDS VIA MITOCHONDRIAL DEPOLARIZATION**R. Singh<sup>1</sup>, M. Rajasagi<sup>1</sup>, A. V. Au<sup>2</sup>, R. Marhaba<sup>1</sup>, M. Zöller<sup>1</sup>

<sup>1</sup>Uni Heidelberg, Dept. of Surgery, Tumor Cell Biology, Heidelberg, Germany, <sup>2</sup>Uni Heidelberg, Heidelberg, Germany

CD44 is a transmembrane molecule that plays an important role in hematopoiesis. During hematopoiesis, CD44 has become assigned with homing, differentiation, apoptosis protection as well as induction, where CD44-induced apoptosis can proceed via different pathways. The question of anti-CD44-induced apoptosis has recently become of interest also for hematological malignancies, where anti-CD44 promoted a reduction in tumor take, obviously due to death of leukaemia-initiating cells.

We have been particularly interested in the role of CD44 in progenitor T cell homing and maturation. We experienced that EL4 thymoma growth becomes retarded by an antibody blockade of CD44 and promotes *in vivo* apoptosis of leukemic T cells. However, in bone marrow cell-reconstituted mice anti-CD44 drives progenitor T cells more efficiently than leukemic cell into apoptosis, such that the survival time is shortened and the incidence of metastasis increases. To explore the underlying mechanism, EL4 cells and thymocytes were cultured for 24h to 72h in medium containing anti-panCD44, which resulted in a 2-4 fold increase in the rate of apoptotic cells. Death receptor expression (CD95, TRAIL, TNFRI) remained unaltered and receptor-mediated apoptosis by CD95 cross-linking was neither inhibited nor strengthened by anti-CD44. Instead, CD44 ligation promoted mitochondrial depolarization that was accompanied by caspase-9 cleavage, upregulation of BAX, low level of BAD phosphorylation and Bcl-2 expression. Apoptosis became initiated by activation of CD44-associated phosphatase 2A (PP2A) and proceeded via ERK1/2 dephosphorylation without evidence for ERK1/2 degradation. Accordingly, CD44-induced apoptosis could be mimicked by ERK1/2 inhibition, that also promoted EL4 cell apoptosis through the mitochondrial pathway.

These findings are in line with our results on the effect of anti-CD44 on thymoma growth and reconstitution *in vivo* and support our interpretation that anti-CD44 can drive thymoma cells and, more efficiently, progenitor T cells into apoptosis, where the association of CD44 with PP2A is of central importance.

**PB04/18 SOLUBLE CD14 PROTECTS HUMAN LYMPHOCYTES FROM APOPTOSIS**B. Tartakovsky<sup>1</sup>, B. Sredni<sup>2</sup>, E. Zigman<sup>3</sup>, G. Senior<sup>1</sup>, E. Naparstek<sup>4</sup>

<sup>1</sup>Tel Aviv Medical Center, Hematology and BMT, Tel Aviv, Israel, <sup>2</sup>Bar Ilan University, The Safdie Institute for AIDS and Immunology Research, Ramat Gan, Israel, <sup>3</sup>Tel Aviv Medical Center and Bar Ilan University, Hematology and BMT, Tel Aviv, Israel, <sup>4</sup>Tel Aviv Medical Center and Tel Aviv University, Hematology and BMT, Tel Aviv, Israel

CD14, a 56 Kd glycoprotein, typically present on myeloid cells, has been traditionally associated with innate immunity and pattern recognition. Recently its membrane bound form has been shown to be involved in apoptosis, as a tethering receptor for apoptotic cells on the surface of phagocytes – in this case with the purpose of removing apoptotic cells, and also as a surface molecule involved in protection from apoptosis of monocytes, neutrophils and enterocytes.

**Objective:** Our aim was to evaluate the possible involvement of the soluble CD14 in the apoptotic pathway of human lymphocytes.

**Methods:** Freshly obtained human peripheral blood lymphocytes were cultured *in vitro* with gliotoxin, an apoptotic inducer. Human recombinant CD14 was added to the culture at physiological concentrations (10 µg/ml – 0.5 µg/ml) and apoptosis was assessed by cell membrane integrity using 7AAD, mitochondrial membrane potential by DiOC6(3) and cytoplasm shrinkage by cell size scatter analysis.

**Results:** Using DiOC6(3) we were able to show that human lymphocytes cultured in the presence of gliotoxin contained 63.8%±21 apoptotic cells, as opposed to 12.2%±11.5 in control cultures. Addition of recombinant human CD14 at a concentration of 10 µg/ml neutralized the apoptotic effect of gliotoxin back to 20.2%±10 (p<0.003). This inhibitory effect was dose dependent and blocked by CD14-specific monoclonal antibodies, but not by control antibodies. We then identified and synthesized the fragment within the CD14 molecule that was responsible for this apoptosis protective effect, and demonstrated its comparable protective efficacy *in vitro*. The results clearly reveal that this specific peptide, as opposed to a scrambled peptide, protected the lymphocytes from apoptosis, similarly to the full CD14 protein. We also demonstrate that this CD14 peptide penetrated *in vitro* the majority of the human lymphocytes.

**Conclusion:** Our data thus suggest that circulating CD14 may play an important role in the prevention of apoptosis of lymphocytes and perhaps of other cells. In addition we identified a peptide of CD14 that mediated this protective activity.

#### PB04/19 ADDRESSING THE FUNCTION OF TGFBR SIGNALLING FOR MATURE T HELPER LYMPHOCYTES BY USE OF A NOVEL CD4-CREERT2 MOUSE STRAIN

A. Sledzinska<sup>1</sup>, S. Hemmers<sup>2</sup>, W. Mueller<sup>3</sup>, A. Waisman<sup>4</sup>, B. Becher<sup>1</sup>, T. Buch<sup>1</sup>

<sup>1</sup>University of Zurich, Department of Pathology, Zurich, Switzerland, <sup>2</sup>Universitaet zu Köln, Institut für Genetik, Cologne, Germany, <sup>3</sup>University of Manchester, Faculty of Life Sciences, Manchester, United Kingdom, <sup>4</sup>Johannes Gutenberg University of Mainz, I Med. Klinik und Poliklinik, Mainz, Germany

TGFβ is a cytokine that plays a major role in regulating final differentiation processes within the T lymphocyte lineage promotes differentiation of thymocytes into NKT cells, regulatory and CD8 positive T cells. As shown by conditional ablation of TGFβ receptor and cytokine in T lymphocytes it also regulates peripheral T cell tolerance and homeostasis. However, a more detailed analysis of TGFβ signalling for these processes was hampered so far by the fact that recombination of the loxP-flanked allele took place already during thymic development and not during final differentiation. To overcome this limitation we have generated by targeted insertion into the CD4 locus a mouse strain that expresses the tamoxifen-inducible CreErt2 protein solely in CD4-expressing T lymphocytes. In initial reporter strain analyses we could obtain recombination frequencies within the T helper compartment as high as 70% after application of tamoxifen. We crossed our CD4-CreErt2 mouse strain with a loxP-flanked conditional TGFβRII allele. After administration of tamoxifen we do not find the protein on the cell surface of 80% of the analysed T helper cells and we investigate currently the functional consequence of the loss of TGFβRII from the surface of mature CD4-expressing T lymphocytes. In contrast to mice that have complete abrogation of TGF-β signalling in both CD4 and CD8 T cells since DP stage of development our mice do not suffer from severe autoimmune diseases. We could observe that CD4 T cells acquire activated phenotype after two weeks since tamoxifen application. The differences in survival of distinct subpopulations of CD4 T cells after abrogation of TGF-β signalling have been reported. We observed increased apoptosis in naïve peripheral CD4 T cell compartment whereas regulatory as well as effector T cells seem to expand.

#### PB04/20 ROLE OF DIPEPTIDYL PEPTIDASE IV (DP IV)-LIKE ENZYMES IN T LYMPHOCYTE ACTIVATION: INVESTIGATIONS IN DP IV/CD26-KNOCKOUT MICE

S. Wrenger<sup>1</sup>, A. Gohl<sup>1</sup>, U. Bank<sup>2</sup>, A. Thielitz<sup>3</sup>, J. Faust<sup>4</sup>, K. Neubert<sup>4</sup>, M. Täger<sup>2</sup>, S. Ansorge<sup>2</sup>, D. Reinhold<sup>1</sup>

<sup>1</sup>Otto-von-Guericke-University Magdeburg, Institute of Molecular and Clinical Immunology, Magdeburg, Germany, <sup>2</sup>IMTM GmbH, Magdeburg, Germany, <sup>3</sup>Otto-von-Guericke-University Magdeburg, University Clinic of Dermatology and Venereology, Magdeburg, Germany, <sup>4</sup>Martin-Luther-University Halle-Wittenberg, Institute of Biochemistry and Biotechnology, Department of Natural Sciences I, Halle, Germany

Dipeptidyl peptidase IV (DP IV, CD26) and DP IV-like enzymes such as dipeptidyl peptidase II (DP II), dipeptidyl peptidase 8 (DP8) or dipeptidyl peptidase 9 (DP9) have been recognized to regulate T lymphocyte activation. Lys[Z(NO<sub>2</sub>)]-thiazolidide (LZNT) and Lys[Z(NO<sub>2</sub>)]-pyrrolidide (LZNP), non-selective inhibitors of DP IV-like activity which target DP IV as well as DP II, DP8 and DP9, induce production of TGF-β1 and subsequent suppression of T lymphocyte proliferation *in vitro*. Moreover, these inhibitors are capable of decreasing the severity of autoimmune diseases like experimental autoimmune encephalomyelitis (EAE), the animal model of multiple sclerosis, and experimental arthritis, a model for human rheumatoid arthritis *in vivo*.

Here, we studied the influence of non-selective and selective inhibitors of DP IV-like enzymes on DNA synthesis in mitogen-stimulated splenocytes from wild-type C57BL/6 mice and DP IV/CD26-knockout (DP IV/CD26-KO) mice.

LZNT and LZNP, the non-selective inhibitors of DP IV-like activity, suppressed the DNA synthesis in stimulated splenocytes from wild-type and DP IV/CD26-KO mice to a comparable extent. Further, a selective inhibitor of DP8/DP9 activity was capable of suppressing DNA synthesis in mitogen-stimulated splenocytes of both wild-type and knockout mice to the same extent. In contrast, selective inhibitors of DP IV and DP II lacked this suppressive activity.

These results support the hypothesis that DP8 and/or DP9 represent additional pharmacological targets for the suppression of T cell proliferation and for anti-inflammatory therapy.

#### PB04/21 AUTOIMMUNE REGULATOR (AIRE) IS ACETYLATED BY TRANSCRIPTIONAL CO-ACTIVATOR P300

M. Saare<sup>1</sup>, A. Rebane<sup>1</sup>, P. Peterson<sup>1</sup>

<sup>1</sup>University of Tartu, Tartu, Estonia

**Objectives:** Aire is a regulator of transcription in the thymic medulla and controls the expression of a large set of genes that are considered to be peripheral-tissue specific, so-called self-antigens. The molecular mechanisms behind the induction of gene expression by Aire are poorly understood. Earlier studies have shown that Aire interacts with the transcription co-activator and acetyltransferase CBP, and together they synergistically activate the transcription of different reporter genes, as well as endogenous Aire target genes. Based on these findings we assumed that the acetylation could stimulate the activity of Aire.

**Methods:** Over expression of Aire with different acetyltransferases in HEK293 cell line followed by immunoprecipitation and mass-spectrometric analysis was used to determine the acetylation of Aire protein. Luciferase assays were conducted to study the transactivational properties of Aire and its mutants, and the results were confirmed with quantitative real-time PCR. Immunofluorescence experiments were carried out to evaluate the morphology of Aire nuclear bodies.

**Results:** We found that Aire is acetylated by CBP and even more highly by p300, a similar protein to CBP. The acetylated lysines are located in close proximity and within the putative DNA-binding domain SAND. We demonstrate that p300 can also cooperate with Aire in transcriptional activation and similarly to CBP localizes into Aire nuclear bodies. We found that the mutants mimicking acetylated Aire SAND domain have reduced transactivational capacity compared to mutants mimicking non-acetylated or wild-type Aire. Mutants mimicking the acetylated Aire SAND domain also display somewhat different localization, accumulating into fewer and larger nuclear bodies compared to mutants mimicking non-acetylated or wild-type Aire, showing that inactive Aire could be the main constituent of Aire nuclear bodies.

**Conclusion:** The results indicate that the ability of p300 to cooperate with Aire in transcriptional activation is separate from its ability to acetylate Aire. Studies with mutated forms of Aire suggest that acetylation may influence the composition of Aire nuclear bodies.

#### PB04/22 ZINC ASPARTATE (UNIZINK®) SUPPRESSES T CELL ACTIVATION *IN VITRO* AND *IN VIVO*

D. Stöve<sup>1</sup>, A. Gohl<sup>1</sup>, S. Wrenger<sup>1</sup>, A. Reinhold<sup>1</sup>, K. Grungriff<sup>2</sup>, D. Reinhold<sup>1</sup>

<sup>1</sup>Otto-von-Guericke University Magdeburg, Institute of Molecular and Clinical Immunology, Magdeburg, Germany, <sup>2</sup>Heydeckstr. 9, Magdeburg, Germany

Zinc is an essential trace element for growth and development of the organism and plays an important role in the maintenance of immune function. Zinc deficiency is shown to impair both, cells of the innate and the adaptive immune system. Diminished immune functions due to zinc deficiency can be normalized by adequate zinc supplementation. In contrast, high dosages of zinc induce negative effects on immune cells, similar to those observed with zinc deficiency.

The aim of the present study was to investigate the effect of Unizink® on T cell activation *in vitro* and on T cell-mediated autoimmunity *in vivo*. The influence of different concentrations of Unizink® on DNA synthesis, IL-2 and IL-17 production was measured in human stimulated T cells and in stimulated splenocytes of C57BL/6 mice. Furthermore, the effect of Unizink® in the experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis, was examined.

We found that Unizink® concentrations >100 µM suppress DNA synthesis and IL-2 production of pokeweed mitogen (PWM)- and anti-CD3-stimulated human T cells of healthy donors in a dose-dependent manner. Moreover, Unizink® decreases DNA synthesis and IL-2 production of PWM-stimulated splenocytes of C57BL/6 mice dose-dependently.

Several studies provided evidence that IL-17-producing T cells (Th17 cells) mainly contribute to the pathogenesis of autoimmune inflammation. Interestingly, we observed that Unizink® is capable of inhibiting IL-17 production in human PWM-stimulated T cells and in PWM-stimulated splenocytes of C57BL/6 mice.

Administration of Unizink® in a therapeutic manner (30 µg per day i.p., from day 11 to day 19) led to a significant reduction of the clinical severity of the EAE during the first relapse of the disease. In contrast, lower (< 10 µg per day) and higher (>100 µg per day) Unizink® concentrations had no therapeutic effects on the severity of the EAE.

Taken together, the data suggest that zinc can modulate activation, proliferation and cytokine production of T cells *in vitro* and *in vivo*.



**PB04/23 GENERATION OF MICE WITH INDUCIBLE T CELL-SPECIFIC EXPRESSION OF CRE**R. Antulov<sup>1</sup>, S. Mandarić<sup>1</sup>, B. Zafirova<sup>1</sup>, J. Arapović<sup>1</sup>, B. Polić<sup>1</sup><sup>1</sup>Rijeka School of Medicine, Department of Histology and Embryology, Rijeka, Croatia

**Objectives:** Inducible Cre/loxP system represents an effective tool to study the role of specific gene *in vivo*, providing spatial (cell type specific) and temporal (inducible) control of gene expression at the same time. Here, we report the generation and characterization of a mouse mutant strain with inducible T cell-specific control of Cre, which we have developed to study the role of several genes (i.e. TCRA, NKG2D) in the activation and homeostasis of mature T cells *in vivo*. We used inducible CreER<sup>T2</sup> system where the activity of CreER fusion protein can be induced by administration of tamoxifen.

**Methods:** We generated CD3-CreERT2 mice by targeting CD3e locus in ES cells. T cell specificity and inducibility of Cre were provided by knocking of the CreER<sup>T2</sup> cassette into the CD3e locus. We designed an appropriate targeting vector that was electroporated into the Bruce4 (C57BL/6) embryonic stem (ES) cells. Upon the selection and screening procedure, we identified several ES cell clones positive for the homologous recombination. Selected ES clones were microinjected into mouse blastocysts and several chimeras were obtained that gave the germline transmission of the mutation. Flow cytometry analysis and Q-PCR of various lymphocyte population were used for the characterization of CD3-CreERT2 mice.

**Results:** In order to examine the efficiency of tamoxifen inducible Cre mediated deletion in T cells, we crossed CD3-CreER<sup>T2</sup> mice to TCRCa<sup>fl</sup> mice, containing loxP flanked TCR Ca region. In this system, we showed conditional TCRA ablation on about 30% of CD4<sup>+</sup> and CD8<sup>+</sup> T cells mediated by Cre recombination upon tamoxifen administration. The specificity of Cre expression and quantification of Cre mediated deletion in different cell types and tissues were determined by quantitative PCR method.

**Conclusions:** Here we report generation and characterisation of mice with T cell specific and inducible control of Cre. We determined specific tamoxifen inducible Cre mediated deletion in T cells and showed deletion on about 30% of CD4<sup>+</sup> and CD8<sup>+</sup> T cells.

**PB04/24 EXPRESSION OF ERYTHROPOIETIN RECEPTOR ON CD4-POSITIVE T LYMPHOCYTES AND APOPTOSIS PROTECTION BY RECOMBINANT HUMAN ERYTHROPOIETIN**K. A. Lisowska<sup>1</sup>, A. Debska-Slizien<sup>1</sup>, A. Jozwik<sup>2</sup>, E. Bryl<sup>2</sup>, J. M. Witkowski<sup>2</sup>, B. Rutkowski<sup>2</sup><sup>1</sup>Medical University of Gdansk, Department of Nephrology, Transplantology and Internal Diseases, Gdansk, Poland, <sup>2</sup>Medical University of Gdansk, Department of Pathophysiology, Gdansk, Poland

Erythropoietin receptor (EPO-R) appears in the early stages of erythropoiesis. It has been also found in neurons, endothelial cells and polymorphonuclear leukocytes, indicating role of EPO beyond erythropoiesis. Previously we have described treatment with recombinant human erythropoietin (rhEPO) influence T lymphocytes function by improving IL-2 production, increasing CD28 and CD69 expression on CD4<sup>+</sup> T lymphocytes. RhEPO has also influence CD4<sup>+</sup> T lymphocytes proliferation. We also have found EPO-R molecules on surface of T lymphocytes, B lymphocytes and monocytes.

In the presented study we further examined expression of EPO-R on CD4<sup>+</sup> T lymphocytes using quantitative flow cytometry to calculate the exact numbers of those receptors and we observed changes in EPO-R expression in cell cultures of peripheral blood mononuclear cells (PBMC) depending on anti-CD3 stimulation and the rhEPO presence. We also studied influence of rhEPO on CD4<sup>+</sup> T lymphocytes apoptosis using annexin-V and 7-AAD staining.

We have detected EPO-R expression on T lymphocytes surface and in their cytoplasm. After 2 days of anti-CD3 stimulation surface EPO-R expression on CD4<sup>+</sup> T lymphocytes was increased. RhEPO presence additionally increased EPO-R expression. Incubation of CD4<sup>+</sup> T lymphocytes with rhEPO protected them from late apoptosis and necrosis cause by hydrogen peroxide but not by camptothecin.

Our results indicate that EPO-R expression on CD4<sup>+</sup> T lymphocytes can be modulated by anti-CD3 stimulation and rhEPO presence and it is important for necrosis and apoptosis protection.

**PB04/25 ROLE OF SPHINGOLIPIDS IN T LYMPHOCYTE ACTIVATION, ENERGY AND APOPTOSIS**E. A. Martinova<sup>1</sup><sup>1</sup>Institute of Nutrition RAMS, Fundamental Investigations, Moscow, Russian Federation

**Introduction:** We were the first who shown an influence of simple sphingolipids (Sphingosine, Sphinganine, C2-Ceramide, and natural inhibitor of Ceramide synthase mycotoxin Fumonisin B1) on T-lymphocyte receptor (TCR/CD3, CD4, CD8, CD25, CD45, etc.) expression and activation of neutral Sphingomyelinase in lymphocyte plasma membrane [Martinova et al, 1995; Martinova, 1996, 1998]. We also found that exogenous sphingolipids altered the Th1 and Th2 differentiation followed by a disruption of primary and secondary immune response and cell memory formation. Sphingolipids inhibited the DNA synthesis in T-lymphocytes, caused the cell cycle arrest or induced an apoptosis dependent on the cell cycle.

**Aims:** We investigated a mechanism of sphingolipid-dependent apoptosis in T-lymphocytes, particularly, a role of mitochondrial ATP-dependent Lon protease. Lon binds the mitochondrial DNA, works as a chaperon, and couples the signals of APT-dependent proteolysis and apoptosis. We also studied a cross-talk between Lon protease and energy-dependent kinase mTOR.

**Methods:** Sphingolipids [1nM – 10microM] were administered intraperitoneal to the BALB/c, CBA or C57BL/6 mice as well as were added in vitro to the primary thymic or spleen lymphocytes. Cells were stained with fluorescence-conjugated monoclonal antibodies and propidium iodide followed by a flow cytometry analysis. Oxygen reactive species release was measured by spectrophotometry. Mitochondrial respiratory chain complexes I, II, III and IV were detected. Dinitrophenol, CCCP, and Oligomycin were used as respiratory chain inhibitors.

**Results:** Primary immune response to T-dependent antigen (sheep red blood cells) does not modulate the Lon protease activity in thymus or spleen T-lymphocytes. Binding of TCR/CD3 complex or CD4 (CD8) receptors by monoclonal antibodies without antigen recognition causes the activation-induced apoptosis in T-lymphocytes accompanied by an elevation of Lon protease activity, mTOR expression, and disruption of cell energy balance. Expression of Lon protease in the non-activated mouse lymphocyte was elevated significantly after sphingolipid exposure in the manner different in the G1 and G2/M phases of cell cycle. Anti-Lon antibodies regulate the apoptotic signals in lymphocyte. Mitochondrial respiration inhibitors modulate the Lon protease expression induced by sphingolipids.

**Conclusions:** Simple sphingolipids modulate the lymphocyte activation, induce the apoptotic-related Lon protease expression, altered the kinase mTOR activity and disrupt the cell energy.N

**PB04/26 GLUCOCORTICOIDS PARTICIPATE IN THE DEVELOPMENT OF THYMUS ATROPHY FOUND DURING INFECTION WITH A HIGHLY VIRULENT STRAIN OF MYCOBACTERIUM AVIUM**M. Borges<sup>1</sup>, M. Flórido<sup>1</sup>, M. Correia-Neves<sup>2</sup>, R. Appelberg<sup>1</sup><sup>1</sup>Institute for Molecular and Cell Biology, University of Porto, Porto, Portugal, <sup>2</sup>Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Braga, Portugal

Immunocompromised patients, namely HIV-infected individuals, are the main targets of infection by *Mycobacterium avium*<sup>1</sup>. A decrease in lymphocyte number, namely in CD4<sup>+</sup> and CD8<sup>+</sup> cells, is observed during infection with *M. avium* and *Mycobacterium tuberculosis* as a consequence of T cell death by apoptosis. Our group has demonstrated that IFN-gamma produced during infection of C57BL/6 mice with a highly virulent strain of *M. avium* (25291) induces profound CD4<sup>+</sup> and CD8<sup>+</sup> cell and B cell depletion<sup>2</sup>. In this work, we show that this peripheral lymphopenia is accompanied by thymic atrophy through a selective depletion of CD4<sup>+</sup>CD8<sup>+</sup> thymocytes dependent on IFN-gamma. Thymectomy and infection with *M. avium* 25291 show additive effects on the development of lymphopenia. To establish the role of glucocorticoids (GCs) on the thymic atrophy and spleen lymphopenia we have determined the profile of corticosterone present in the blood throughout infection and the effect of blocking the GC receptors by the steroid type II GC receptor antagonist RU486 in the thymus and spleen cell populations. We found that GC blood levels are not increased during *M. avium* 25291 infection or dependent on IFN- gamma production. Treatment with RU486 was able to reverse the depletion of CD4<sup>+</sup>CD8<sup>+</sup> thymic cells but not peripheral lymphopenia. These results indicate that despite no increased GCs blood levels are found in infected compared to control mice the GC pathway may be involved in the thymus atrophy observed in our model of study.

<sup>1</sup> Appelberg R. Pathogenesis of *Mycobacterium avium* infection: typical responses to an atypical mycobacterium? *Immunol. Res.* 2006. 35: 179-90.

<sup>2</sup> Flórido M, Pearl JE, Solache A, Borges M, Haynes L, Cooper AM, Appelberg R. Gamma interferon-induced T-cell loss in virulent *Mycobacterium avium* infection. *Infect. Immun.* 2005. 73: 3577-86.

**PB04/27 HERPES VIRUSES HHV6, HHV7 AND LYMPHOCYTE SUB-POPULATIONS IN GASTROINTESTINAL CANCER PATIENTS AT DIFFERENT STAGES OF THE DISEASE**S. Donina<sup>1</sup>, S. Chapenko<sup>1</sup>, I. Jaunalksne<sup>2</sup>, M. Murovska<sup>1</sup>, A. Sultanova<sup>1</sup>, S. Kozireva<sup>3</sup><sup>1</sup>RSU A. Kirshenstein Institute of Microbiology and Virology, Riga, Latvia, <sup>2</sup>VISA Paula Stradina Clinical University Hospital, Clinical Immunology Center, Riga, Latvia, <sup>3</sup>RSU A. Kirshenstein Institute of Microbiology and Virology, Clinical Immunology Center, Riga, Latvia

**Introduction:** Impairment of cellular immune reactions contribute development of malignant tumors, but defect in afferent phase of immunity also may lead to host bearing herpes viruses activation and malignant process spread.

**Objectives:** of the study was to evaluate the frequency of HHV-6 and HHV-7 activation depending from the lymphocyte subsets in patients with gastrointestinal cancer at various stages of the disease before any specific treatment modalities were applied.

**Methods:** We examined 100 gastrointestinal cancer patients with histological conformed adenocarcinoma at stage I-IV of the disease. 36 patients were diagnosed at stage I -II and 64 patients – at stage III-IV of digestive tract cancer. Nested PCR with the PBL DNA as a template was used to detect latent/persistent infection, with the plasma DNA as template – to detect active viral infection (viremia). Lymphocyte subsets CD3+, CD4+, CD8+, CD16+, CD19+ as well as CD25+ and CD95+ in peripheral blood of gastrointestinal cancer patients were determined by laser flow-cytometer using corresponding monoclonal antibodies (Becton Dickinson, USA).

**Results:** Latent/persistent beta-herpes viruses' infection was found in 54/100 (54%) patients: HHV-6 – 14/100 (14%); HHV-7 – 39/100 (39%). In 25/100 (25%) patients active HHV-7 infection, in 2/100 (2%) – active HHV-6 infection was detected. Frequency of the viruses' activation was three times higher in patients with advanced disease in comparison to the stage I-II patients. Number of CD3+ and CD4+ cells decreased in patients with active viral infection at stage I-II as well as at stage III-IV in comparison to those who had no virus activation. Number of CD16+ cells was decreased in advanced stages group. Absolute count of immunocompetent cells bearing markers of activation on cell surface was elevated in patients with active viral infection independently of stage of the disease. CD4/CD8 ratio was decreased in both patients groups with active viral infection.

**Conclusions:** HHV6 and HHV7 infection was found in gastrointestinal cancer patients leading to cellular immune insufficiency. HHV7 infection was more frequent than HHV 6. CD4+, CD8+, CD16+ cell absolute count reflect impairment of induction phase of immune answer, affecting prognoses of malignant process spread.

#### PB04/28 VASCULAR ENDOTHELIAL GROWTH FACTOR AS ANTI-APOPTOTIC FACTOR IN ACUTE PANCREATITIS

S. Chooklin<sup>1</sup>, I. Bihalsky<sup>1</sup>

<sup>1</sup>Medical University, Lviv, Ukraine

One of principal causes of lethality of patients with severe acute pancreatitis is multiorgan dysfunction. Apoptosis may be involved in the mechanism of multiple organ dysfunction syndrome. Vascular endothelial growth factor (VEGF), also known as vascular permeability factor, is a glycoprotein with potent angiogenic, mitogenic, and vascular permeability-enhancing activities. VEGF can also stimulate cell migration and inhibit apoptosis. Apoptosis of liver and kidney was significantly inhibited by the administration of recombinant VEGF in experimental acute pancreatitis. The role of VEGF in acute pancreatitis patients is not clear.

Applying the ELISA technique, levels of VEGF were studied in 27 patients with acute pancreatitis. According the Atlanta criteria the mild pancreatitis was established in 10 patients and severe – in 17 patients.

The mean value of serum VEGF levels in patients with acute pancreatitis was significantly higher than that in healthy volunteer. It was a clear correlation between levels of VEGF and severity of pancreatitis. Serum VEGF levels with hepatic dysfunction were higher than those without hepatic dysfunction. Serum VEGF levels with renal insufficiency were significantly higher than those without renal insufficiency.

VEGF levels are significantly elevated in acute pancreatitis patients. VEGF is closely related to organ dysfunction in this disease. VEGF may function as not a vascular permeability factor, but a anti-apoptotic factor against the organ injuries in severe acute pancreatitis. Administration of VEGF or reinforcement of production of VEGF may be useful as a new therapeutic option in severe acute pancreatitis.

#### PB04/29 IN VITRO STUDIES TO EVALUATE A POTENTIAL IMMUNOMODULATORY ACTIVITY OF QUINTON SOLUTION ON HUMAN PBMC

J.E. Martínez-López<sup>1</sup>, P. Martínez-Peinado<sup>1</sup>, P. Maseres-Javaloy<sup>1</sup>, R. Córcoles-Córcoles<sup>1</sup>, R. López-Úbeda<sup>1</sup>, J.M. Sempere-Ortells<sup>1</sup>

<sup>1</sup>Universidad de Alicante, Group of Immunology, Biotechnology Department, San Vicente del Raspeig, Spain

**Objectives:** To check a potential immunomodulatory activity of Quinton Solution (ultrafiltrated cold processed seawater; Quinton Isotonic®) *in vitro*.

**Methods:** Isolated PBMC from 10 healthy volunteers were cultured in 96 well-plates under the following conditions: RPMI, Quinton Isotonic (ISO+) and Saline solution (SS), all of them supplemented with 1% antibiotic, 1% glutamine and 10% FCS; Quinton Isotonic without any supplement (ISO-) was also tested. Cells were stimulated for 4 days in a CO<sub>2</sub> incubator with PHA, PMA+Ionomycin or anti-CD3+anti-CD28. Cell viability, morphological changes and aggregates were analysed by optical/inverted microscope. CFSE assay and different monoclonal antibodies (CD3, CD4, CD8, CD19, CD25) were used to analyse proliferation and/or activation on several lymphocyte subsets by Flow Cytometry. Released haemoglobine from erythrocytes in every condition was measured by a modified-Drabkin procedure.

**Results:** Concerning cell parameters, the most important aspect to be considered is that ISO+ always had a similar behaviour to RPMI; even some aggregates were found in unstimulated cells that could reflect a certain ability of the solution to stimulate itself.

It is also interesting to observe that ISO+ vs. ISO- and SS, tended to increase the percentage of CD3+ lymphocytes coexpressing CD25, especially for the CD4 subset, although in a lesser extent than RPMI. Proliferation was also observed for the same stimulated subsets in ISO+ cultures vs. ISO- and SS, although always lower than that observed for RPMI.

Regarding haemolysis, released haemoglobin from ISO+ erythrocytes remained constant all along the 96 hours of culture, being always lower than that observed for RPMI, in which a constant increase of haemoglobin was detected since t=8h reaching its highest value at t=96h.

**Conclusion:** QUINTON solution is very well tolerated by cultured cells; morphology and cell viability is perfectly conserved all along the culture. When minimally supplemented, is able to emulate many of the results observed when using conventional culture media such as RPMI, in terms of activation, proliferation and aggregation. Furthermore, it seems to exert a possible protective effect on erythrocytes, avoiding spontaneous lysis. These results although preliminary, open new interesting fields to be evaluated with the product, both *in vitro* and *in vivo*.

#### PB04/30 IN VIVO DEMONSTRATION THAT THE NUMBER OF MHC/PEPTIDE COMPLEXES ON THE APC DETERMINES THE INITIAL BURST OF CD4 T CELL PROLIFERATION

M. Dusseaux<sup>1</sup>, G. Dorothee<sup>1</sup>, O. Lantz<sup>1</sup>

<sup>1</sup>INSERM U932 Institut Curie, Paris, France

Contrary to CD8+ T cells, CD4+ T cells seem to require a continuous presence of Antigen (Ag) to keep proliferating (Obst et al, J Exp Med, 2005). However, in some cases an autonomous proliferation has also been described (Lee et al, J Immunol, 2002). It is likely that the number of MHC/peptide complexes seen by the naïve CD4+ T cells during its first encounter with the antigen presenting cells (APC) determines the number of cell divisions. Some *in vitro* data support this hypothesis but *in vivo* data are scarce.

Using a model system recapitulating a localized primary immune response (Helft et al, Blood, 2008), we found that the cell division pattern of CFSE labelled CD4+ T cells is dependent upon the number of MHC/peptide complexes per APC but is independent of DC numbers. However, due to the asynchronized nature of our experimental system, we were not able to rigorously demonstrate the relationship between the number of MHC/peptide complexes per APC and the number of T cell divisions.

To answer this question, we used two experimental procedures: (i) *in vitro* synchronized stimulation followed by *in vivo* transfer: naïve CD4+ T cells are stimulated *in vitro* by dendritic cells (DC) loaded with different amount of peptide during a few hours before being sorted and injected back into mice; (ii) *in vivo* synchronized stimulation: Diphtheria toxin (DT) receptor bearing DCs loaded with different amounts of peptides are injected into the foot-pad before the transfer of naïve CD4 T cells. After 24 hours, DCs are killed with injection of DT and the entrance of new CD4 T cells into the lymph node is blocked with anti-CD62L antibody.

By studying the pattern of proliferation of these fully synchronized CD4 T cell populations, we can precisely determine *in vivo* the relationship between the amount of MHC/peptide complexes on the APC and the number of cell divisions. We found a direct correlation between the number of MHC/peptide complexes and the number of cell divisions indicating that the antigenic stimulation strength during the initial T/DC encounter directly determines the initial burst of CD4+ T cell proliferation.

### PB05 – T CELL MEMORY

#### PB05/1 CONTROL OF CD4 T CELL MEMORY RESPONSES BY THE NKG2D RECEPTOR

M. Lucas<sup>1</sup>

<sup>1</sup>Universität Freiburg, Institut für Medizinische Mikrobiologie und Hygiene, Freiburg, Germany

It is a newly evolving paradigm that activated and memory T cells upregulate the expression of germline-encoded receptors previously believed to be exclusively expressed by cells of the innate immune system. However, the role of these receptors for T cell function is largely unknown. We propose to probe the role of the stimulatory NKG2D receptor for the function of CD4 T cells during infection and autoimmunity. NKG2D associates with the signaling adaptor molecule DAP10 and recognizes a group of stress-inducible ligands which are not expressed on most normal cells but which are strongly upregulated by infected and stressed cells. These findings make the NKG2D receptor/ligand system likely to be involved in the immune response to infections as well as during autoimmunity. Although it is widely appreciated that activated CD8 T cells express NKG2D, expression of this activating receptor by CD4 T cells has not been thoroughly investigated. In order to probe the role of NKG2D for CD4 T cell responses, we have developed mouse models that allow us to study immune responses of CD4 T cells lacking NKG2D expression. To this end, we have crossed TCR transgenic mice (OT-II, SMARTA) to mice genetically lacking DAP10 or NKG2D. My preliminary data for the first time establish that after T cell stimulation, a subpopulation of mouse Th1 but not Th2 cells expresses the NKG2D receptor, which is able to potentially costimulate Th1 cells but fails to directly activate these cells. During memory responses against pathogens, the NKG2D<sup>+</sup> sub-population was the one most vividly responding and the lack of DAP10 expression at this stage may lead to a decreased proliferation of the antigen specific T cells. Our data indicate that NKG2D-mediated signals are important for the maintenance and function of memory CD4 T cells during infections. These studies will have important implications for developing more powerful vaccines.

**PB05/2 EFFECTS OF INTERFERON-G ON ANTIGEN-DEPENDENT CD8<sup>+</sup> T CELL HOMEOSTASIS**Ö. Sercan<sup>1,2</sup>, G.J. Hämmerling<sup>2</sup>, B. Arnold<sup>2</sup>, T. Schüller<sup>1,2</sup><sup>1</sup>Institute of Immunology, Charité, Berlin, Germany, <sup>2</sup>German Cancer Research Center (DKFZ), Molecular Immunology, Heidelberg, Germany

CD8<sup>+</sup> T cell homeostasis and memory generation are still poorly understood and are therefore major targets of immunological research aiming for the development of new vaccination procedures. Upon primary antigen (Ag) contact, CD8<sup>+</sup> T cells expand and produce interferon-gamma (IFNγ). IFNγ plays important roles in host defense against viral and bacterial pathogens. Recent evidence demonstrates that IFNγ also regulates Ag-dependent CD8<sup>+</sup> T cell homeostasis. However, it remains unclear which cells contribute to the regulation of IFNγ-associated CD8<sup>+</sup> T cell responses. In order to address this question, IFNγ/IFNγR-competent or -deficient T cell receptor transgenic CD8<sup>+</sup> T cells were adoptively transferred into IFNγ/IFNγR-knockout recipients. At different time points following immunization, effector and memory CD8<sup>+</sup> T cell development was evaluated. Here, we show that direct IFNγ action on Ag-specific CD8<sup>+</sup> T cells is not necessary for their optimal response. However, IFNγ that is produced both by T and host cells is sufficient to limit the numbers of effector and memory CD8<sup>+</sup> T cells via its action on host cells. Furthermore, we show that IFNγR signaling in host but not CD8<sup>+</sup> T cells regulates central and effector memory T cell differentiation. In the early phase of CD8<sup>+</sup> T cell responses, IFNγ-responsive CD11b<sup>+</sup> cells appear to regulate memory T cell differentiation. In conclusion, we show that host- and CD8<sup>+</sup> T cell-derived IFNγ can control the magnitude of CD8<sup>+</sup> T cell responses and memory differentiation via its action on host cells.

**PB05/3 EFFECTOR AND MEMORY CD8<sup>+</sup> T CELLS SHARE THE SAME PRECURSORS IN THE NAÏVE T CELL POOL**C. Gerlach<sup>1</sup>, J. van Heijst<sup>1</sup>, E. Swart<sup>1</sup>, D. Sie<sup>2</sup>, N. Armstrong<sup>3</sup>, R. Kerkhoven<sup>3</sup>, K. Schepers<sup>1</sup>, T. Schumacher<sup>1</sup><sup>1</sup>Netherlands Cancer Institute, Immunology, Amsterdam, Netherlands, <sup>2</sup>Netherlands Cancer Institute, Central Microarray Facility, Amsterdam, Netherlands, <sup>3</sup>Netherlands Cancer Institute, Bioinformatics and Statistics, Amsterdam, Netherlands

**Objectives:** Antigen-driven activation of naïve CD8<sup>+</sup> T cells results in the development of effector and memory T cell populations. However, the mechanism by which the immune system produces effector and memory T cells is largely unclear. In this project we set out to determine whether effector and memory CD8<sup>+</sup> T cells are derived from the same pool of naïve T cells or not.

**Method:** To allow a large-scale assessment of the development of single naïve T cells into different subsets, we have developed a technology that introduces unique genetic tags (barcodes) into naïve T cells.

**Results:** By comparing the barcodes present in different antigen-specific T cell populations in a *Listeria monocytogenes* infection model, we demonstrate that CD8<sup>+</sup> T cells that are present during the effector and memory phase, as well as memory precursor cells and short lived effector cells, are progeny of the same individual naïve T cells.

**Conclusion:** These data indicate that in this infection model, T cell differentiation towards effector or memory subsets is not determined by the nature of the priming APC or the time of T cell priming. In addition, the technology described here should be of value for a series of other questions related to the formation of different T cell subsets.

**PB05/4 MAINTAINED MEMORY INFLATION BUT DECREASED CD8<sup>+</sup> T CELL FUNCTION DURING CYTOMEGALOVIRUS INFECTION OF B CELL DEFICIENT MICE**V.S. Tchgang<sup>1</sup>, A. Mekker<sup>1</sup>, L. Häberli<sup>1</sup>, U. Karrer<sup>1</sup><sup>1</sup>Division of Infectious Diseases and Hospital Epidemiology, Department of Internal Medicine, Zurich, Switzerland

Infection with Cytomegalovirus (CMV) leads to life long viral persistence with the need for constant immune surveillance to prevent reactivation and disease. During long term infection, CMV-specific T cells slowly accumulate leading to large populations of CMV-specific CD8<sup>+</sup> T cells in ageing hosts occupying a significant part of the T cell compartment. Thus, CMV-driven memory inflation has been implicated in premature immune senescence. However, the crucial factors responsible for memory inflation have not yet been elucidated although antigen availability during CMV-reactivation is likely to be involved. Here we tested, whether memory inflation and immune senescence after mouse (M)CMV infection are influenced by increased viral dissemination in the absence of MCMV-control by B cells and antibodies using B cell-deficient μMT and JHT mice. In B cell-deficient mice, viral clearance was delayed in lung, spleen, salivary gland and liver after primary MCMV-infection. The kinetics of the CD8<sup>+</sup> T cell response specific for inflating (M38) epitopes was similar, whereas the frequency of non-inflating, M45-specific T cells was significantly reduced in B cell deficient mice. Moreover, the total number of M45-specific cells was reduced in spleen, lung and liver during MCMV-infection. In contrast, M38-specific T cell numbers were only reduced early after infection. However, functionality of M38 and M45-specific T cells concerning cytokine secretion (INFγ) and degranulation (CD107a) was reduced in B cell deficient mice, particularly during phases of active MCMV-replication.

**PB05/5 NAB2 REGULATES THE SURVIVAL OF REACTIVATED CD8<sup>+</sup> T CELLS BY AFFECTING THE EXPRESSION OF TRAIL**M.C. Walkers<sup>1</sup>, C. Gerlach<sup>2</sup>, E.M. Janssen<sup>3</sup>, P. Fitzgerald<sup>4</sup>, T.N. Schumacher<sup>2</sup>, D.R. Green<sup>4</sup>, S.P. Schoenberger<sup>1</sup><sup>1</sup>La Jolla Institute for Allergy & Immunology, Cellular Immunology, La Jolla, United States, <sup>2</sup>Netherlands Cancer Institute, Immunology, Amsterdam, Netherlands, <sup>3</sup>Cincinnati Children's Hospital Medical Center, Immunology, Cincinnati, United States, <sup>4</sup>St Jude Children's Research Hospital, Immunology, Memphis, United States

The generation of durable CD8<sup>+</sup> T cell immunity requires CD4<sup>+</sup> T cell help during the initial antigen encounter. Albeit primary CD8<sup>+</sup> T cell responses can proceed normally in the absence of T cell help, antigen-specific recall responses are severely compromised in these 'helpless' T cells via a transcriptional program that results in TNF-related apoptosis inducing ligand (TRAIL)-mediated apoptosis. Here we have studied the molecular mechanisms that regulate TRAIL expression in CD8<sup>+</sup> T cells. Micro-array analysis of polyclonal CD8<sup>+</sup> T cells revealed that the transcriptional co-repressor/co-activator Nab2 was differentially expressed in reactivated 'helped' versus 'helpless' CD8<sup>+</sup> T cells. We show that exogenous expression of Nab2 in helpless CD8<sup>+</sup> T cells blocks the induction of TRAIL upon restimulation. Furthermore, the dominant negative Nab2E51K suppresses the capacity of helped CD8<sup>+</sup> T cells to expand upon a secondary antigenic challenge, demonstrating a significant role of Nab2 for the survival of CD8<sup>+</sup> T cells during reactivation. We found that Interleukin-2, the survival factor for helpless CD8<sup>+</sup> T cells, induces the expression of Nab2, suggesting a link of Nab2 expression to TRAIL regulation through IL-2-mediated signaling. Our findings should help elucidate how the imprinting of proficient long-term reactivity in CD8<sup>+</sup> T cells can be achieved.

**PB05/6 HOW CHRONIC ANTIGEN PRESENTATION COMPROMISES CD4<sup>+</sup> T CELL MEMORY**S. Han<sup>1</sup>, R. Obst<sup>1</sup><sup>1</sup>Ludwig Maximilians-University Munich, Institute for Immunology, Munich, Germany

Within days after acute viral or bacterial infection, T cells expand and differentiate into effector cells that contribute to the pathogens' elimination. A fraction of the cells survives the following phase of apoptotic death and differentiate into mostly antigen-independent memory cells that express effector functions in response to secondary infections faster. This scenario is significantly altered in diseases where the pathogen cannot be completely eliminated and antigen persists. In a tetracycline-based transgenic mouse system where dose and time of antigen presentation by dendritic cells can be regulated in vivo, we tested how antigen presentation prolonged for one to four weeks beyond the priming phase affects memory cell differentiation of CD4<sup>+</sup> T cells. The cells were adoptively transferred into recipients whose dendritic cells expressed the respective antigen for different times and were analysed for surface markers, proliferation using BrdU and CFSE, and effector cytokine expression. We have found that proliferation, IL-2, TNFα and IFNγ secretion are differentially compromised by persisting antigen. The suppression of memory functions is detectable already by 10 days and are differentially reversible following antigen removal. The ability to proliferate can be almost completely suppressed by persisting antigen and appears to recover quickly after antigen removal. IL-2 and TNFα production, however, do not recover, while IFNγ secretion, which can hardly be suppressed, recovers easily. To our knowledge, this is the only system where the consequences of timed and reversible antigen presentation by dendritic cells can be studied in vivo without the complicating effects of innate immune effects of infection. The data indicate a stepwise induction of and recovery from exhaustion by persisting antigen only.

**PB05/7 CD70 DRIVEN CO-STIMULATION IMPAIRS MEMORY CD8 T CELL FORMATION**K. van Gisbergen<sup>1</sup>, R. van Olfen<sup>1</sup>, J. van Beek<sup>2</sup>, K. van der Sluijs<sup>3</sup>, R. Arens<sup>4</sup>, M. Nolte<sup>1</sup>, R. van Lier<sup>1</sup><sup>1</sup>Academic Medical Center, Experimental Immunology, Amsterdam, Netherlands, <sup>2</sup>Sanquin Research and Landsteiner Laboratory, Academic Medical Center Amsterdam, Amsterdam, Netherlands, <sup>3</sup>Academic Medical Center, Pulmonology, Amsterdam, Netherlands, <sup>4</sup>La Jolla Institute for Allergy and Immunology, La Jolla, United States

**Objectives:** The immune system responds to infection through the generation of short-lived effector T cells that eradicate pathogens and memory T cells that are maintained after pathogen clearance and protect against secondary infection. Co-stimulation has a positive effect on the strength of CD8 T cell responses, but its role in the development of effector versus memory CD8 T cells has not been examined. We analyzed the role of the co-stimulatory receptor-ligand pair CD27 and CD70 in effector versus memory T cell formation.

**Methods:** Therefore, we generated transgenic mice using a construct containing the murine CD70 gene under control of the human CD2 promoter. These mice constitutively express CD70 specifically on T cells and this transgenic CD70 is functional and able to trigger CD27.

**Results:** CD70 transgenic mice had an increased population of effector rather than memory CD8 T cells compared to wild-type mice. Similar to CD70 on APCs, CD70 on T cells provided co-stimulation that enhanced primary CD8 T cell responses against influenza infection. In contrast, memory CD8 T cell responses against influenza were severely impaired. CD70 did not impair differentiation into memory precursor CD8 T cells, but induced gradual disappearance of CD8 memory T cells in the memory phase after infection. Expression of CD70 during the primary infection and not thereafter was sufficient to trigger the elimination of memory



CD8 T cells. In vitro and in vivo experiments showed that induction of the Fas pathway of apoptosis through CD70 is the underlying mechanism that enables removal of memory CD8 T cells.

**Conclusion:** Thus, CD70-driven co-stimulation results in continual formation of effector CD8 T cells and in failure of memory CD8 T cell development, which are both hallmark events of chronic infection. CD70 induces FAS-mediated apoptosis of activated T cells, which may act as a pathway of negative feedback on CD70-driven T cell activation. Since CD70 is highly expressed in chronic infection, this qualifies CD70 as a potential target for intervention in chronic disease to prevent excessive effector formation and to re-establish memory formation.

#### PB05/8 LACK OF POLYFUNCTIONALITY OF GAG-SPECIFIC T LYMPHOCYTE RESPONSE IN HIV+ PATIENTS DEFINED “LONG TERM NON PROGRESSORS”

A. Cossarizza<sup>1</sup>, E. Nemes<sup>1</sup>, E. Lugli<sup>1</sup>, L. Bertoncelli<sup>1</sup>, M. Nasi<sup>1</sup>, L. Gibellini<sup>1</sup>, S. Manzini<sup>1</sup>, S. De Biasi<sup>1</sup>, M. Pinti<sup>1</sup>, L. Manzini<sup>2</sup>, L. Bisi<sup>2</sup>, V. Borghi<sup>2</sup>, C. Mussini<sup>2</sup>

<sup>1</sup>Univ. of Modena and Reggio Emilia, Dept. of Biomedical Sciences, Modena, Italy, <sup>2</sup>Azienda Ospedaliero-Universitaria, Infectious Diseases Clinics, Modena, Italy

Polyfunctional CD8+ T cells specific for HIV are supposed to play a role in controlling the production of the virus, measured by the plasma viral load (VL), and thus in delaying the progression of the infection. Less is known about T regulatory cells (Treg) and HIV-specific CD4+ in “long-term non progressors” (LTNP), i.e., those infected from at least 10 years, with a stable number of CD4+ T cells (>500 CD4+ T cells/uL), and who never took antiretroviral therapy.

We analyzed 10 LTNP, 8 treatment-naïve HIV+ patients with progressive disease (PROG) and 8 patients who underwent CD4-guided Structured Treatment Interruption and had to restart therapy (STI, 11-52 months without highly active antiretroviral therapy). By polychromatic flow cytometry (16 parameters flow cytometer CyFlow ML from Partec, Germany) in peripheral blood we evaluated HLA-DR+/- Treg (CD3+, CD4+, CD25+, FoxP3+, CD127-), and detected HIV-specific CD4+ and CD8+ T cells by simultaneously evaluating the expression of CD107a, CD40L, IL-2, IFN-gamma after stimulation with Gag overlapping peptides and exclusion of dead cells.

We observed that all groups displayed similar levels of Treg; in comparison with LTNP, STI patients showed a higher percentage of Treg and CD4+ that expressed HLA-DR+. LTNP had more CD4+ lymphocytes expressing CD127 if compared to PROG and STI patients. STI patients presented a higher HIV-specific CD4+ total response vs. LTNP, while HIV-specific CD8+ frequency was comparable in all groups. The overall quality of specific T cell response was similar in all patients. The majority of Gag-responding CD4+ cells were CD40L+ or CD107a+; cytokine production was detected in few responding CD4+. Gag-specific CD8+ response was dominated by CD107a+ cells, some of them producing also IFN-gamma+, while IL-2 was detected only in one LTNP. We could not highlight any association between the parameters we analyzed and CD4 count or VL, or between HIV-specific response and Treg frequency in LTNP.

The polyfunctionality of T cells specific for gag was very rare, even in LTNP. Most specific CD4+ cells did not produce TH1 cytokines, but were CD107a+ or CD40L+. The most striking difference between LTNP and PROG or STI patients was the expression of CD127 on CD4+ lymphocytes.

#### PB05/9 CD127 (IL-7 RECEPTOR ALPHA) DOWN-MODULATION BY MEMORY CD8 T CELLS IN THE BONE MARROW: COMPARISON OF CD127 TRANSGENIC AND WILD-TYPE C57BL/6 MICE

E. Parretta<sup>1</sup>, A.C. Quinci<sup>1</sup>, A. Santoni<sup>1</sup>, F. Di Rosa<sup>1</sup>

<sup>1</sup>Institute of Molecular Biology and Pathology, National Research Council, and Department of Experimental Medicine, Sapienza University of Rome, Rome, Italy

**Objectives:** Interleukin 7 (IL-7) is a master regulator of lymphopoiesis and T cell homeostasis. The IL-7 Receptor comprises an alpha-chain (CD127) and a gamma-chain (CD132), which is shared by receptors for IL-2, IL-4, IL-9, IL-15, IL-21. Membrane CD127 is down-modulated by CD8 T cells upon priming and in response to pro-survival cytokines. It has been proposed that CD127 is a marker of memory CD8 T cell differentiation. Our aim is to better understand the mechanisms regulating CD127 expression by CD8 T cells and the effects of CD127 modulation on CD8 T cell homeostasis.

**Methods:** Using flow cytometry, we analyzed CD127 membrane expression by CD8 T cells from different organs of either wild-type C57BL/6 (B6) or CD127 transgenic (CD127tg) mice. In CD127tg mice, early expression of high levels of CD127 by T cells leads to impaired thymic development and reduced peripheral T cell numbers, with high percentage of memory CD44high cells. Based on our previous results demonstrating that CD8 T cells are activated and proliferate in the bone marrow (BM) of B6 mice, we compared CD8 T cells from spleen, lymph nodes (LN) and BM in each mouse strain. We also performed adoptive transfer experiments to discriminate between CD8 T cell intrinsic factors versus the role played by the organ environment.

**Results:** In B6 mice, BM memory CD8 T cells always expressed lower levels of CD127 than corresponding cells from spleen and LN, either during an antigen-specific response or under steady-state conditions. In CD127tg mice, CD127 membrane expression by CD8 T cells was lower in BM than in spleen and LN, in the case of both naive CD44int/low and memory CD44high subsets. When splenic CD44high CD8 T cells from CD127tg mice were transferred into B6 hosts, CD127 membrane expression by donor cells was not reduced in the BM, as compared to spleen and LN. In contrast, CD127 down-regulation in the BM was observed when wild-type CD44high CD8 T cells were transferred into B6 hosts.

**Conclusion:** Our results suggest that CD127 surface expression not only depends on the differentiation stage of CD8 T cells, but is also regulated by the organ environment.

#### PB05/10 ABSENCE OF MEMORY FORMATION DESPITE POTENT CYTOTOXIC FUNCTION OF $\gamma$ C-DEFICIENT CD8+ EFFECTOR T CELLS

H. Decaluwe<sup>1,2</sup>, M. Taillardet<sup>1,2</sup>, Y. Rivière<sup>3</sup>, I. Munitić<sup>4</sup>, B. Rocha<sup>4</sup>, J.P. Di Santo<sup>1,2</sup>

<sup>1</sup>Institut Pasteur, Unité de Cytokines et Développement Lymphoïde, Paris, France, <sup>2</sup>INSERM U668, Paris, France, <sup>3</sup>Institut Pasteur, Laboratoire d'Immunopathologie Virale, Paris, France, <sup>4</sup>INSERM U591, Faculté de Médecine René Descartes, Paris, France

**Objectives:** Cytokines signaling through receptors sharing the common  $\gamma$ c chain ( $\gamma$ c), including IL-2, IL-7, IL-15 and IL-21, are critical for the generation and peripheral homeostasis of naive and memory T cells. However, their precise function in the initial proliferation and subsequent differentiation of CD8+ T cells after a viral challenge has not been thoroughly studied. Furthermore, in light of the current knowledge on the ontogeny of memory T cells, it is unclear at which step of the differentiation process these cytokines impact.

**Methods:** In order to define the role of  $\gamma$ c-dependent cytokines in the differentiation of CD8+ T cells, we compared the response of  $\gamma$ c+ or  $\gamma$ c- CD8+ T cells from P14 TCR transgenic mice after challenge with the lymphocytic choriomeningitis virus (LCMV Armstrong strain). The intrinsic survival defect of  $\gamma$ c- naive CD8+ T cells (due to their inability to respond to IL-7) was corrected by transgenic human Bcl-2 over-expression.

**Results:** Despite equal precursor numbers and identical kinetic of proliferation in the first days of infection, Bcl-2+  $\gamma$ c- CD8+ T cells generated ten-fold less effector cells at the peak of the response compared to their  $\gamma$ c+ counter-parts. Moreover, CD8+ memory T cells failed to develop in LCMV-infected mice in the absence of  $\gamma$ c. This lack of memory T cell generation could be linked to significant differences in the single-cell gene expression profile of a key transcription factor, T-bet, as well as numerous cytokines, cytokine receptors and cytolytic molecules. In spite of those important transcriptional differences, only discrete protein changes could be confirmed intracellularly (T-bet and granzyme B), or at the cell surface (KLRG1, CD27, CD43). Interestingly, despite their aberrant differentiation,  $\gamma$ c- cells could normally eliminate target cells, and could prevent the development of LCMV-induced hemophagocytic lymphohistiocytosis after adoptive transfer to perforin-deficient recipients.

**Conclusion:** These results demonstrate that although  $\gamma$ c-dependent signals are dispensable for the acquisition of adequate cytotoxic function, they condition the proliferation and complete differentiation of CD8+ effector T cells and are required for the appropriate generation of memory CD8+ T cells.

#### PB05/11 T CELL CYTOKINE RESPONSES IN *B. PERTUSSIS* EXPOSED AND INFECTED CHILDREN AND ADULTS

R.-M. Schure<sup>1</sup>, G. Berbers<sup>1</sup>, E. Sanders<sup>1</sup>, A.-M. Buisman<sup>1</sup>, Lis-Imm

<sup>1</sup>RIVM, LIS, Bilthoven, Netherlands

**Introduction:** Whooping cough remains a worldwide problem despite vaccination, resulting in the Netherlands in three yearly epidemics. Since long-term cellular immunity might play a major role in protection against *B. pertussis*, we studied *B. pertussis*-specific T-cell cytokine responses.

**Methods:** PBMCs were isolated from *B. pertussis* exposed or clearly infected adults and children as well as vaccinated adults. The exposed or infected persons were family members of clinically diagnosed and laboratory confirmed neonates.

PBMCs, depleted of B-cells, were stimulated with tetanus toxoid (TT) and the pertussis proteins: inactivated pertussis toxin (PTx), filamentous hemagglutinin (FHA) or pertactin (Prn) for 5 days. IFN- $\gamma$  was measured by ELISPOT-assay. The cytokines TNF- $\alpha$ , IFN- $\gamma$ , IL-12 IL-5, IL-13, IL10 and IL-17 were determined in culture supernatants by fluorescent bead-based multiplex immunoassay. A fourfold higher production of cytokines induced by *B. pertussis* antigens compared to the medium control was considered a responder.

**Results:** Infected children and adults showed a higher number of responders producing IFN- $\gamma$  spots to FHA and PTx compared to exposed children and adults or vaccinated adults (the controls). In exposed adults in comparison to vaccinated adults a high number of responders was found after stimulation with Ptx for IL-12, TNF- $\alpha$  and IFN- $\gamma$ , with Prn for IL-5, IL-13 and IL-10 and with FHA for all cytokines. When comparing exposed adults to exposed children higher IFN- $\gamma$  responders were found in adults for all *B. pertussis* antigens. The same high numbers of IFN- $\gamma$  responders were observed in infected children.

In exposed and infected adults a high number of responders to IL-17 was found for all three *B. pertussis* antigens compared to children, in adults this number is even higher after infection.

**Conclusion:** Adults induce better IL-17 responses compared to children. Adult persons exposed to or infected with *B. pertussis* trigger Th1, Th2 and IL-17 responses. Infection induces more Th1, Th2 and IL-17 responders in children, and only more IL-10 and IL-17 responders in adults. This might suggest that adults induce better T cell responses that protect to whooping cough in comparison with children.

**PB05/12 OPTIMAL IL-15-DEPENDENT HOMEOSTATIC MAINTENANCE OF CD8 T CELL MEMORY RELIES ON DENDRITIC CELLS AND MACROPHAGES AND IS AMPLIFIED BY CD27-CD70 AND CD137-CD137L CROSS-TALK**L. Frasca<sup>1</sup>, S. W. Stonier<sup>2</sup>, W. W. Overwijk<sup>2</sup>, K. S. Schluns<sup>2</sup><sup>1</sup>Istituto Superiore di Sanità, Infectious, Parasitic, Immune Mediated Diseases, Rome, Italy, <sup>2</sup>MD Anderson Cancer Center, Immunology, Houston, United States

Dendritic cells (DCs) support interleukin (IL)-15-mediated homeostatic proliferation of memory CD8 T cells *in vivo*; however, IL-15 trans-presentation by DCs insufficiently recovers normal homeostatic maintenance suggesting other cell types involvement. Furthermore, whether all DC subsets have equivalent abilities to mediate homeostatic proliferation is unknown. To evaluate the ability of various myeloid cells to trans-present IL-15, an *in vitro* model of homeostatic proliferation was developed by coculturing memory phenotype CD8 T cells with various types of myeloid cells. DCs and macrophages were best at mediating IL-15-dependent maintenance of memory CD8 T cells with DCs uniquely inducing IL-15-driven proliferation. DC subsets were equivalent, but plasmacytoid DCs (pDCs) were less efficient. Monocytes and granulocytes induced the lowest IL-15-mediated effects. Blocking CD70 and, to a lesser extent CD137L costimulation by DCs, inhibited the *in vitro* homeostatic maintenance, suggesting these molecules facilitate the mechanism of IL-15 trans-presentation to T cells. Factors that increase both IL-15 trans-presentation and CD70 expression by DCs, such as Type I interferon (IFN), enhanced homeostatic maintenance of memory CD8 T cells. Overall, this study demonstrates that myeloid cells other than DCs can provide homeostatic support to memory CD8 T cells; however, DCs are superior, which may relate to a more optimal source of costimulatory molecules such as CD70 and CD137L. The capacity of Type I IFN to increase memory CD8 T cells homeostatic proliferation is discussed for the potential pathological implications in autoimmune diseases characterized by high systemic and local enhancement of this immune regulatory cytokine.

**PB05/13 CONTROL OF VACCINIA VIRUS-SKIN LESION CALLS UP LONG-TERM MAINTAINED IFN $\gamma$ +TNF $\alpha$ + EFFECTOR/MEMORY CD4 LYMPHOCYTES IN HUMANS**B. Puissant<sup>1,2</sup>, P. Bossi<sup>3</sup>, F. Gay<sup>3</sup>, J.-M. Crance<sup>3</sup>, O. Bonduelle<sup>1,2</sup>, D. Garin<sup>3</sup>, B. Autran<sup>1,2,4</sup>, B. Combadière<sup>1,2,5</sup>, IMMUVAR study group<sup>1</sup>Institut National de la Santé et de la Recherche Médicale, INSERM U953, Paris, France, <sup>2</sup>Hôpital Pitié Salpêtrière, Université Pierre et Marie Curie, INSERM U945, Laboratoire d'Immunologie Cellulaire, Paris, France, <sup>3</sup>Laboratory of Virology, Centre de Recherche du Service de Santé des Armées – CRSSA Émile-Pardé, Grenoble, France, <sup>4</sup>Centre d'Investigation Biomédicales, Hôpital Pitié-Salpêtrière Assistance Publique-Hopitaux de Paris (AP-HP), Paris, France, <sup>5</sup>Interface, Hôpital Pitié-Salpêtrière Assistance Publique-Hopitaux de Paris (AP-HP), Paris, France

The persistence of a long-lasting immune memory against vaccinia and smallpox viruses has been a subject of considerable debate and its efficacy in protection remains unclear in the absence of circulating smallpox virus throughout the world. A significant proportion of the population has never been vaccinated against smallpox and the level of immunity remains variable in people who have been vaccinated. Question remains about the efficacy of such long-term memory in human. Historically, efficacy of vaccinia virus (VV) vaccination was considered with the presence of a skin lesion at the vaccination site. While, antibody responses have been widely proposed as correlate of efficacy and protection in human, the role of cellular and humoral immunity in VV-associated skin lesion formation remained to be determined. We asked whether long-term residual humoral and cellular immune memory to VV persisting 30 years after vaccination, could control VV-induced skin lesion. We showed for the first time that the residual VV-specific IFN $\gamma$ +TNF $\alpha$ + or IFN $\gamma$ +IL-2+ CD4 but not CD8 effector/memory lymphocytes expressing a skin-homing marker control the size of the skin lesion. Indeed, we showed that high residual effector T cells were significantly associated with lower VV-skin lesion size after re-vaccination. In contrast, long-term residual anti-VV neutralizing antibodies (NABs) did not affect the skin lesion formation, which in turn strongly correlated with high levels of NABs boost post-revaccination. These findings represent a qualitative change in existing knowledge of the immune response to VV and should help measurement of vaccination efficacy for clinical trials. It demonstrates the potential protective role of VV-specific CD4 responses against VV dissemination at the site of VV-associated skin lesion.

**PB05/14 HHV-8-SPECIFIC CD8 T CELL RESPONSES IN ACTIVE AND REMISSION HIV-RELATED KAPOSI SARCOMA**A. Guihot<sup>1</sup>, N. Dupin<sup>2</sup>, H. Moins-Teisserenc<sup>3</sup>, J. Cadranet<sup>4</sup>, A. G. Marcelin<sup>5</sup>, E. Oksenhendler<sup>6</sup>, B. Autran<sup>1</sup>, G. Carcelain<sup>1</sup><sup>1</sup>UMR U945, Pitié Salpêtrière, Paris, France, <sup>2</sup>Dermatology, Hôpital Cochin, Paris, France, <sup>3</sup>Immunology, Hôpital Saint Louis, Paris, France, <sup>4</sup>Pneumology, Hôpital Tenon, Paris, France, <sup>5</sup>UMR U720, Pitié Salpêtrière, Paris, France, <sup>6</sup>Clinical Immunology, Hôpital St Louis, Paris, France

**Background:** We previously demonstrated a lack of T cell responses to Human Herpesvirus-8 (HHV-8) in patients with active Kaposi sarcoma (KS), when compared to asymptomatic HHV-8 carriers who display higher frequencies of HHV-8-specific T cells in ELISpot IFN $\gamma$  assay (Guihot et al, J Infect Dis 06).

**Methods:** To further characterize this defect, we quantified HHV-8-specific T cells in ELISpot IFN $\gamma$  assays before and after *in vitro* HHV-8 peptide-specific T cell expansion, in patients with active KS (AKS, n=17) or remission KS (RKS, n=16). We further looked for HHV-8-specific CD8+ T cells using HLA-A2-peptide/tetramer in CMF and analyzed their differentiation and activation phenotype (CD28, CD27, CD38) in AKS and RKS patients with HLA-A2 genotype. MHC-HHV-8-peptide/tetramers used 3 HHV-8-specific peptides on K12, K15 and LANA-1 proteins.

**Results:** Frequencies of HHV-8-specific T cells were low in ELISpot IFN $\gamma$  assays, in both AKS and RKS groups of patients (n=2/16 and 3/17 responders, respectively), while EBV-specific T cells were largely detected in both groups as positive control. In non-responders, ELISpot assays with HHV-8-peptides expanded PBMC showed 8/9 responders (2 AKS and 6 RKS), demonstrating that HHV-8-specific functional T cells are very few but present in PBMC. Among A2-non responders in standard ELISpot assay, 1/2 AKS had significant CD8+K15-tetramere+ cells, mainly of late CD28-CD27- phenotype with 18% CD38+ cells; 1/7 RKS had significant CD8+K15-tetramere+ cells, mainly of early CD28+CD27+ phenotype with 2% CD38+ cells. More results of ELISpot IFN $\gamma$  assays with antagonized IL-10 and TGF $\beta$  to prevent inhibition of HHV-8-specific CD8 cells by regulatory T cells will be presented.

**Conclusions:** HHV-8-specific T cells lack but are present in PBMC of active and remission HIV-related KS patients. Some of these CD8+ cells are non-functional cells for being marked with tetramers, are non activated cells, and their differentiation phenotype are late in active KS but early in remission KS. This results offer new insights in the immunopathology of KS.

**PB05/15 CHARACTERISATION OF MYCOBACTERIUM TUBERCULOSIS – SPECIFIC MEMORY T-CELLS: A COMPARATIVE STUDY IN A HIGH ENDEMIC AREA BETWEEN PATIENTS UNDERGOING THERAPY AND HEALTHY LATENTLY INFECTED CONTACTS**H. Müller<sup>1</sup>, H. J. Golakai<sup>2</sup>, K. Stanley<sup>2</sup>, T. Roberts<sup>2</sup>, A. Loxton<sup>2</sup>, G. Walzl<sup>2</sup>, S. H. E. Kaufmann<sup>1</sup>, M. Jacobsen<sup>3</sup><sup>1</sup>Max-Planck-Institute for Infection Biology, Immunology, Berlin, Germany, <sup>2</sup>Stellenbosch University, Stellenbosch, South Africa, <sup>3</sup>Bernhard-Nocht Institute for Tropical Medicine, Hamburg, Germany

Previous work in the mouse model has provided evidence for a potential association of polyfunctional memory T cells with the protection against intracellular pathogen. Based on these findings we addressed the question whether this also holds true for human tuberculosis (TB). We established a field site in Cape Town, South Africa, recruited TB patients and followed them up during anti-tuberculosis drug therapy. We then compared our findings with results from healthy latently infected contacts (LTBI) using multi color flow cytometry for the measurement of intracellular cytokines. We found that after short-term PBMC stimulation with PPD, multifunctional memory T cells (CD45RO+) co-expressing IFN $\gamma$ , TNF $\alpha$ , Interleukin 2 (IL-2) and GM-CSF were strongly represented in both treated and untreated TB patients. Interestingly, the proportion of polyfunctional memory T cells was also found in LTBI. Furthermore, no significant differences between the cytokine profile of TB patients and LTBI were observed after antigen stimulation. In order to have a closer look at the potential of the CD4+ T cells to produce cytokines we incubated PBMCs with a superantigen. In this case the profile was significantly different between these two groups and it changed during therapy. While the expression of IFN $\gamma$  was significantly lower in TB patients in comparison to LTBI and increased under therapy, the expression of TNF $\alpha$  showed a significant decrease under therapy. Also the expression of IL-2 was significantly higher in TB patients compared to LTBI. The potential of CD4+ T cells to express Interleukin 4, Interleukin 17 and GM-CSF did not differ between these two study groups and remained stable during therapy. In an additional experiment, we determined the degranulation in CD8+ T cells, by measuring CD107a, but could not detect any differences between TB patients and LTBI whether they were undergoing therapy or not.

To conclude, it can be said that upon PPD stimulation, polyfunctional memory T cells are found in TB patients pre- and post therapy as well as in LTBI. Our data is not consistent with the notion that multifunctionality of memory T cells is a correlate of protection against TB infection.

**PB05/16 CRYPTIC AND IMMUNODOMINANT EPITOPES FROM ANTHRAX LETHAL FACTOR: COMPARISON OF T CELL EPITOPE RECOGNITION FOLLOWING PROTEIN IMMUNISATION AND LIVE BACTERIAL CHALLENGE**S. Ascoug<sup>1</sup>, R. J. Ingram<sup>1</sup>, K. Chu<sup>1</sup>, E. D. Williamson<sup>2</sup>, B. Maillere<sup>3</sup>, S. Srisakandan<sup>3</sup>, D. M. Altmann<sup>1</sup><sup>1</sup>Imperial College, Hammersmith Hospital, Infectious Diseases, London, United Kingdom, <sup>2</sup>Dstl, Porton Down, Biomedical Sciences, Salisbury, United Kingdom, <sup>3</sup>CEA Saclay, Service d'Ingénierie Moléculaires des Protéines, Saclay, France

The causative agent of the infectious disease anthrax, *Bacillus anthracis*, mediates pathogenicity through the action of three immunomodulatory toxins. One of these toxins, the metalloprotease, lethal factor (LF), is a principle antigen of *B. anthracis* and a source of vaccine candidate epitopes. T cells responding to immunisation with a complex protein antigen such as LF are usually specific for a limited number of peptide epitopes. In contrast to these immunodominant epitopes, responses to cryptic epitopes are only revealed following immunisation with the individual peptide antigen. The immunodominance hierarchy of these bacterial antigens has a direct relevance to the epitopes recognised by host T cells during vaccination and natural infection. Using a humanised HLA class II transgenic mouse strain, we have defined HLA-DR4 restricted CD4+ T cell responses to the immunodominant and cryptic epitopes processed and presented following immunisation. This was compared to the T cell epitopes identified in the context of live *B. anthracis* infection. There was minimal overlap of immunodominant epitopes between immunisation and infection. However there was remarkable parity between cryptic epitopes and infection specific epitopes. Our data suggest that infection influences the epitopes that are processed and presented by APCs, shifting the repertoire towards the unveiling of previously cryptic epitopes.

**PB05/17 ANTIGEN SPECIFIC T-T INTERACTIONS REGULATE CD4 T CELL EXPANSION**J. Helft<sup>1</sup>, A. Jacquet<sup>1</sup>, N. Joncker<sup>1</sup>, M. Dusséaux<sup>1</sup>, I. Grandjean<sup>1</sup>, G. Dorothée<sup>1</sup>, A. Kissenpfennig<sup>2</sup>, B. Malissen<sup>2</sup>, P. Matzinger<sup>3</sup>, O. Lantzi<sup>1</sup><sup>1</sup>Institut Curie-Inserm, Laboratoire d'immunologie et U932, Paris, France, <sup>2</sup>Inserm, U631, CIML, Marseille, France, <sup>3</sup>The Ghost Lab, LCMi, NIAID, NIH, Bethesda, United States

The mechanisms limiting T cell expansion during an immune response are poorly understood. The regulation of CD4 T cell numbers should take account of the amount of antigen (Ag), the initial frequency of Ag-specific T cells, the mix of naïve versus experienced cells, and (ideally) the diversity of the repertoire. As Ag lasts in vivo much longer than anticipated, competition for Ag, which is the main mechanism usually proposed for CD4 T cell proliferation regulation, would lead to a rapid narrowing of the T cell repertoire. In fact, this is not observed.

Here we describe a novel mechanism of T cell regulation that potentially deals with all of these parameters. Using a 2-cohort CD4 T cell system, we studied the recruitment of naïve or Ag-experienced CD4 T cells into a localized immune response.

We found that the recruitment of Ag-experienced CD4 T cells is selectively inhibited compared to that of naïve T cells. CD4 T cells establish a negative feedback loop by capturing their cognate MHC/peptide complexes from Ag-presenting cells and presenting them to Ag-experienced CD4 T cells, thereby inhibiting their recruitment into the response while allowing recruitment of naïve T cells. The inhibition is Ag specific, begins at day 2 (long before Ag disappearance), and cannot be overcome by providing new Ag-loaded dendritic cells. This inhibition is observed at physiological number of T cells and can be mediated by polyclonal CD4 T cells. This preferential inhibition of Ag-experienced CD4 T cells can be transferred in vivo by Ag bearing CD4 T cells but not by non-specifically activated T cells. This specific inhibition of Ag-experienced T cell recruitment from an ongoing immune response explains the so-called transgenic artifact observed when the frequency of responding T cells is very high. This mechanism may also be involved in the regulation of memory versus effector CD4 T cell differentiation. Thus, in comparison with mere competition for Ag, this mechanism enables a regulation of the CD4 T cell proliferation in a functional relationship to the amount of Ag, while allowing naïve T cells to generate repertoire variety.

**PB05/18 CD40L EXPRESSION ON CD4<sup>+</sup> T-CELLS EX VIVO – A TOOL FOR ANALYSIS OF THE DIFFERENTIATION OF ENDOGENOUS MEMORY CELLS**M. Rudolph<sup>1,2</sup>, K. Knieke<sup>1</sup>, M. C. Brunner-Weinzierl<sup>1</sup><sup>1</sup>Otto-von-Guericke-University Magdeburg, Department of Pediatrics, Magdeburg, Germany, <sup>2</sup>Charité-University Hospital and Deutsches Rheuma-Forschungszentrum, Berlin, Germany

The generation of endogenous memory T-cells *in vivo* has so far been challenging as no reliable surface molecule was available for *ex vivo* monitoring of antigen-specific CD4<sup>+</sup> T-cells. Recently it was shown, that expression of the surface glycoprotein CD40L identifies activated T-cells after antigen-specific stimulation. We evaluated the CD40L expression as a tool for analysis of the differentiation of CD4<sup>+</sup> cells from effector to memory T-cells *in vivo*. For this purpose animals were immunized at the outer part of the ear pinna and the antigen was removed at various time points. Total spleen or lymphnode cells were restimulated *in vitro* for the induction of the CD40L expression. We assessed triple-, double- and single-production of IFN- $\gamma$ , IL2 and TNF $\alpha$  of individual cells to define the quality of CD4<sup>+</sup> T-cells during the effector as well as memory response.

At early time points after immunization CD40L<sup>+</sup> CD4<sup>+</sup> T-cells produce one or two but only a small fraction produced all of the three analyzed cytokines. The frequency of the multifunctional CD40L<sup>+</sup> CD4<sup>+</sup> T-cells subset, which was able to produce IFN- $\gamma$ , IL2 and TNF $\alpha$  simultaneously, increased two months after removal of the antigen. Furthermore we detected heterogeneity in the median fluorescence intensity for IFN- $\gamma$  and CD40L among the subsets of single, double or triple cytokine producers, reflecting differences in cytokine production and CD40L expression. Almost all of the CD40L<sup>+</sup> CD4<sup>+</sup> T-cells express CD127 and CD44 and show a fast recall response two months after antigen removal, a typical feature of memory T-cells.

Using CD40L as marker for endogenous antigen-specific T-cells, provides now the opportunity to analyze easily the influence of various factors (e.g. time-period of immunization, antigen character, antibody-treatment) on the development and quality of *in vivo* generated memory CD4<sup>+</sup> T-cell.

**PB05/19 MEMORY T CELL RESPONSES AFTER MEASLES BOOSTER VACCINATION**J.A.J. Sonnsma<sup>1</sup>, H.I. ten Hulscher<sup>1</sup>, G.A. Berbers<sup>1</sup>, H.C. Rumke<sup>2</sup>, R.S. van Binnendijk<sup>1</sup><sup>1</sup>National Institute for Public Health and The Environment, Laboratory for Infectious Diseases and Perinatal Screening, Bilthoven, Netherlands, <sup>2</sup>Vaxinostics b.v., University Vaccine Center, Rotterdam, Netherlands

**Introduction:** Although it is assumed, that measles vaccination leads to lifelong immune protection, specific antibody titers decline in adults who had been previously vaccinated against measles. Traditionally, measles immunity is expressed in specific antibody titers or plaque reduction neutralization titers (PRNT). Titers below 0.2 International Units per ml (IU/ml) are considered non-protective for measles, but this value actually lacks a firm scientific basis. We investigated vaccinated adults and immunised them with measles vaccine. We studied T cell precursor frequencies longitudinally in peripheral blood and their secreted cytokine profiles.

**Methods:** Measles immunised healthy adults were divided into three groups: low (< 0.2 IU/ml), medium (0.2-0.8 IU/ml) or high titers (>0.8 IU/ml). PBMCs isolated from these groups were stimulated in vitro with inactivated measles antigen, tetanus toxoid, influenza antigen and autologous measles infected B-LCLs for 5 days. Cells were transferred to pre-coated ELISPOT plates and analyzed for IFN- $\gamma$  producing T cells. Culture supernatants were analyzed by fluorescent bead-based multiplex immunoassay for the following cytokines: IFN- $\gamma$ , IFN- $\alpha$ 2, TNF- $\alpha$ , IL-1 $\alpha$ , IL-4, IL-5, IL-10, IL-13 and IL-17. The significance of the T cell response was calculated by comparing the outcome of specific versus control stimulation.

**Results:** Individuals from the low titer group showed approximately in 25% of the cases significant T cell response at day 0, increasing to 50% at time points 3 and 9 months post-vaccination. Medium and high titer groups already demonstrated the presence of specific T cells in at least 50% of the individuals before vaccination, which persisted after vaccination. Individuals in the medium and high titer group showed overall 2 to 3 times higher measles virus specific IFN- $\gamma$  response than persons in the low titer group. Remarkably, all groups had sustained their specific T cell responses after 9 months. Furthermore, the significance of the Th2 cytokine IL-13 in response to measles antigen was detected in all groups.

**Conclusion:** Our data clearly indicate that individuals with critical antibody titers against measles do have memory T cells, which sustain at least over a period of 9 months after measles vaccination. The indication of a mixed Th1/Th2 T cell response needs further investigation.

**PB05/20 DIFFERENTIAL EXPRESSION OF SUPPRESSOR OF CYTOKINE SIGNALING (SOCS) MOLECULES IN T CELLS BETWEEN TUBERCULOSIS PATIENTS AND HEALTHY LATENTLY M. TUBERCULOSIS INFECTED CONTACTS**M. Jacobsen<sup>1,2</sup>, D. Repsilber<sup>3</sup>, K. Kleinstaubert<sup>2</sup>, A. Gutschmidt<sup>1</sup>, S. Leitner<sup>1</sup>, S. Parida<sup>1</sup>, G. Black<sup>4</sup>, G. Walzl<sup>4</sup>, S.H.E. Kaufmann<sup>1</sup><sup>1</sup>Max-Planck-Institute for Infection Biology, Berlin, Germany, <sup>2</sup>Bernhard-Nocht-Institute for Tropical Medicine, Hamburg, Germany, <sup>3</sup>Research Institute for the Biology of Farm Animals, Genetics and Biometry, Dummerstorf, Germany, <sup>4</sup>Faculty of Health Sciences, University of Stellenbosch, Cape Town, South Africa

In the vast majority (about 90%) of individuals infected with *Mycobacterium tuberculosis* (*M. tuberculosis*) the pathogen is controlled by an efficacious immune response leading to latent *M. tuberculosis* infection (LTBI) without symptoms of disease. Although essential mediators of protection, i.e., T cells, TNF $\alpha$ , and IFN $\gamma$ , have been identified, the underlying mechanisms of protection are ill defined and reliable biomarkers of protection are missing. The aim of this study was to identify novel T-cell factors from the blood of tuberculosis (TB) patients, LTBI, and non-infected (Quantiferon and TST negative; QFTneg) household contacts.

We compare gene expression profiles of enriched CD3<sup>+</sup> T cells from 41 TB patients, 41 LTBI, and 11 QFTneg household contacts by microarray analyses. After adjustment for multiple testing we determine significantly differentially expressed genes between TB patients and LTBI (set X), as well as between TB patients and QFTneg household contacts (set Y). Genes which are part of both groups (set X AND set Y) are then subtracted from set X and the remaining genes are chosen as biomarker candidates of protection. Functional categorization revealed a subgroup of genes involved in cytokine receptor signaling including three suppressor of cytokine signaling (SOCS) family members including SOCS2, SOCS3, and CISH. Differential expressed candidates are verified by quantitative PCR analyses and assessed for the capacity to discriminate members from both study groups. A minimal group of genes could be identified that is optimally suited for classification of TB patients, LTBI, and QFTneg household contacts.

This study identifies SOCS family members as possible T-cell factors relevant for protection against TB and raises the question about the mechanisms how differential SOCS molecule expression influences T-cell immunity against *M. tuberculosis*.

**PB05/21 DELAYED ANTIBODY RESPONSE TO TICK-BORNE ENCEPHALITIS VIRUS VACCINATION AND DECREASED CD4<sup>+</sup> NAIVE T-CELLS IN CHILDREN AFTER THYMECTOMY**M. Zlamy<sup>1</sup>, C. Gögele<sup>1</sup>, R. Würzner<sup>2</sup>, C. Wilk<sup>1</sup>, R. Geiger<sup>3</sup>, L.-B. Zimmerhackl<sup>1</sup>, M. Prelog<sup>1</sup><sup>1</sup>Medical University Innsbruck, Department of Pediatrics I, Innsbruck, Austria, <sup>2</sup>Department of Hygiene, Microbiology and Social Medicine, Innsbruck, Austria, <sup>3</sup>Medical University Innsbruck, Department of Pediatrics III, Innsbruck, Austria

**Objective:** Thymectomy in early childhood due to open heart surgery leads to immunosenescence characterized by a decrease of naïve T-cells. The aim of our study was to investigate whether children after thymectomy may show a poor antibody response to new antigens like vaccines.

**Methods:** Thus, 44 thymectomized and 56 non-thymectomized healthy age-matched children were vaccinated with tick-borne encephalitis virus (TBEV) vaccine (FSME Immun junior, Baxter, Vienna, Austria) following the standard 3 dose vaccination schedule. IgG levels were evaluated each 4 weeks after the second and third vaccination. Testing of TBEV IgG levels and IgG avidity with a commercial test kit (Euroimmun, Lübeck, Germany) was performed 3 years after the first vaccination. T-cells were gated for naïve (CD28+CD45RO+) and memory T-cells (CD28+CD45RA+) by flow cytometry.



**Results:** Thymectomized children showed 2.2-fold lower TBEV IgG antibody levels after the second vaccination when compared to controls ( $p=0.03$ ), but a normal response after the third vaccination. Two years after the third vaccination, there was neither a statistical difference comparing IgG antibody levels of patients and controls, nor any differences according to avidity. When considering total counts of CD4+ T-cells, patients showed a significant decrease of naive CD4+ T-cells with age ( $p=0.002$ ) and an increase of CD4+ memory T-cells ( $p=0.004$ ) compared to controls.

**Conclusion:** Our results showed a delayed increase of TBEV IgG antibody levels after vaccination in thymectomized children and quantitative changes in T-cell subsets. This may indicate alterations of the primary T-cell immune response to new antigens but a normal memory function. Thus, it is mandatory to monitor antibody responses to other vaccinations as well as infection rates in thymectomized children to avoid long-term complications.

#### PB05/22 EFFECTOR AND MEMORY DEVELOPMENT OF CD8<sup>+</sup> T LYMPHOCYTES DIFFERENTIALLY REGULATED BY N-RAS

S. Iborra<sup>1</sup>, E. Fernández<sup>2</sup>, M. Ramos<sup>1</sup>, E. Santos<sup>3</sup>, S. Lázaro<sup>1</sup>, F. Aguilar<sup>1</sup>, D. López<sup>1</sup>, M. Del Val<sup>1,4</sup>

<sup>1</sup>Instituto de Salud Carlos III. Centro Nacional de Microbiología, Immunología Viral, Majadahonda, Spain, <sup>2</sup>Universidad Complutense de Madrid. F. Medicina, Inmunología, Madrid, Spain, <sup>3</sup>Centro de Investigación del Cáncer. Universidad de Salamanca-CSIC, Salamanca, Spain, <sup>4</sup>Centro de Biología Molecular, CSIC-UAM, Madrid, Spain

Signal transduction down the Ras/MAPK pathway is critical to T cell activation, proliferation and differentiation. Mammalian genomes encode three *ras* genes, *H-ras*, *K-ras*, and *N-ras*, their products have very similar structures, yet their specific functions are poorly understood. All three *ras* genes are expressed in T lymphocytes, but several evidences indicate that *N-ras* isoform has a particular significance in T cell signalling. This study sought to determine if *N-ras* protein is required for CD8<sup>+</sup> T-lymphocyte function. *In vitro* analyses were performed using mice deficient in *N-ras* that express the transgenic OT-I TCR, which recognizes the ovalbumin<sup>257</sup>SIINFEKL<sup>264</sup> epitope presented by H-2K<sup>b</sup>. Our data suggest that *N-ras* is not essential for activation and CTL effector functions. In *N-ras* deficient mice, we have determined the *ex vivo* cytokine response and *in vivo* cytotoxic function of CD8<sup>+</sup> T lymphocytes induced by vaccinia infection or dendritic cell immunization. Our results support a dual role for *N-ras* as a survival factor during CD8<sup>+</sup> T contraction following viral infection and in memory responses.

#### PB05/23 ROLE OF HISTONE MODIFICATIONS IN MEMORY CD8 T CELL RESPONSE

Y. Araki<sup>1</sup>, N.-P. Weng<sup>1</sup>

<sup>1</sup>NIA/NIH, Immunology, Baltimore, United States

Memory CD8 T cells derived from naïve CD8 T cells after antigenic stimulation are able to mount a more rapid and robust recall immune response than the primary response. Differential expression of the effector molecules and their master regulators provides a key base of memory CD8 T cell functions. How memory CD8 T cells acquire and maintain this differential gene expression is currently unknown. Histone modifications has been recognized as a major player in regulating chromatin states and gene expression yet little is known about their involvement in the function of memory CD8 T cells. We examined three histone modifications (H3K9ac, H3K4me3, H3K27me3) in the gene loci of several effector molecules and their master transcription factors in naïve, central (T<sub>CM</sub>) and effector (T<sub>EM</sub>) memory CD8 T cells. Gene expressions were positively correlated with the levels of H3K9ac and H3K4me3 and negatively correlated with the levels of H3K27me3 in these gene loci. Furthermore, the patterns and the time of histone modifications in these gene loci differ in the resting and activated naïve and memory CD8 T cells, reflecting an additional layer of the regulation corresponding to their function. Finally, reduction of H3K9Ac level resulted in a decrease expression of these differentially expressed genes in memory CD8 T cells. Together, these findings suggest that change of chromatin structure mediated by histone modifications may serve a fundamental basis for the rapid and robust response of memory CD8 T cells.

#### PB05/24 CHARACTERIZATION OF DENGUE VIRUS NS3 PROTEIN SPECIFIC T CELL RESPONSES IN HEALTHY IMMUNE DONORS WITH VARYING SEVERITY OF PAST DENGUE INFECTION

G.N. Malavige<sup>1,2</sup>, L. T. Rohanachandra<sup>2</sup>, L. Crack<sup>1</sup>, N. Fernando<sup>2</sup>, M. Peelawatta<sup>2</sup>, S. D. Jayaratne<sup>2</sup>, G. S. Ogg<sup>1,3</sup>

<sup>1</sup>University of Oxford, Oxford, United Kingdom, <sup>2</sup>University of Sri Jayawardenapura, Colombo, Sri Lanka, <sup>3</sup>Churchill Hospital, Department of Dermatology, Oxford, United Kingdom

**Objectives:** Dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS) usually occur during secondary dengue infections. However, DHF/DSS only occur in a minority of individuals experiencing secondary dengue infections. Cross reactive memory T cells of previous dengue infections, which are of lower avidity to the infecting virus, are thought to cause severe disease by altering the cytokine profiles during secondary dengue infections. Therefore, we set out to investigate memory T cell responses in adult individuals with a past history of varying severity of dengue infection.

**Methods:** Based on epidemiological data, as DEN2 and DEN3 viruses were responsible for almost 95% of the infections during the last 2 decades in Sri Lanka, overlapping 20mer peptides of NS3 of these 2 viruses were used. *Ex vivo* IFN $\gamma$  ELISpots were used to investigate DEN2 and DEN3, NS3 specific T cell responses in healthy donors from Sri Lanka, with a past history of DHF ( $n=9$ ) and healthy individuals with who never had a symptomatic dengue infection or had dengue fever ( $n=9$ ).

**Results:** All individuals responded to the majority of the peptide pools of both DEN2 and DEN3 NS3 protein. IFN $\gamma$  responses to these peptide pools varied from 0 spot forming units (SFU)/1 million PBMCs to 3345SFU/1 million PBMCs. There was no significant difference between the overall IFN $\gamma$  responses to NS3 of either DEN2 (mean 1479, SD $\pm$ 532.4) or DEN3 (mean 1622, SD $\pm$ 799.4). Although individuals with a past history of DHF had higher IFN $\gamma$  responses to DEN2 NS3 (mean 1618, SD $\pm$ 683.3) and DEN3 NS3 peptides (mean 1776, SD $\pm$ 898.5), when compared to DEN2 (mean 1196, SD $\pm$ 406.6) and DEN3 (1158, SD $\pm$ 593) IFN $\gamma$  responses in individuals who had mild or asymptomatic dengue infections, this difference was not statistically significant.

**Conclusion:** Our data show that NS3 specific T cells are seen at a high frequency in individuals with past dengue infection and show rapid effector function. However, other functional T cell profiles and T cell phenotypes need to be investigated in order to address how dengue specific memory T cells contribute to disease pathogenesis.

#### PB05/25 NMDAR-DEPENDENT CALCIUM SIGNALING IN CD4+ MEMORY CELLS

A. Tadmouri<sup>1,2,3</sup>, P. Affaticati<sup>1,2,3</sup>, E. Gouadon<sup>1,2,3</sup>, I. Klingel-Schmitt<sup>1,2,3</sup>, N. Alossey<sup>1,2,3</sup>, F. Jambou<sup>1,2,3</sup>, J.-F. Renaud<sup>1,2,3</sup>, S. Cohen-Kaminsky<sup>1,2,3</sup>

<sup>1</sup>CNRS UMR8162, Université Paris Sud, IPSC, Recherche Médicale, Le Plessis Robinson, France, <sup>2</sup>Université Paris Sud 11, Biologie -Santé, Orsay, France, <sup>3</sup>Centre Chirurgial Marie Lannelongue, Recherche Médicale, Le Plessis Robinson, France

Memory formation represents a shared feature between immune and neuronal systems. It consists of information acquisition, and storage. Immunological memory is defined by the ability to generate a highly accelerated and intense immune response during the secondary encounter with a pathogen. Such as the nervous system functioning, glutamate signaling seems to play a pivotal role in the immune system. Studies have described several transport machineries for glutamate release and uptake as well as specific receptors in immune system cells, which classify glutamate as an immunoregulator, till now considered as neurotransmitter. We recently demonstrated a role for glutamate in communication at the immunological synapse between dendritic cells and lymphocytes, through the NMDA receptor. Here, we reveal a role for NMDA receptor signaling in immune memory cells generated during immunization of TCR transgenic OTII mice. NMDA receptors are highly expressed and phosphorylated in purified CD4+ memory T-cells comparing to naive cells, with the highest levels of expression and phosphorylation observed in CD4+ central memory cells as compared to effector memory cells. Furthermore CD4+ effector memory T-cells exhibit sustained calcium signals in response to antigen-specific synaptic contact with dendritic cells, such as the long-term potentiation (LTP), a cellular mechanism of memory and learning in the nervous system. Our data show that specific blockade of NMDA receptors reverses the sustained calcium signal into naive-like transient, and subsequently inhibits the proliferation of effector memory cells and their differentiation into central memory cells *in vitro*.

Similarly to nervous memory, an involvement of NMDA receptor signaling in the immune memory formation, through the immunological synapse, is suggested, with potential application in immunoregulation and vaccine strategies.

#### PB05/26 DIFFERENTIAL PROLIFERATIVE RESPONSES OF PBMCs AMONG TUBERCULOSIS PATIENTS TO PPD AND IFN-GAMMA

T. E. Kissina<sup>1</sup>, I. S. Freidlin<sup>1</sup>, B. E. Knoring<sup>2</sup>, T. S. Baski<sup>1</sup>, A. V. Elkin<sup>1</sup>, T. Ulrichs<sup>3</sup>

<sup>1</sup>Institute for Experimental Medicine, Immunology, St. Petersburg, Russian Federation, <sup>2</sup>Institute for Phthisiopulmonology, Medical Microbiology, St. Petersburg, Russian Federation, <sup>3</sup>Koch-Metchnikov-Forum, Tuberculosis, Berlin, Germany

One characteristic feature of human tuberculosis is the phenomenon of hypo-response to specific antigens, anergy. Low proliferation to specific antigens is correlated to decreased IFN-gamma, but increased IL-4 and IL-10 secretion in anergic tuberculosis patients. Regulatory T cells were recently described to play a substantial role in coordinating a balanced immune response in tuberculosis.

Therefore, we aimed at studying different manifestations of the response of PBMCs from tuberculosis patients to specific antigens from *Mycobacterium tuberculosis* PPD and to IFN-gamma.

Though the overall proliferation to PPD was elevated in patients, a high percentage of non-responders to PPD (21 patients out of 34) was detected. On this basis, we divided patients into two sub-groups: A – non-responders to PPD, and B – responders to PPD. There were no differences of PBMCs' oxidative burst between A and B tuberculosis patient sub-groups. However, patients from both subgroups showed diminished oxidative burst to PPD or IFN-gamma, when compared to healthy donors. Tuberculosis patients from group A also presented decreased capacity to secrete IL-2 and IFN-gamma in response to PPD, compared to patients from group B. Though there was no correlation of weak oxidative burst with diminished proliferation to PPD correlated with IL-2 and IFN-gamma production in patients' PBMCs. We found that oxidative burst in response to PPD was positively correlated with IL-4 in the patient group, but negatively correlated in the healthy donor group.

**PB05/27 IMMUNOLOGICAL MEMORY WITHOUT A MEMORY CD4<sup>+</sup>-T-CELL AND THE ADJUVANT EFFECT OF SLEEP**I. Braumann<sup>1</sup>, J. Westermann<sup>1</sup><sup>1</sup>University of Lübeck, Institute of Anatomy, Lübeck, Germany

**Objectives:** Most investigators assume that CD4<sup>+</sup>-T-cell memory – just like B-cell memory – is based on a specialized memory T-cell. But in contrast to B-cells, where antigen induced changes are fixed on the DNA-level, antigen contact in T-cells leads to only temporary and reversible changes making the identification of a memory T-cell challenging. Critical assembly of published data now leads to the suggestion that CD4<sup>+</sup>-T-cell memory is not based on a specialized cell type but on increased numbers of antigen specific naïve-like T-cells.

**Methods:** Evaluating the current literature did not reveal decisive evidence that antigen encounter increases the life span of CD4<sup>+</sup>-T-cells or permanently imprints a memory phenotype. Further conclusive evidence was found to favour a reversion of T-cells that had encountered antigen to a long lived naïve like state.

**Results:** Based on the evaluated data a new CD4<sup>+</sup> memory model was established: A non immunized individual possesses only low numbers of CD4<sup>+</sup>-T-cells with specificity for a given antigen. If this individual is immunized the contact with antigen will lead to proliferation of antigen specific CD4<sup>+</sup>-T-cells. We now propose that after infection is resolved the antigen specific CD4<sup>+</sup>-T-cells revert from a primed state back into a naïve state. Cells that already had contact with antigen phenotypically will be indistinguishable from naïve T-cells. The more effective T-cell response after secondary antigen contact is simply due to increased numbers of antigen specific CD4<sup>+</sup>-T-cells.

**Conclusions:** Following our model, establishment of immunological memory, e.g. in vaccination, is dependant on the ability of CD4<sup>+</sup>-T-cells to revert from the primed state into a naïve state. Therefore hormones or cytokines, that influence the reversion of primed T-cells, should have an effect on consolidation of immunological memory. As sleep is known to have a positive impact on vaccination, sleep regulated hormones and cytokines shall be tested whether they influence T-cell reversion.

**PB05/28 THE EFFECTS OF “THIRD SIGNALS” DURING PRIMING ON PHENOTYPE AND FUNCTION OF HUMAN CD8<sup>+</sup> T CELLS**Y.J. Ho<sup>1</sup>, A.E.S. Brooks<sup>1</sup>, P.R. Dunbar<sup>1,2</sup><sup>1</sup>University of Auckland, School of Biological Sciences, Auckland, New Zealand, <sup>2</sup>University of Auckland, The Maurice Wilkins Centre for Molecular Biodiscovery, Auckland, New Zealand

The establishment of functional CD8<sup>+</sup> T cell memory is essential for effective immunity against pathogens and tumours. Corrupted differentiation may impair crucial functional properties of CD8<sup>+</sup> T cells and recent evidence has suggested that priming conditions are critical in determining the differentiation programme of these cells. IL-12 is secreted by APCs during immune reactions and is reported to enhance effector functions of CD8<sup>+</sup> T cells, whereas IL-21 is thought to boost their proliferation while limiting their differentiation. To investigate the effect of these “third signals” during T cell priming, we have established a new *in vitro* model of human CD8<sup>+</sup> T cell differentiation that allows comparison of the properties of CD8<sup>+</sup> T cells primed under different conditions. CD8<sup>+</sup> T cells primed in the presence of IL-12 and IL-21 have a less differentiated cell surface phenotype and a different transcription factor profile compared to those primed in their absence. These molecular markers are associated with faster proliferation kinetics after re-stimulation, and an enhanced expression of effector molecules such as IFN- $\gamma$  and granzyme B. This suggests that the differentiation program of memory CD8<sup>+</sup> T cell is altered by the availability of IL-12 and IL-21 during priming and that the design of effective vaccines and adoptive immunotherapy needs to consider the likely exposure of naïve cells to “third signals” such as IL-12 and IL-21 during priming.

**PB05/29 CHARACTERIZATION OF CD8<sup>+</sup> HELPER T CELLS**R. Stark<sup>1,2</sup>, M. Frentsch<sup>2</sup>, S. Meier<sup>2</sup>, A. Thiel<sup>2,3</sup><sup>1</sup>International Max Planck Research School for Infectious Diseases and Immunology (IMPRS-IDI), Berlin, Germany, <sup>2</sup>Berlin-Brandenburg Center for Regenerative Therapies (BCRT), Regenerative Immunology and Aging, Berlin, Germany, <sup>3</sup>German Rheumatism Research Center (DRFZ), Clinical Immunology, Berlin, Germany

**Objectives:** T-cell immunity is exerted by CD4<sup>+</sup> T cells providing help for antigen-presenting cells as well as B cells, and CD8<sup>+</sup> T cells primarily regarded as cytotoxic effector cells. We have identified and characterized a subset of CD8<sup>+</sup> T cells which is capable of performing potent helper functions in immunity and lacks cytotoxic properties. These CD8<sup>+</sup> T cells are characterized by the expression of CD40L, which is also one essential feature mediating the function of activated CD4<sup>+</sup> T helper cells.

**Methods:** For the analysis of CD40L expression in human CD8<sup>+</sup> T cells, CD8<sup>+</sup> T cells were enriched from PBMC using MACS and CD8<sup>+</sup> T-cell subsets were sorted by FACS as CD3<sup>+</sup>/CD4<sup>+</sup> and by the expression of CCR7 and CD45RA. Cells were stimulated for 6h with PMA/ Ionomycin with or without addition of Brefeldin A. Subsequently cells were stained with fluorescent antibodies on the surface or intracellular and analyzed by FACS.

**Results:** We found that on average 25% of peripheral human memory CD8<sup>+</sup> T cells express CD40L after activation. Stimulated human CD40L<sup>+</sup> CD8<sup>+</sup> T cells can activate B cells and dendritic cells *in vitro* as effectively as CD4<sup>+</sup> T cells and were consequently named according to their functional properties CD8<sup>+</sup> Helper T cells. CD8<sup>+</sup> Helper T cells do not express CD56 or CD57 and display no cytotoxicity, shown by lack of Granzyme B, Perforin and degranulation (CD107a). Furthermore they express high amounts of IL-2, TFN $\alpha$  and partly IFN $\gamma$ . Interestingly all IL-4 and IL-13 expressing CD8<sup>+</sup> T cells coexpress CD40L.

**Conclusion:** CD8<sup>+</sup> Helper T cells, characterized by the expression of CD40L after activation, represent a new and versatile facet of the T-cell response. According to their unique functional capacities CD8<sup>+</sup> Helper T cells have the capability to exert essential helper responsibilities in immunity independent of classical CD4<sup>+</sup> T cells and MHC-II antigen presentation. CD8<sup>+</sup> Helper T cells might be potent candidate T cells to execute or support effective anti-tumor or anti-pathogen immune therapies.

This work was supported by IMPRS-IDI, the EC network MUVAPRED and the SFB TR36.

**PB05/30 ANTIGEN SPECIFICITY OF CD8<sup>+</sup> T HELPER CELLS**S. Meier<sup>1</sup>, M. Frentsch<sup>1</sup>, R. Stark<sup>1,2</sup>, A. Thiel<sup>1,3</sup><sup>1</sup>Berlin-Brandenburg Center for Regenerative Therapies (BCRT), Berlin, Germany, <sup>2</sup>International Max Planck Research School for Infectious Diseases and Immunology (IMPRS-IDI), Berlin, Germany, <sup>3</sup>German Rheumatism Research Center (DRFZ), Berlin, Germany

**Objectives:** Activated CD4<sup>+</sup> T cells induce APC maturation and B cell differentiation particularly through CD40L (CD154). We now identified a subset of activated CD8<sup>+</sup> T cells from healthy donors able to express CD40L as well. After 6h polyclonal stimulation on an average 25% of the memory CD8<sup>+</sup> T cells express CD40L. CD8<sup>+</sup> CD40L<sup>+</sup> T cells exert similar functions as CD4<sup>+</sup> T cells and were accordingly assigned CD8<sup>+</sup> Helper T cells. In the current study we assessed the antigen specificities of human CD8<sup>+</sup> Helper T cells.

**Methods:** PBMC obtained from healthy volunteers were used for *in vitro*-stimulation with overlapping peptide pools of immunodominant antigens from the common pathogens CMV, EBV or influenza. During the 6h stimulation  $\alpha$ CD28, CD107a and monensin were present and Brefeldin A was added for the last 4h. After fixation and permeabilisation cells were analyzed for CD3, CD4, CD8, CD40L, CD107a, IL-2 and IFN $\gamma$  by 9-color-flow cytometry.

**Results:** We observed that around 30% of donors showing a response to the pathogens, exhibited a CD8<sup>+</sup> CD40L<sup>+</sup> population after stimulation with peptide pools and therefore can be identified as CD8<sup>+</sup> Helper T cells. These CD8<sup>+</sup> Helper T cells co-express cytokines such as IL-2 and/or IFN $\gamma$ .

**Conclusion:** We have identified CD8<sup>+</sup> Helper T cells, capable to express CD40L after short-time activation specific for common pathogens such as CMV, EBV and influenza. Human CD8<sup>+</sup> Helper T cells in fact play a role in physiological immune responses and potentially are essential for control of infectious diseases where reinforced T-helper cell function is necessary.

**PB05/31 LACK OF THE “ORIGINAL ANTIGENIC SIN” PHENOMENON IN RECALL CD8 T CELL RESPONSES**D. Zehn<sup>1</sup>, M.J. Bevan<sup>1</sup><sup>1</sup>University of Washington, Department of Immunology, Seattle, United States

Neither mice nor human are immunologically naïve, having been exposed to numerous and varied antigenic challenges. The infectious history of an individual not only changes its T cell repertoire but it also may impact the response to a subsequent, cross-reactive pathogen. The “original antigenic sin” concept proposes that memory T cells that respond with high affinity to a previously encountered peptide MHC-ligand and with low affinity to a cross-reactive second ligand, would impair the response of other T cells that recognize the second ligand with high affinity. We investigated this proposed phenomenon with a series of related MHC class I-peptide epitopes expressed by bacterial and viral pathogens. We find that, in all cases, high affinity CD8<sup>+</sup> T cell precursors, whether naïve or memory, out-compete lower affinity memory cells. This holds true when even more than 10% of the CD8 T cell compartment consists of memory T cells that weakly cross-react with the rechallenge ligand. In line with these observations we demonstrate that unlike in primary responses, where even very low affinity T cells are recruited and expand, during recall responses only T cells with higher affinity for the recall ligand expand and form secondary effector T cells. In conclusion and in contrast to prior reports, during secondary pathogen challenges that induce a profound infection and extensive T cell expansion, we do not observe the original antigenic sin phenomenon.

## PB06 – REGULATORY T CELLS

PB06/1 MYCOBACTERIUM TUBERCULOSIS PEPTIDES PRESENTED BY HLA-E MOLECULES ARE TARGETS FOR HUMAN CD8<sup>+</sup> T-CELLS WITH CYTOTOXIC AS WELL AS REGULATORY ACTIVITYS.A. Joosten<sup>1</sup>, K.E. van Meijgaarden<sup>1</sup>, P.C. van Weeren<sup>1</sup>, F. Kazi<sup>1</sup>, A. Geluk<sup>1</sup>, N.D. Savage<sup>1</sup>, J.W. Drijfhout<sup>2</sup>, D.R. Flower<sup>3</sup>, W.A. Hanekom<sup>4</sup>, M.R. Klein<sup>1</sup>, T.H. Ottenhoff<sup>1</sup><sup>1</sup>Leiden University Medical Center, Infectious Diseases, Leiden, Netherlands, <sup>2</sup>Leiden University Medical Center, Immunohematology and Blood Transfusion, Leiden, Netherlands, <sup>3</sup>The Jenner Institute, University of Oxford, Berkshire, United Kingdom, <sup>4</sup>South African Tuberculosis Vaccine Initiative, University of Cape Town, Cape Town, South Africa

**Objectives:** Non-classical HLA molecules like the highly conserved class Ib family member HLA-E can present both self and non-self peptides to the immune system, including pathogen-derived peptides. Recognition of HLA-E bound foreign peptides may lead to activation of CD8<sup>+</sup> T cells, although their functional properties in humans are very poorly defined. We have studied whether peptide sequences derived from *Mycobacterium tuberculosis* (Mtb), can be presented by the non-classical HLA-molecule HLA-E, and analysed the phenotypical and functional properties of responding CD8<sup>+</sup> T cells.

**Methods:** We screened the Mtb genome for potential HLA-E binding epitopes using HLA-E peptide binding algorithms. Peptides identified were tested for binding to HLA-E and for recognition by T cells from healthy Mtb responsive donors. Further phenotypical and functional analyses were performed on T cell lines generated against HLA-E binding peptides, including cytotoxicity and suppression assays.

**Results:** We identified 69 peptides from the Mtb genome with putative HLA-E binding motifs. We observed CD8<sup>+</sup> T cell proliferation in Mtb responsive donors and in BCG vaccinated infants in response to the majority of peptides, demonstrating recognition by sensitized individuals. These CD8<sup>+</sup> T cells killed peptide loaded target cells when the exact peptide was presented by HLA-E. Besides CTL activity, human HLA-E restricted CD8<sup>+</sup> T cells also inhibited proliferation of other T cells, thus demonstrating an immunoregulatory function. Suppression of proliferation was cell-cell contact-dependent and was inhibited by addition of the latency associated peptide (LAP), indicating a role for TGFβ. TGFβ was indeed detected on the surface of the CD8<sup>+</sup> T cells in a membrane bound form.

**Conclusion:** We show for the first time that Mtb derived peptides can be presented by HLA-E molecules to human CD8<sup>+</sup> T cells; furthermore, we find that these cells have both cytolytic and suppressive activities, the latter mediated via membrane-bound TGFβ.

Supported by grants from the European Union, Netherlands Organization for Scientific Research and the Leiden University Medical Center.

PB06/2 THE TYROSINE PHOSPHATASE SHP-1 REGULATES THE SUPPRESSIVE FUNCTION OF CD4<sup>+</sup>CD25<sup>+</sup> TREG CELLST. Iype<sup>1</sup>, M. Sankarshanan<sup>1</sup>, D.W. Mullins<sup>1</sup>, U. Lorenz<sup>1</sup><sup>1</sup>University of Virginia, Microbiology, Charlottesville, United States

While the importance of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> regulatory T (Treg) cells for immune tolerance has been increasingly appreciated, our mechanistic and molecular understanding of their function is still very limited. The tyrosine phosphatase SHP-1 is a well-recognized negative regulator of TCR-mediated signaling, and we and others have previously shown that SHP-1 affects the function of conventional T cells. We therefore asked whether SHP-1 also plays a role in Treg function. Throughout these studies we made use of mutant mice that lack SHP-1, the co-called *motheaten* (*me/me*) mice. A comparison of the suppressive activities of wild type and *me/me* Treg cells demonstrated increased suppressive activity of *me/me* Treg cells both *ex vivo* as well as *in vivo*. Our micro-array analyses showed no developmental disparities between *+/+*, *me/+* and *me/me* Treg cells, which suggests that SHP-1 directly regulates the suppressive activity of Treg cells in a cell intrinsic manner further supporting the hypothesis that SHP-1 plays a regulatory role in Treg function. Moreover, pharmacological inhibition of SHP-1 activity increased Treg activity similarly as observed in SHP-1-deficient Treg cells. While this suppression is dependent on stimulation via the TCR, our data indicate that additional factors, which are critical for an effective Treg function, are regulated by SHP-1. To gain a better mechanistic understanding of Treg-mediated suppression, standard and imaging flow cytometric analyses were performed. These studies showed that in the absence of SHP-1, Treg cells display changes in the expression levels of a number of surface and intracellular molecules consistent with a more active phenotype. Furthermore, SHP-1 deficiency promoted increased conjugate formation between Treg cells and antigen presenting cells. Conjugate formation with SHP-1-deficient Treg cells causes a more efficient down-modulation of the costimulatory CD80/CD86 molecules on the antigen presenting dendritic cells further demonstrating the more potent suppressive activity. Moreover, SHP-1-deficient Treg cells were more effective in out-competing conjugate formation between the dendritic cells and effector T cells suggesting that these are mechanisms of Treg-mediated suppression.

Taken together, our data indicate that SHP-1 regulates the function and activation status of Treg cells and that under conditions of SHP-1 deficiency, Treg cells become more potent.

## PB06/3 NATURAL REGULATORY T-CELL EPITOPE INDUCTION IN AUTOIMMUNE EAE SUGGESTS NEW THERAPY FOR MULTIPLE SCLEROSIS

A.S. De Groot<sup>1,2,3</sup>, L. Moise<sup>1,2</sup>, J. Desrosiers<sup>1</sup>, Y. Yang<sup>1</sup>, W. Martin<sup>1</sup><sup>1</sup>EpiVax, Inc, Providence, United States, <sup>2</sup>University of Rhode Island, Institute for Immunology and Informatics, Providence, United States, <sup>3</sup>Brown University School of Medicine, Pediatric Infectious Disease, Providence, United States

Immunoglobulin (IgG) therapy expands regulatory T cells and protects against disease development in experimental autoimmune encephalitis (EAE) in mice (Ephrem et al., Blood, 2008). This protective effect appears to be mediated by regulatory T cells (Tregs). We have shown that IgG-derived-regulatory T cell epitopes activate natural Tregs and suppress immune response in human PBMCs, and that the corresponding murine epitopes suppress *in vivo* immune response in HLA DR4 transgenic mice (De Groot et al., Blood 112:3303-3311, 2008). We have now performed three separate studies to evaluate the effect of the Tregitopes in EAE. In the first study, two regulatory T-cell epitopes (mouse Tregitope 167 and mouse Tregitope 289) were administered individually at the time of MOG peptide-induced disease induction. In the second study, EAE-induced mice were treated with a combination of mTregitopes 167 and 289 at disease induction, SC, for the first day and then intraperitoneally for totally five days, or at disease onset, IP, for five days. In the third study we administered the mTregitopes in liposomes according to the previous schedule, so as measure the impact of improved Tregitope delivery.

In the first study, symptoms were initially suppressed in mice treated with mTregitopes in comparison with untreated mice, suggesting a potential therapeutic effect, but by day sixty, disease progression was indistinguishable in all groups. In the second study, Tregitope treatment delayed onset of disease and, when administered at the peak of disease, resulted in reduced severity of symptoms as compared to untreated controls. The transient effect was likely due to the very short half-life of the peptides *in vivo*. We therefore formulated the Tregitopes in liposomes. This improved the effect: Tregitope treated mice demonstrated a dramatic reduction in disease severity overall, when compared to mice that did not get treated. The discovery of natural regulatory T-cell epitopes in the Fc fragment of IgG may lead to a disease-management paradigm shift, allowing clinicians to safely harness the potential of natural regulatory T cells to regulate immune responses in a wide range of health conditions.

PB06/4 CD25<sup>+</sup> REGULATORY T CELLS SPECIFICALLY SUPPRESS AUTO-ANTIBODY GENERATION AGAINST PANCREATIC TISSUE AUTOANTIGENSI. Ludwig-Portugall<sup>1</sup>, E.E. Hamilton-Williams<sup>2</sup>, J. Goto<sup>1</sup>, C. Gottschalk<sup>1</sup>, C. Kurts<sup>1</sup><sup>1</sup>Institute of Molecular Medicine and Experimental Immunology, Rheinische Friedrich Wilhelms Universität Bonn, Bonn, Germany, <sup>2</sup>The Scripps Research Institute, La Jolla, United States

To study B cell tolerance against non-lymphoid tissue autoantigens, we generated transgenic RIP-OVA/HEL mice expressing the model antigens, OVA and HEL, in pancreatic islets. Their vaccination with OVA or HEL induced far less auto-antibody titers compared to non-transgenic controls. Depletion of CD25<sup>+</sup> cells during immunization completely restored auto-antibody production, but did not affect antibodies against a foreign control antigen. Depletion at later time-points was not effective. OVA-specific CD25<sup>+</sup> FoxP3<sup>+</sup> regulatory T cells were more frequent in the autoantigen-draining pancreatic LN than in other secondary lymphatics. Consistently, B cells were suppressed in that LN and also in the spleen, which is known to concentrate circulating antigen, such as the antigens used for vaccination. Suppression involved preventing expansion of autoreactive B cells in response to autoantigen, reducing antibody production per B cell and isotype changes.

Using ELISPOT analysis and NP hapten linked protein immunisation to determine direct B cell suppression we showed that suppressive CD25<sup>+</sup> cells needed to possess the same specificity as the Th cells that supported autoantibody production and that CD25<sup>+</sup> cells acted also by suppressing B cells directly. These findings demonstrate that CD25<sup>+</sup> regulatory T cells suppress auto-antibody production against non-lymphoid tissue antigens in an antigen-specific manner.

## PB06/5 REGULATORY T-CELLS IN BH3-ONLY PROTEIN DEFICIENT MICE

J.G. Wieggers<sup>1</sup>, I. Peschel<sup>1</sup>, M. Kaufmann<sup>1</sup>, G. Böck<sup>1</sup>, D. Tischner<sup>1</sup>, V. Labi<sup>1</sup>, A. Villunger<sup>1</sup><sup>1</sup>Innsbruck Medical University, Biocenter, Division of Developmental Immunology, Innsbruck, Austria

Regulatory T-cells (Treg) are critical for maintaining self-tolerance and controlling inflammatory responses. They are generated within the thymus as a separate lineage and retain a stable phenotype following export into the periphery (natural Treg). TCR diversity is comparable in Treg and conventional T-cells (Tconv) although there appears to be only a modest overlap in the TCRs shared by Treg and Tconv. How Treg are generated in the thymus and what factors determine their survival, however, is still not known. One view is that Foxp3<sup>+</sup> cells resist negative selection.



Mice deficient for the BH3-only protein Bim display a defect in thymocyte negative selection and suffer from autoimmune kidney disease on a mixed genetic background. Therefore, we investigated whether Treg development and function might be affected in these mice. The results were compared with two other BH3-only protein deficient mice lacking Puma or Bmf. Compared to normal WT mice, the number of CD4<sup>+</sup>Foxp3<sup>+</sup> Treg in the thymus of *Bim*<sup>-/-</sup> mice was increased whereas thymi of *Puma*<sup>-/-</sup> and *Bmf*<sup>-/-</sup> mice contained normal Treg cell numbers. Similar results were obtained when the number of CD4<sup>+</sup>Foxp3<sup>+</sup> cells in the spleen were analyzed. Subset analysis revealed that the increased number of CD4<sup>+</sup>Foxp3<sup>+</sup> Treg in the thymus and spleen of Bim deficient mice was largely due to an increase in the CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> subset. In addition, Foxp3 expression on a per cell basis was moderately decreased in Treg of *Bim*<sup>-/-</sup>, but not of *Puma*<sup>-/-</sup> and *Bmf*<sup>-/-</sup> mice. Thus, our results reveal that the BH3-only protein Bim may play an important role in the development and homeostasis of Foxp3<sup>+</sup> Treg cells.

#### PB06/6 INCREASED FOXP3<sup>+</sup> REGULATORY T CELLS IN POLY(ADP-RIBOSE) POLYMERASE-1 DEFICIENT MICE

F. Laudisi<sup>1</sup>, F. Nasta<sup>1</sup>, M. Sambucci<sup>1</sup>, M. M. Rosado<sup>2</sup>, C. Pioli<sup>1</sup>

<sup>1</sup>ENEA (Italian Agency for New Technologies, Energy and Environment), Department of Biotechnology, Section of Toxicology and Biomedicine, Rome, Italy, <sup>2</sup>IRCCS Research Center – Ospedale Pediatrico Bambino Gesù, Rome, Italy

**Objective:** Growing evidence is unveiling a role for poly(ADP-ribose) polymerase-1 (PARP-1) in the regulation of inflammatory/immune responses. The present study investigated the role of PARP-1 in regulatory T cell differentiation.

**Methods:** Thymocytes and peripheral lymphocytes from C57BL/6 wild type and C57BL/6 PARP-1KO mice were characterized both phenotypically and functionally (cell proliferation, cytokine production, antibody production, ...). Helper T (CD4<sup>+</sup>CD25<sup>-</sup>, Th), Treg (CD4<sup>+</sup>CD25<sup>+</sup>) and naïve CD4 T (CD4<sup>+</sup>CD62L<sup>high</sup>) cells were purified by immuno-magnetic cell sorting. Natural regulatory T cells (Treg) were functionally challenged in an *in vitro* suppressive assay. Naïve CD4 cells were induced to differentiate *in vitro* Foxp3<sup>+</sup> iTreg cells with anti-CD3, anti-CD28 mAb or recombinant CD86, and graded concentrations of TGFβ<sub>1</sub>. Frequency of iTreg (Foxp3<sup>+</sup> cells) and CTLA-4 and GITR expression were evaluated by flow cytometry; Foxp3 mRNA expression by RT-real time PCR. Data were analyzed by a two-tailed unpaired student's T test.

**Results:** An expansion of the regulatory T cell population was observed in thymus, spleen and lymph nodes of PARP-1KO mice as compared to WT controls. Stimulation of spleen cells or purified CD4 cells showed a decreased proliferative response and a reduced IL-2 production in PARP-1KO cells as compared to WT control cells. Depletion of CD25<sup>+</sup> cells from purified CD4 cells induced an increase in cell proliferation and IL-2 production vanishing the difference between PARP-1KO and WT CD4 cells. Thus, it was the higher number of Treg cells in the CD4 cell population of PARP-1KO mice to inhibit their activity. Inhibitory function of PARP-1KO Treg cells was confirmed in an *in vitro* suppressive assay. On a per cell basis WT and KO Treg cells were equally able to suppress cell proliferation and IL-2 production of WT Th cells. *In vitro* stimulation with TGFβ<sub>1</sub> induced a higher percentage of Foxp3<sup>+</sup> (iTreg) cells in PARP-1KO naïve CD4 T cell cultures than in WT control cells. This finding was confirmed by an increase in Foxp3 mRNA expression. WT and PARP-1KO iTreg displayed a similar phenotype.

**Conclusion:** Our data provide the first evidence that PARP-1 plays a negative role in regulatory T cell differentiation.

#### PB06/7 QUANTITATIVE PHOSPHOKINOME ANALYSES OF SIGNALING NETWORKS IN T-LYMPHOCYTES

T. Reinl<sup>1</sup>, S. König<sup>1</sup>, M. Probst-Kepper<sup>2</sup>, J. Wissing<sup>1</sup>, C. Hundertmark<sup>1</sup>, B. Schraven<sup>3</sup>, J. Wehland<sup>1</sup>, L. Jänsch<sup>1</sup>

<sup>1</sup>Helmholtz Center for Infection Research (HZI), Department of Cell Biology, Braunschweig, Germany, <sup>2</sup>Clinical Center Braunschweig, Institute for Microbiology, Immunology and Hospital Hygiene, Braunschweig, Germany, <sup>3</sup>Otto-von-Guericke University, Institute for Molecular and Clinical Immunology, Magdeburg, Germany

**Aims:** T-cell receptor (TCR) stimulation of CD4<sup>+</sup>CD25<sup>hi</sup>FOXP3<sup>+</sup> regulatory T (T<sub>reg</sub>) cells and conventional CD4<sup>+</sup>CD25<sup>-</sup> effector T (T<sub>eff</sub>) cells leads to the mutually exclusive activation of their suppressor and effector functions, respectively. Substrate phosphorylation by active protein kinases might strongly influence the underlying molecular network. In the present study, we introduce a proteome approach to investigate the kinome following TCR activation of human T<sub>reg</sub> and T<sub>eff</sub> cells by quantitative phospho-site determination.

**Methods:** To achieve access to a comprehensive portion of the human T-cell kinome, we apply a combination of highly unspecific small molecule ATP analogues for affinity purification of protein kinases. Subsequent phosphopeptide enrichment by IMAC and quantitative peptide sequencing (iTRAQ<sup>TM</sup>/SCX/nHPLC-HCD-Orbitrap) is used to extract information of both expression and phosphorylation state of protein kinases from anti-CD3/-CD28 stimulated T<sub>reg</sub> and T<sub>eff</sub> cells. Statistical evaluation and visualization of phosphopeptide and protein regulation is enabled by an in-house developed software package called iTRAQassist.

**Results:** We provide for the first time a comprehensive expression profile of the T-cell kinome and describe differences in FoxP3 dependent protein kinase expression in T<sub>reg</sub> compared to T<sub>eff</sub> cells. Our quantitative phosphoproteome approach gains access to phosphorylation sites from well described key kinases of the TCR signaling network like LCK, FYN and ITK serving as proof of concept. Moreover, we enable quantitative expression data for approximately 100 protein kinases and phosphosite information of approximately 90 phosphorylation sites derived from 50 protein kinases from TCR stimulated FoxP3 positive T<sub>reg</sub> cells compared to T<sub>eff</sub> cells.

**Conclusions:** We demonstrate a new strategy to define unique signaling features of human T<sub>reg</sub> and T<sub>eff</sub> cells associated with their mutually exclusive functions. Our results implicate novel signaling components and will probably open new perspectives specifically target T cell functions to overcome aberrant immune responses.

#### PB06/8 THE IMMUNOSUPPRESSIVE CAPACITY OF FOXP3<sup>+</sup> T REGULATORY CELLS IS AUGMENTED BY HEAT SHOCK PROTEIN 70

J. Wachstein<sup>1</sup>, S. Lukis<sup>1</sup>, D. Rokitta<sup>1</sup>, C. Figueiredo<sup>1</sup>, M. Wittmann<sup>2</sup>, R. Blaszczak<sup>1</sup>, B. Eiz-Vesper<sup>1</sup>

<sup>1</sup>Hannover Medical School, Institute for Transfusion Medicine, Hannover, Germany, <sup>2</sup>University of Leeds, Institute of Molecular and Cellular Biology, Leeds, United Kingdom

Human CD25<sup>+</sup> FoxP3<sup>+</sup> T regulatory cells (Tregs) have been found to control effector T cells and therefore are pivotal of the maintenance of peripheral tolerance and immune homeostasis. Heat shock protein 70 (HSP70) has gained plenty of attention because of its adjuvant capability to induce CD4<sup>+</sup> T cell responses but the mechanism how HSP70 affects the immunosuppressive function of Tregs is still unknown. To determine its influence we treated Tregs with endotoxin-free HSP70 to survey their function in a target-dependent and -independent expression of cytokines, regulation of proliferation and cytotoxicity.

Treatment of Tregs with HSP70 significantly inhibited proliferation of CD4<sup>+</sup>CD25<sup>-</sup> target T cells and downregulated the secretion of IFN-γ and TNF-α by these cells. The observed effects were underlined by increased levels of Treg suppressor cytokines IL-10 and TGF-β<sub>1</sub> as determined by Real Time PCR and Multiplex Bead Assays using Luminex technology. The influence of HSP70 can be further enhanced in the presence of low-dose IL-2.

To gain insight into the cellular events Tregs were exposed to inhibitors of signalling before HSP70 treatment and then co-cultured with CD4<sup>+</sup>CD25<sup>-</sup> target T cells. Treatment of Tregs with Wortmannin (PI3K inhibitor), GF109203X (PKC inhibitor), SB203580 (p38 inhibitor) and Pertussis toxin (G protein inhibitor) blocked the immunosuppressive function of HSP70-preactivated Tregs as quantified by not affected target cell cytokine levels (IFN-γ; TNF-α). In contrast, inhibition of MAP Kinase ERK1/2 using PD98059 led to an even higher activation of HSP70-mediated Treg functions, indicating that this signalling pathway negatively influences HSP70 effects on Tregs.

Our data clearly showed that extracellular HSP70 enhance suppressive functions of Tregs to neutralise target immune cells. Recently it has been shown that HSP70 is upregulated in autoimmune diseases, allergies, allogeneic transplant rejection and GvHD. It seems to be likely that HSP70-induced suppressive capacity of Tregs can prevent overshooted immune responses and help to maintain immune homeostasis.

#### PB06/9 CD8<sup>+</sup> T CELL RESPONSE INDUCTION BY DENDRITIC CELLS IS INHIBITED BY MHC CLASS II-DEPENDENT DX5<sup>+</sup>CD4<sup>+</sup> REGULATORY T CELLS

W. Han<sup>1</sup>, D. Schuurhuis<sup>1</sup>, N. Fu<sup>1</sup>, M. Camps<sup>1</sup>, L. van Duivenvoorde<sup>1</sup>, P. Louis-Plence<sup>2</sup>, K. Franken<sup>1</sup>, T. Huizinga<sup>1</sup>, C. Melief<sup>1</sup>, R. Toes<sup>1</sup>, F. Ossendorp<sup>1</sup>

<sup>1</sup>Leiden University Medical Center, Leiden, Netherlands, <sup>2</sup>Institut National de la Santé et de Recherche Médicale, Montpellier, France

**Objectives:** CD4<sup>+</sup> T cells are important for CD8<sup>+</sup> T cell-priming by providing cognate signals for DC maturation. Although activated DC are able to prime CD8<sup>+</sup> T cells *in vivo* without CD4<sup>+</sup> T cell help, it is unclear whether CD4<sup>+</sup> T cells influence this process. We analyzed the capacity of CD4<sup>+</sup> T cells to influence CD8<sup>+</sup> T cell responses induced by activated DC.

**Methods:** C57BL/6 mice were depleted for CD4<sup>+</sup> cells by i.p. injection of 50 µg purified anti-CD4 (GK1.5) in PBS at days 3 and 1 before and day 5 after DC injection. CD25<sup>+</sup> cells were depleted by i.p. injection of 100 µg anti-CD25 antibody (PC61) at day 6 before DC injection. DC were generated from C57BL/6 and MHC class II<sup>-/-</sup> mice bone marrow or a long term growth factor-dependent immature splenic DC line (D1) was used. LPS-activated DC pulsed with OVA<sub>257-264</sub> or E1A<sub>234-243</sub> were injected i.v. and OVA<sub>257-264</sub> or E1A<sub>234-243</sub>-specific CD8<sup>+</sup> T cell responses were assessed by tetramer staining. For *in vitro* analysis of CD8<sup>+</sup> T cell response modulation by DX5<sup>+</sup>CD4<sup>+</sup> cells, OT-1 cells were co-cultured with OVA<sub>257-264</sub>-pulsed DC in the presence or absence of DX5<sup>+</sup>CD4<sup>+</sup> T cells.

**Results:** Surprisingly, mice depleted for CD4<sup>+</sup> cells were able to generate stronger antigen-specific CD8<sup>+</sup> T cell responses after DC vaccination than non-depleted mice. The same observation was made when mice were vaccinated with MHC class II<sup>-/-</sup> DC, indicating the presence of a MHC class II-dependent CD4<sup>+</sup> T cell population inhibiting CD8<sup>+</sup> T cell responses. Recently we described the expansion of DX5<sup>+</sup>CD4<sup>+</sup> T cells, a T cell population displaying immune regulatory properties, upon vaccination with DC. Intriguingly, we now observe an inverse correlation between CD8<sup>+</sup> T cell induction and expansion of DX5<sup>+</sup>CD4<sup>+</sup> T cells as the latter cells did not expand after vaccination with MHC class II<sup>-/-</sup> DC. *In vitro*, DX5<sup>+</sup>CD4<sup>+</sup> T cells were able to limit proliferation, modulate cytokine production and induce FoxP3<sup>+</sup> expression in OVA-specific CD8<sup>+</sup> T cells.

**Conclusion:** Together, our data show an inhibitory role of CD4<sup>+</sup> T cells on the induction of CD8<sup>+</sup> T cell responses by activated DC and indicate the involvement of DX5<sup>+</sup>CD4<sup>+</sup>, but not CD4<sup>+</sup>CD25<sup>+</sup>, T cells in this process.

**PB06/10 THE ROLE OF T CELL RECEPTOR AVIDITY IN THYMIC REGULATORY T CELL DEVELOPMENT**G. Kalodimos<sup>1</sup>, E. Shklovskaya<sup>1</sup>, B. Fazekas de St. Groth<sup>1</sup>  
<sup>1</sup>Centenary Institute, T Cell Laboratory, Sydney, Australia

Natural regulatory T cells (nTregs) are derived from the thymus and play a key role in the maintenance of tolerance and in suppressing excessive immune responses. Tregs are thought to develop in the thymus as result of higher affinity T cell receptor (TCR)- self-peptide MHC interactions relative to conventional T cells. Previous studies have shown the effects of ligand affinity on nTreg development but to date there is little information on how TCR signal strength (avidity) directly influences this process. We hypothesized that the generation of nTregs in the CD4<sup>+</sup>CD8<sup>+</sup> compartment occurs within a specific window of TCR avidity that lies between positive and negative selection. To investigate this, we studied nTreg selection in double transgenic (DTg) models that co-express the 5C.C7 TCR and its neo self-antigen and compared with a TCR transgenic (TCRTg) model expressing the 5C.C7 TCR only. In DTg mice, selection of T cells expressing the Tg TCR into the nTreg compartment peaked at approximately 3-4 weeks of age and then declined. In contrast, in TCRTg mice that did not express neo-self antigen, very few nTregs expressed the Tg TCR and the number was stable with age. To test whether the age-dependent changes in selection of nTregs resulted from changes in thymic TCR expression, we measured expression of CD3 and Tg TCR between birth and 4 months of age in DTg mice. Expression of CD3 in the thymic CD4<sup>+</sup>CD8<sup>+</sup> compartment declined over that time, whereas all other thymic compartments were stable. At the same time, a decrease in the percentage and absolute numbers of Tg TCR-expressing cells was observed in the thymic CD4<sup>+</sup>CD8<sup>+</sup> and peripheral CD4<sup>+</sup> T cell compartments. These changes were likely due to a decrease in Tg TCRalpha chain expression, allowing increased expression of endogenously rearranged TCRalpha chains. These data are consistent with the selection of nTregs at the CD4<sup>+</sup>CD8<sup>+</sup> stage as a result of self-antigen recognition with an intermediate avidity.

**PB06/11 GPR83 ISOFORM-4 BUT NOT GPR83 ISOFORM-1 IS INVOLVED IN THE INDUCTION OF REGULATORY T CELLS *IN VIVO***W. Hansen<sup>1</sup>, A. M. Westendorf<sup>1</sup>, J. Buer<sup>1</sup><sup>1</sup>University Hospital Essen, Institute of Medical Microbiology, Essen, Germany

Regulatory T cells (Tregs) are well known key players in the maintenance of homeostasis, keeping the balance between tolerance and immunity. CD4<sup>+</sup>CD25<sup>+</sup> Tregs are either thymus-derived or emerge from naïve CD4<sup>+</sup> T cells under various conditions in the periphery. Most recently, we have described the G protein-coupled receptor 83 (GPR83), which is highly expressed by CD4<sup>+</sup>CD25<sup>+</sup> Tregs to be involved in the induction of CD4<sup>+</sup>Foxp3<sup>+</sup> Tregs in the course of an ongoing immune response. Until now, four GPR83 isoforms have been described. In the present study, we show that GPR83 isoform-4, which differs from GPR83 isoform-1 by 20 additional aminoacids in the second cytoplasmatic loop, is predominantly expressed by CD4<sup>+</sup>CD25<sup>+</sup> Tregs. To examine the role of these 20 aminoacids in the course of peripheral Treg induction, we have overexpressed GPR83 isoform-1 and GPR83 isoform-4 in naïve T cells by retroviral gene transfer. Interestingly, GPR83 isoform-4 but not GPR83 isoform-1 transduced T cells are able to interfere with the effector phase of severe contact hypersensitivity (CHS) reaction of the skin and to suppress the development of disease in a T cell transfer model of colitis. Re-analysis of GPR83 transduced T cells reveals that this *in vivo* acquisition of suppressive activity is associated with the induction of Treg associated molecules including Foxp3 in GPR83 isoform-4 but not GPR83 isoform-1 transduced CD4<sup>+</sup> T cells under inflammatory conditions. Our results suggest that the 20 additional aa within GPR83 isoform-4 are involved in Treg induction during inflammatory immune responses.

**PB06/12 SUPPRESSION OF ALLOGENEIC T-CELL RESPONSES BY HUMAN TCRαβ<sup>+</sup> CD4<sup>+</sup> CD8<sup>+</sup> DOUBLE-NEGATIVE T CELLS**S. Völkl<sup>1</sup>, R. Garyl<sup>1</sup>, A. Mackensen<sup>1</sup><sup>1</sup>University Hospital Erlangen, Department of Internal Medicine 5, Erlangen, Germany

Regulatory T lymphocytes play an important role in the maintenance of immune tolerance to self antigens and are involved in downregulating immune responses in autoimmunity, transplant rejection and tumor immunity. Numerous studies have demonstrated the existence of distinct T cell subsets with immunoregulatory properties. Recently, a novel subset of TCRαβ<sup>+</sup> CD4<sup>+</sup> CD8<sup>+</sup> (double-negative, DN) T cells has been characterized to specifically suppress immune responses in both mice and humans. Here we demonstrate for the first time that human DN T cells are highly potent suppressor cells of allogeneic CD4<sup>+</sup> or CD8<sup>+</sup> T cell responses. A prerequisite for DN T cells to acquire its immunosuppressive activity is the repetitive priming with allogeneic dendritic cells whereas stimulation with artificial antigen presenting cells (anti-CD3/CD28 beads) has no effect. DN T cell-mediated suppression requires cell contact and cannot be abrogated by neutralizing antibodies to the immunosuppressive cytokines TGF-β and IL-10. In contrast to murine DN T cells, which eliminate effector T cells via a fas/fasL or perforin/granzyme pathway, human DN T cells do not induce apoptosis in responder cells but suppress the proliferation of alloreactive T cells in an active manner. Furthermore we demonstrate that the suppressive effect of DN T cells is not mediated by modulation of antigen presenting cells. Taken together, our data indicate that human DN T cells exert strong immunosuppressive activity on alloreactive CD4<sup>+</sup> or CD8<sup>+</sup> T cells. DN T cells may serve to limit clonal expansion of alloantigen-specific T cells after allogeneic peripheral blood stem cell transplantation.

**PB06/13 DE NOVO GENERATION OF TREG CELLS AND DIFFERENTIAL EXPRESSION OF TREG-RELATED CHEMOKINES DURING PREGNANCY**A. Teles<sup>1,2</sup>, P. Wafula<sup>2</sup>, M. Popovic<sup>2</sup>, H. D. Volk<sup>3</sup>, A. C. Zenclussen<sup>2,3</sup><sup>1</sup>Centro de Neurociências e Biologia Celular – Universidade de Coimbra, Coimbra, Portugal, <sup>2</sup>Medical Faculty, Otto-von-Guericke-University, Magdeburg, Germany,<sup>3</sup>Charité Institute of Medical Immunology, Berlin, Germany

To maintain fetal tolerance along pregnancy, the maternal immune system developed regulatory mechanisms. Therefore, a number of regulatory T (T reg) cells, including the Foxp3<sup>+</sup> T reg cells were shown to exhibit a protective effect towards paternal alloantigens. The origin of these cells as well as their mechanisms of recruitment at the fetal-maternal interface are still unknown.

We hypothesized the occurrence of extrathymic T reg cell development by the uterine environment. Like in other systems, the involvement of chemokines during the recruitment process of these cells in the materno-fetal environment is to be expected.

Using an animal model of cell transfer and by analyzing the populations of Treg cells by flow cytometry at day 5 and 10 of pregnancy we show that an increased peripheral conversion of CD4<sup>+</sup> T cells to T reg cells occurs in the iliac lymph nodes (which drain the uterus) during murine pregnancy when compared with non pregnant animal controls.

Additionally, given the existent concordance between the expression of chemokines in the uterus/placenta and their receptors in Treg, we selected several receptor-ligand pairs that may be involved in the recruitment of these cells into the fetal-maternal tissues along pregnancy. By real time RT-PCR we investigated the expression patterns of the selected chemokine ligands in different phases of pregnancy in uterus or decidua and placenta from normal pregnant C57/BL6-mated BALB/c mice. We observed a gradual up-regulation of TCA3 (homologous of CCL1 in mice) in decidua, beginning on day 8 of pregnancy, and peaking on day 10 in placenta. CXCL12 was further found to be up-regulated on day 12 in placenta. CCL4 expression was significantly increased in placental tissue on day 10 while CCL21 was up-regulated in the decidual tissue beginning on day 8 of pregnancy.

These results suggest that the maternal immune system evolved a strategy to promote T reg cell neoconversion during pregnancy and that trafficking of Treg to the fetal-maternal interface at different pregnancy stages is probably mediated by different chemokine signals.

**PB06/14 GENERATION OF ALLERGEN-SPECIFIC T REGULATORY CELLS BY MULTICISTRONIC VECTOR BASED RETROVIRAL TRANSFER OF T CELL RECEPTOR ALPHA AND BETA CHAINS AND FOXP3 OR TGF-BETA**K.G. Schmetterer<sup>1</sup>, D. Haiderer<sup>1</sup>, V.M. Leb<sup>1,2</sup>, A. Neunkirchner<sup>1,2</sup>, B. Jahn-Schmid<sup>3</sup>, H.J. Küng<sup>1</sup>, C.P. Manta<sup>1</sup>, P. Steinberger<sup>1</sup>, B. Bohle<sup>2,3</sup>, W.F. Pickl<sup>1</sup><sup>1</sup>Institute of Immunology, Medical University of Vienna, Vienna, Austria, <sup>2</sup>Christian Doppler Laboratory for Immunomodulation, Medical University of Vienna, Vienna, Austria, <sup>3</sup>Medical University of Vienna, Department of Pathophysiology, Vienna, Austria

**Background:** Ectopic overexpression of transgenes in T-cells can modify T-cell function. Recently, we have shown that transfer of T-cell receptor (TCR) alpha and beta chains specific for allergen-derived epitopes can transfer allergen-specificity to peripheral blood T-cells. Similarly, retro- and lentiviral overexpression of Foxp3 has been shown to generate T-cells with regulatory function. Our aim is to combine these two approaches to generate allergen-specific regulatory T-cells (Treg) that might become useful in immunotherapeutic approaches.

**Methods:** Multicistronic expression constructs containing the respective alpha and beta chains of two TCRs specific for the Arvt1<sub>25-36</sub> (TRAV17/TRBV18) and Bctv1<sub>142-153</sub> (TRAV6/TRBV20) and the cDNAs encoding human Foxp3 and TGF-beta1 (TGFβ) were generated by using internal ribosomal entry sites (IRES) and picornaviral 2A sequences. Subsequently, retrovirally transduced peripheral blood T-cells of non-allergic individuals were assessed for their regulatory capacity using either transduced HEK-293 as artificial antigen presenting cells or anti-CD3/CD28 coated microbeads as polyclonal stimulus.

**Results:** Both Foxp3<sup>+</sup> and TGFβ<sup>+</sup> T-cells showed the typical characteristics of Treg: hyporesponsiveness to polyclonal and antigen-specific activation, low cytokine secretion and proliferation in response to exogenous IL-2. Furthermore, TCR<sup>+</sup>Foxp3<sup>+</sup> transgenic T-cells could inhibit T-cell proliferation of antigen-specific responder T-cells in a dose-dependent fashion both in response to antigen-specific and polyclonal activation. Importantly, Foxp3<sup>+</sup> transgenic T-cells expressing their endogenous or a non-specific T-cell receptor could only inhibit activation of antigen-specific responder T-cells after polyclonal activation, supporting the view that Foxp3-mediated regulation is an active mechanism requiring activation of the regulatory T-cell. In contrast, both in an activated and resting state TGFβ<sup>+</sup> T-cells produced sufficient amounts of TGFβ to inhibit T-cell activation.

**Conclusions:** Our results show the feasibility to generate T regulatory cells, which exert their function only in response to allergen-specific activation. Such approaches might become useful for tolerance induction in allergic and other immune-mediated diseases.

This work was supported by grants SFB F1816-B13 and SFB F1807-B04 of the Austrian Science Foundation, the Austrian Research Promotion Agency (Forschungsförderungs-gesellschaft) Bridge grant 812079 & Biomay AG, and the Christian Doppler Laboratory for Immunomodulation.

**PB06/15 SELECTIVE DEPLETION OF FOXP3<sup>+</sup> CELLS DURING INTESTINAL NEMATODE INFECTION FAVOURS IMMUNOPATHOLOGY AND RESULTS IN ACCELERATED, STRONGER TH2 RESPONSES**

S. Rausch<sup>1</sup>, J. Hühn<sup>2</sup>, C. Loddenkemper<sup>3</sup>, T. Sparwasser<sup>4</sup>, R. Lucius<sup>1</sup>, S. Hartmann<sup>1</sup>

<sup>1</sup>Humboldt University of Berlin, Department of Molecular Parasitology, Berlin, Germany, <sup>2</sup>Helmholtz Center for Infection Research, Experimental Immunology, Braunschweig, Germany, <sup>3</sup>Charité – Benjamin Franklin, Department of Pathology, Berlin, Germany, <sup>4</sup>Hannover Medical School, Institute for Infection Immunology, Hannover, Germany

A characteristic of intestinal nematode infections is the provocation of highly biased Th2 responses with protective immunity being essentially dependent on interleukin (IL)-4 and IL-13. However, intestinal nematode infections in humans and animal models are often long lasting with modulated immune responses. We have previously shown that infection with the intestinal nematode *Heligmosomoides polygyrus* leads to quantitative and qualitative differences in Tregs in the intestine and gut draining mesenteric lymph nodes. Using the transgenic DEREK mouse model we further examined the role of Foxp3<sup>+</sup> Tregs in this model. Foxp3<sup>+</sup> cells expressing the diphtheria toxin receptor were selectively depleted during the early phase of infection. Treg removal led to increased inflammation of the parasitized small intestine. Systemic and local Th2 cytokine responses were increased after Treg removal. Furthermore, the depletion resulted in an accelerated parasite-specific cytokine response, pointing out that the early commitment to Th2 effector cells is controlled by Tregs. IL-10 levels were also strongly increased after Treg depletion, arguing for effector T cells or innate cells as major IL-10 sources during intestinal nematode infection. Finally, Treg-depletion had no effect on adult worm burden. Hence, our data indicate that during intestinal worm infection, Tregs control the magnitude and onset of Th2 responses and are essentially needed to control immunopathology.

**PB06/16 FOXP3 DIRECTLY UPREGULATES CD25 EXPRESSION IN HUMAN CD4+CD25- T LYMPHOCYTES CONVERTED TO CD4+CD25+FOXP3+ T LYMPHOCYTES BY CD28/B7 INTERACTION**

M. Soligo<sup>1</sup>, C. Camperio<sup>1</sup>, C. Scotti<sup>1</sup>, P. Del Porto<sup>1</sup>, E. Piccolella<sup>1</sup>

<sup>1</sup>Sapienza – University of Rome, Cellular and Developmental Biology, Rome, Italy

Considerable evidence supports the prediction that CD25 is directly regulated by FOXP3. However, given that CD25 is normally upregulated in all activated T cells, regardless of whether they express FOXP3, and the difficulty of analyzing in vitro signal transduction pathways that specifically target FOXP3 to CD25 promoter, this issue has yet to be definitively demonstrated. Starting from our previous results showing that CD28 unique signals were sufficient to induce FOXP3 in human CD4+CD25- T cells that become CD4+CD25+FOXP3+ T cells, we have analyzed the possible mechanisms through which this occurs. We started by analyzing the activation signals induced by the engagement of CD28 with the natural ligand B7 and their implications on the post-translational modification of FOXP3 function. We found that CD28 signals induced the phosphorylation of Akt and GSK3 and the binding of NFAT1 to FOXP3 promoter, measured by chromatin immunoprecipitation (ChIP), in the presence of CsA. Moreover, CD28 unique signals were able to mediate the translocation of FOXP3 from the cytoplasm into the nucleus and this occurred between 24–48 h from CD28 engagement. In addition we demonstrated by ChIP analysis that CD28 signals induced the recruitment of FOXP3 and not of NFAT1 on CD25 promoter, suggesting a direct effect FOXP3 on CD25 expression. This hypothesis was verified: i) by comparing the MFI of CD25 expression on CD28-induced CD4+CD25+FOXP3- and CD28-induced CD4+CD25+FOXP3+ T cells and ii) by observing the effect of the knock down of FOXP3 gene by siRNA in CD28-induced CD4+CD25+FOXP3+ T cells. The results have shown a significant increase of CD25 on CD28-induced CD4+CD25+FOXP3+ T cells and a significant reduction of the transcription and translation of CD25 molecules in FOXP3 silenced cells. Taken together, our data strongly support the view that FOXP3, induced independently of calcineurin activation, is a critical regulator of CD25 in CD4+CD25- T cells committed to expressing FOXP3.

**PB06/17 HIGH EXPRESSION OF PRDM1/BLIMP-1 IN HUMAN PERIPHERAL ACTIVATED/MEMORY BUT NOT IN NAÏVE OR THYMIC REGULATORY T CELLS**

N. Seddiki<sup>1,2</sup>, C. Phetsouphanh<sup>1,2</sup>, T. Juelich<sup>3</sup>, G. Denyer<sup>4</sup>, Y. Xu<sup>1,2</sup>, S. Rao<sup>3</sup>, S. Alexander<sup>5</sup>, D. Cooper<sup>1,2</sup>, A. Kelleher<sup>1,2</sup>

<sup>1</sup>St Vincents for Applied Medical Research, Darlinghurst, Australia, <sup>2</sup>University of New South Wales, Kensington, Australia, <sup>3</sup>Australian National University, Canberra, Australia, <sup>4</sup>University of Sydney, Sydney, Australia, <sup>5</sup>Children's Hospital at Westmead, Westmead, Australia

Regulatory T cells (Tregs) are important for maintaining T cell homeostasis. We have previously reported the existence of two bona fide subsets of naïve CD25<sup>+</sup>CD127<sup>lo</sup>CD45RA<sup>+</sup>/RO<sup>-</sup> (nveTregs) and activated CD25<sup>+</sup>CD127<sup>hi</sup>CD45RA<sup>+</sup>/RO<sup>+</sup> (actTregs) Foxp3<sup>+</sup> Tregs in peripheral blood. Here we report that subsets of actTregs, in contrast to nveTregs, express significantly increased levels of B lymphocyte-induced maturation protein-1 (Blimp-1/*Prdm1*) as determined by microarray (13.7-fold difference), reverse transcriptase polymerase chain reaction (RT-PCR) (100-fold difference,  $p < 0.05$ ) and flow cytometric studies, whereas no difference in Foxp3 expression was observed. Subsets of CD25<sup>+</sup>CD127<sup>lo</sup>62L<sup>+</sup>/RO<sup>+</sup> and CD25<sup>+</sup>CD127<sup>hi</sup>62L<sup>+</sup>/RO<sup>+</sup> effector and memory CD4<sup>+</sup> T cells showed intermediate expression of *Prdm1*/*Blimp-1* compared to actTregs (2.5-fold difference in microarray and 10-fold difference by RT-PCR), but Foxp3 expression was significantly higher in actTregs. The microarray data showed expected increased expression of genes specific for bona fide Tregs, such as *Ctla4*, *Il2ra*, *Icos*, *cd58*, *Tnfrsf9*, *Ccr8* as well as expression of new recently reported genes such as *Lrrc32*, *Irf-4* and *Il12rb2*. In contrast to peripheral actTregs, *Blimp-1* mRNA was not detected in single positive (SP) CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup>CD25<sup>+</sup>CD127<sup>lo</sup> thymocytes or in peripheral CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>lo</sup> Tregs from cord blood by RT-PCR. However, after *in vitro* stimulation with a-CD3 mAb in the presence of IL-2 *Prdm1*/*Blimp-1* could be detected in Tregs from cord blood by RT-PCR and by flow cytometry. Taken together, these data show that *Blimp-1*/*Prdm1* has a significantly increased expression in human actTregs compared to nveTregs, thymic Tregs and Tregs isolated from cord blood. IL-2 seems to play a role in its induction, suggesting that *Prdm1*/*Blimp-1* is preferentially expressed in subsets of Tregs with an antigen-experienced phenotype. *Prdm1*/*Blimp-1* can therefore be considered as a marker for peripheral activated Tregs but not for peripheral naïve or thymic Tregs.

**PB06/18 GENERATION OF PERIPHERAL TREGS BY HOMOTYPIC T CELL INTERACTION**

K. Thüemmler<sup>1</sup>, A. Ramming<sup>1</sup>, J. Leipe<sup>1</sup>, H. Schulze-Koops<sup>1</sup>, A. Skapenko<sup>1</sup>

<sup>1</sup>University of Munich, Med. Poliklinik, Division of Rheumatology, Munich, Germany

**Objective:** Several T cell subsets with a regulatory phenotype that differentiate in the periphery have been described. We have recently shown that homotypic interaction of CD4 memory T cells with activated T cells results in the development of IFN $\gamma$ /IL-10- or IL-4-producing cells. Given the immunomodulatory potential of IL-4 and IL-10, we tested the hypothesis that T-T cell interaction results in the generation of T cells with a regulatory capacity and investigated mechanisms contributing to their development.

**Methods:** CD4 memory T cells from healthy individuals or from Balb-c mice were cultured together with activated effector T cells in the presence or absence of neutralizing antibodies to surface receptors and their phenotype and function was analyzed *in vitro* and *in vivo*.

**Results:** *In vitro*, blocking LFA-1 in cocultures of activated effector T cells and resting memory T cells completely prevented the development of cytokine-producing cells from the later. Interestingly, however, the neutralization of each particular ICAM lead to different phenotype alterations of the developing cytokine-producing T cells. Blocking ICAM-1 diminished the production of IFN $\gamma$  but not of IL-4 and IL-10, whereas ICAM-3 was important for IL-4 secretion. Neutralization of ICAM-2 did not alter cytokine production at all, together indicating that ICAM/LFA-1 interaction was not only responsible for the occurrence of T-T-cell contact but had also a decisive impact on the phenotype of developing cytokine-producing cells. The developing cytokine-producing cells, both from man and mouse, strongly inhibited proliferation of CD25 negative T cell responses to T cell receptor stimulation in a dose dependent manner *in vitro*. The extend of inhibition was comparable to that of naturally occurring CD25 positive regulatory T cells. The inhibitory effect was contact dependent and could be abrogated by exogenous IL-2. *In vivo*, the expansion of OVA specific effector T cells in Balb-c mice upon antigen challenge could be inhibited by OVA specific memory T cells cocultured with activated Th1 or Th2 cells.

**Conclusion:** Homotypic interaction of resting and activated T cells induced the development of regulatory T cells and might, thus, provide a novel negative feedback mechanism to control sustained T cell driven immunity.

**PB06/19 THE REGULATORY T CELL RESPONSE DURING ACUTE VIRAL INFECTION IS LOCALLY DEFINED AND CONTROLS THE MAGNITUDE AND DURATION OF THE VIRUS-SPECIFIC CYTOTOXIC T CELL RESPONSE**

K. K. Dietze<sup>1</sup>, G. Zelinsky<sup>1</sup>, U. Dittmer<sup>1</sup>

<sup>1</sup>Universität Duisburg-Essen, Institut für Virologie, Essen, Germany

Cytotoxic T-cells (CTL) facilitate control of acute viremia in many viral infections, including retroviruses like HIV or HTLV. However, viruses that establish chronic infections have developed mechanisms to evade destruction by CTL. We have used the Friend Virus (FV) model to investigate these mechanisms. In the acute infection FV induces a strong CTL response but the mice become persistently infected. However, regulatory CD4<sup>+</sup> T cells (Treg) that expand in the spleen of infected mice suppress the production of cytotoxic molecules in CD8<sup>+</sup> T cells and the cytotoxic function of CTL. The aim of our current work was to analyse the compartmentalisation of the Treg response and the subsequent local suppression of CD8<sup>+</sup> T cells by Tregs during an ongoing retroviral infection. We found, that expansion of effector CD8<sup>+</sup> T cells, production of cytotoxic molecules and degranulation was directly linked to viral loads in lymphatic organs. Consequently the expansion of induced Treg correlated with the number and function of virus-specific CD8<sup>+</sup> T cells. For the expansion of Treg the presence of CTL was obligatory, what was shown by CD8 depletion experiments. Furthermore, depleting Treg in DEREK mice resulted in enhanced expansion of effector CD8<sup>+</sup> T cells and improved the production of cytotoxic molecules leading to reduced viral loads in lymphatic organs. In summary, during acute retroviral infection Treg downregulated the expansion and function of virus-specific CTL. The immunosuppressive activity of Tregs was locally defined to the organs in which efficient viral replication followed by a strong CD8<sup>+</sup> effector cell response took place.



**PB06/20 T CELL REGULATION IN PATIENTS WITH WHIPPLE'S DISEASE**K. Schinnerling<sup>1</sup>, A. Geelhaar<sup>1</sup>, V. Moos<sup>1</sup>, K. Conrad<sup>1</sup>, K. Allers<sup>1</sup>, T. Schneider<sup>1</sup><sup>1</sup>Charité – Universitätsmedizin Berlin (CBF), Medizinische Klinik für Gastroenterologie und Infektiologie, Berlin, Germany

**Objectives:** Whipple's disease (WD) is an infectious disease characterized by intestinal and articular manifestations, which are caused by the ubiquitous actinomycet *Tropheryma whippelii*. WD-patients show a reduced *T. whippelii*-specific Th1 response and decreased levels of serum IL-12. It is unclear whether dendritic cells (DC) of WD-patients are capable to induce potent T cell responses or if T cell regulatory mechanisms are responsible for the persistence of *T. whippelii*.

**Methods:** Blood and serum samples for comparative studies were collected from WD-patients and healthy control subjects. Phenotypic characterization of DC and T cells was performed in whole blood by flow cytometry. DC were generated from peripheral blood to assess phagocytosis by the uptake of FITC-coupled dextran- or transferrin-beads and to study the stimulatory capacity by proliferation assays with autologous CFSE-labeled T cells. Levels of regulatory cytokines in serum samples were measured by a cytometric bead assay (CBA). Regulatory T cells were detected immunohistochemically in duodenal biopsies. Statistical analysis was performed by Student's *t* test.

**Results:** Here we demonstrate that peripheral blood DC from WD-patients show reduced IL-12 levels and increased amounts of IL-10 after LPS-stimulation but comparable expression of costimulatory molecules (CD86, CD83, CD40, CD80) and HLA-DR to healthy controls. There are no differences in phagocytosis of *in vitro* generated immature DC from healthy controls and WD-patients, however, stimulation with *T. whippelii* reduces the unspecific uptake of dextran-beads and enhances the receptor-mediated uptake of transferrin-beads in both. DC from WD-patients are able to stimulate proliferation of *T. whippelii*-specific CD4<sup>+</sup> T cells, but fail to induce IFN- $\gamma$  production. The impaired Th1 response goes along with higher serum levels of IL-10 and TGF- $\beta$  in WD-patients. While we found similar numbers of CD25<sup>high</sup> FoxP3<sup>+</sup> regulatory T cells (Treg) in peripheral blood of WD-patients and healthy controls, there are increased numbers of FoxP3<sup>+</sup> Treg in the duodenum of untreated WD-patients that decrease to the level of controls upon antibiotic treatment.

**Conclusion:** Our data suggest that *T. whippelii*-specific Th1 responses might rather be inhibited by an at least in part cytokine-induced Treg activity than caused by an absent costimulatory capacity of DC.

**PB06/21 STUDIES OF IMMUNE RESPONSES TO PRP DURING PRION INFECTION: ROLE OF REGULATORY T CELLS**A. Sacquin<sup>1</sup>, D.A. Gross<sup>2</sup>, J. Davoust<sup>2</sup>, P. Aucouturier<sup>1</sup>, M. Bruley Rosser<sup>3</sup><sup>1</sup>UMR 938, Paris, France, <sup>2</sup>Inserm 580, Paris, France, <sup>3</sup>UMR 938, Hôpital St Antoine, Paris, France

Infections are usually diagnosed by detecting the pathogenic agent and/or a specific immune response. In Creutzfeldt-Jakob disease, most of the efforts have focused on the detection of pathological PrP (PrP<sup>Sc</sup>) in body fluids before brain invasion. Indeed, the absence of a specific antibody response to PrP<sup>Sc</sup>, due to the strong immune tolerance to self-PrP, prevents early diagnosis and precludes the analysis of immunological events that develop during the incubation period. Natural or induced CD4<sup>+</sup>CD25<sup>+</sup> Foxp3<sup>+</sup> regulatory T (Treg) cells are one mechanism of peripheral tolerance that may explain specific unresponsiveness by impairing the initiation of effector T cell responses. Our recent findings suggest that accumulation of PrP<sup>Sc</sup> in the spleen during infection interferes negatively with the development of T and B cell responses to a class II-restricted PrP peptide.

In this work, we investigated the impact of Tregs on the development of T and B-cell anti-PrP effectors either during the natural course of disease progression or after vaccination. Such a phenomenon has never been explored in prion diseases. We showed that *in vivo* and *in vitro* depletion of Tregs lead to a complete recovery of the T cell response to a major CD4<sup>+</sup> PrP epitope. We further demonstrated that CD4<sup>+</sup>Foxp3<sup>+</sup> T cells expand and accumulate in secondary lymphoid organs but not in the blood of mice injected intraperitoneally with murine scrapie-infected brain but not with normal brain homogenates. Moreover, the CD25<sup>+</sup> cells accumulate preferentially over CD25<sup>+</sup> within the CD4<sup>+</sup> Foxp3<sup>+</sup> compartment. The magnitude and specificity of the suppressive activity of Tregs derived from infected and non-infected mice is currently tested *in vitro*. In addition, the direct impact of Tregs on the natural course of scrapie infection is studied either by depleting or transferring these cells in newly infected mice. Our results suggest that the immune system reacts to prion infection. Knowledge of interaction between immune system and prions and of the possible role of Tregs on prion progression may be useful for studying the mechanisms of peripheral tolerance to PrP<sup>Sc</sup> and designing possible immunotherapies.

**PB06/22 ROLE OF THYMIC INVOLUTION AND RECENT THYMIC EMIGRANTS IN EXTRA-THYMIC REGULATORY T CELL DE NOVO GENERATION**J. Buchweitz<sup>1</sup>, K. Kretschmer<sup>1</sup><sup>1</sup>Immunotolerance in Regeneration, CRTD/ DFG-Center for Regenerative Therapies Dresden, Dresden, Germany

Maintenance of peripheral T cell populations throughout life depends on balancing the influx of recent thymic emigrants (RTEs) with the homeostatic regulation of mature peripheral T cells. In young individuals, the thymus exports sufficient numbers of naïve T cells to maintain the naïve T cell pool. As the thymus atrophies with age, RTE numbers decrease and homeostatic proliferation of residual T cells is required to maintain the size of the pool. Thus, although thymic involution in an aging immune system results in the gradual decline of the RTE pool, the size of the peripheral T cell pool remains relatively stable over time. The consequence is a progressive increase in memory T cell numbers, and a predisposition to impaired T cell function, reduced T cell immunity, and increased autoimmunity.

Here, we address the question whether thymic involution and the resulting changes in the peripheral T cell pool influences extrathymic *de novo* generation of Foxp3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells (Treg). Furthermore, we ask whether RTEs, which continue to undergo functional maturation in the lymphoid periphery as they are incorporated into the mature naïve T cell compartment, may represent particularly suitable targets for Treg *de novo* generation outside the thymus. We took advantage of a reporter mouse to mark RTEs in mice carrying a GFP transgene driven by the recombination-activating gene 2 promoter (*RAG2*-GFP) in CD4<sup>+</sup> T cells expressing transgenic T cell receptors. Thus, differential GFP expression levels in peripheral T cells allow faithful discrimination of antigen-specific RTEs (GFP<sup>hi</sup>) and mature naïve cells (GFP<sup>low</sup>) in peripheral lymphoid organs. We show that RTEs and mature naïve CD4<sup>+</sup> T cells are indistinguishable in their capacity to convert into Foxp3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> Treg when low doses of the antigenic peptide is presented by steady state dendritic cells *in vivo*. Furthermore, we report on cell-intrinsic molecular differences underlying the capacity of RTEs to efficiently induce Foxp3 expression under conditions that prevent Treg *de novo* generation from mature naïve CD4<sup>+</sup> T cells. These findings may have implications for the development of novel strategies to promote Treg *de novo* generation in clinical settings of autoimmune diseases such as type 1 diabetes.

**PB06/23 REGULATORY T CELLS REDUCE INFLUENZA VIRUS SPECIFIC T CELL RESPONSE**A. Vogel<sup>1</sup>, K. Droebner<sup>1</sup>, K. Klingel<sup>1</sup>, O. Planz<sup>1</sup><sup>1</sup>Friedrich-Loeffler-Institut Tübingen, Institute of Immunology, Tübingen, Germany, <sup>2</sup>Eberhard-Karls-Universität Tübingen, Institute of Pathology, Tübingen, Germany

The highly pathogenic avian influenza A viruses (HPAIV) H5N1 cause severe disease in humans, still the basis for their virulence remains unclear. Various investigations suggest that both virus-induced cytokine dysregulation (hypercytokinemia) and reduction of the lymphocyte population (lymphopenia) may contribute to disease severity. The presence of various cytokines and chemokines in the lung of infected individuals results in an influx of immune mediators into the lung and consequently to a massive organ damage. Lymphopenia leads to a reduced antiviral T-cell mediated immune response. In this study we investigated the role of Regulatory T-cells (Tregs) during an HPAIV infection in mice to observe whether lymphopenia correlates with changes in the Regulatory T-cell population. Our results show that during an infection with HPAIV in mice the number of Tregs produced in the thymus was drastically increased. This result led us to the presumption that influenza viruses play an active role in the T-cell maturation. We therefore raised the question, whether viral antigen could be present in the thymus. Using immunohistology, standard infectivity assay and *in situ* hybridisation, we were able to detect viral footprints in the thymus. Moreover, the presence of cytotoxic CD8<sup>+</sup> T cells in the thymus could be demonstrated. We assume that influenza viruses infect epithelial cells in the thymic leading to presentation of viral antigens that interfere with T-cell selection in the thymus. Consequently next to a virus specific immune response, thymocytes specific against viral antigens are eliminated already in their production side or become Regulatory T cells. The reduction of specific T cells in combination with the increase of immune suppressive Tregs might influence the lymphopenia and consequently develop a missing and adequate T cell response against the chemoattractants produces by respiratory epithelial cells.

**PB06/24 THE ANERGIC PHENOTYPE OF HUMAN REGULATORY T CELLS IS SUPPORTED BY LOW EXPRESSION OF NFATC2, NF- $\kappa$ B AND AP-1**H. Bendfeldt<sup>1</sup>, S. Frischbutter<sup>1</sup>, M. Benary<sup>2</sup>, H. Herzl<sup>2</sup>, R. Baumgras<sup>1</sup><sup>1</sup>DRFZ (Deutsches Rheumaforschungszentrum), Berlin, Germany, <sup>2</sup>Humboldt University, Institute for Theoretical Biology, Berlin, Germany

T cell receptor signaling and subsequent IL-2 production have been shown to be impaired in regulatory T (Treg) cells. Whether the expression levels and activation of the main TCR- dependent transcription factors are also different in Treg cells, is not known so far.

Using transcription factor analysis on single cell level by flow cytometry, we compared the expression and activation of NFATc2, NF- $\kappa$ B, AP-1 and FOXP3 in human Treg and memory Th cells. We found, that the NFATc2, NF- $\kappa$ B and AP-1 activation was not impaired in Treg cells after stimulation with PMA/Ionomycin. However, the expression levels of all three transcription factors were strikingly lower in Treg compared to memory Th cells. Surprisingly, we found a small subset of Treg cells that was able to produce IL-2 after stimulation. These IL-2 producing Treg cells were characterized by higher total amounts of NFATc2, AP-1 and NF- $\kappa$ B but lower amounts of FOXP3 compared to the IL-2 non-producing Treg cells.

Using these data, we developed a mathematical model showing the importance of the amounts and ratios of the different transcription factors for the decision whether a single Treg cell is able to produce IL-2 or not.

In summary, our results indicate that IL-2 expression in Treg cells is not switched off by genetic imprinting but rather inhibited by the low amounts of the transcription factors NFATc2, AP-1 and NF- $\kappa$ B and their relation to FOXP3. This might be an important precondition to protect Treg cells from achieving effector functions and thereby maintain their anergic phenotype.

**PB06/25 CONTRIBUTION OF PLASMACYTOID DENDRITIC CELLS TO THE GENERATION OF NATURAL REGULATORY T CELLS IN THE HUMAN THYMUS**E. Martín Gayo<sup>1</sup>, M.L. Gaspar<sup>2</sup>, B. De Andrés<sup>2</sup>, M.L. Toribio<sup>1</sup><sup>1</sup>Centro de Biología Molecular 'Severo Ochoa', Cellular Biology and Immunology, Madrid, Spain, <sup>2</sup>Centro Nacional de Microbiología (ISCIII), Majadahonda, Spain

**Objectives:** The thymus plays a key role in the education of T-lymphocytes and in the establishment of self-tolerance. In addition, the thymus provides the appropriate niche for the generation of CD4+CD25+Foxp3+ "natural" regulatory T cells (Treg), which are fundamental for the maintenance of immunological tolerance. However, little is known about the molecular and cellular events that regulate Treg cell generation in the thymus, especially in humans, and about the developmental origin of Treg cells. Dendritic cells (DCs) have emerged as key players in the regulation of the immune system. Two distinct types of DCs are generated in the human thymus: conventional DCs (cDCs) and plasmacytoid DCs (pDCs). TSLP-activated cDCs from human thymus were recently shown to induce the generation of allogeneic natural Treg cells, but the contribution of pDCs to that process has not been investigated as yet. The objective of this study has been to determine the tolerogenic features of human thymic pDCs.

**Results:** We show here that human thymic pDCs activated with CD40L and IL-3, but not immature pDCs, were able to induce the generation of functional CD4+CD25+Foxp3+ Treg cells from CD4+ thymocytes and peripheral T lymphocytes after 7-9 days of culture under allogeneic conditions. More importantly, activated pDCs were potent inducers as well of autologous Treg cells, which differentiated from a TCR<sup>hi</sup> CD69<sup>hi</sup> progenitor subset included within the double positive (DP) thymocyte compartment. Also, mature thymic pDCs (MpDCs) were proved as efficient as thymic cDCs activated in response to TSLP in promoting this Treg developmental pathway. Interestingly, while thymic pDCs were not able to mature in the response to TSLP, they became activated upon specific interaction with anti-CD3-stimulated TCR<sup>hi</sup> CD69<sup>hi</sup> DP thymocytes showing upregulated CD40L expression. The physiological relevance of these findings is demonstrated by the observation that Treg cells and pDCs lie in close proximity *in vivo* in the steady-state human thymus.

**Conclusion:** Human thymic pDCs may play an important role in the establishment and maintenance of immunological tolerance through the generation of natural Treg cells.

**PB06/26 NOVEL FOXP3.EGFP-DTR-LUCIFERASE MICE FOR ANALYSIS AND IMAGING OF TREG HOMEOSTASIS**J. Suffner<sup>1</sup>, K. Hochweller<sup>1</sup>, M.-C. Kühnle<sup>1</sup>, X. Li<sup>1</sup>, N. Garcia Garbi<sup>1</sup>, G.J. Hämmerling<sup>1</sup><sup>1</sup>Deutsches Krebsforschungszentrum (DKFZ), Department of Molecular Immunology, Heidelberg, Germany

**Objective:** Foxp3+CD4+CD25+ T cells, so called regulatory T cells (Tregs), play a fundamental role in the maintenance of self-tolerance. For prevention of autoimmunity the number of Tregs needs to be tightly controlled. In the present study we investigated the homeostatic regulation of the Treg compartment.

**Methods:** In order to analyze Treg homeostasis *in vivo*, we generated a set of novel BAC-transgenic mice, expressing under control of the Foxp3 promoter eGFP, the diphtheria toxin receptor (DTR), and luciferase (designated Foxp3.GDL mice). The DTR/DT suicide system allows selective depletion of Tregs with diphtheria toxin (DT), so that Treg homeostasis can be studied in mice in which the compartments for conventional CD4+ and CD8+ T cells is unaltered. Expression of luciferase allows imaging of the homeostatic process by non-invasive bioluminescence imaging (BLI).

**Results:** Treatment of Foxp3.GDL mice with DT resulted in depletion of Tregs. For further studies, a Foxp3.GDL line was selected in which a small percentage of Tregs escaped depletion. These surviving Tregs underwent rapid homeostatic proliferation and replenished the Treg compartment in about two to three weeks. This homeostatic expansion could be visualized by BLI and flow cytometric analysis of blood, lymph nodes, and spleen. The proliferation occurred in the presence of all other lymphocyte subpopulations such as B cells, CD4+, and CD8+ T cells, and was thymus-independent, as demonstrated in thymectomized mice. When CFSE-labeled CD4+ T cells were transferred into Treg depleted Foxp3.GDL mice, only the Foxp3+ cells proliferated strongly but not conventional Foxp3-CD4+ cells. Studies addressing the mechanism revealed a role for dendritic cells in the homeostatic proliferation of Tregs.

**Conclusions:** The present study shows that following depletion of Tregs, the small percentage of surviving Tregs expands vigorously by homeostatic proliferation and replenishes the Treg compartment. Dendritic cells were found to be an important factor in this process.

**PB06/27 FOXP3-DEPENDENT NETWORKS OF TRANSCRIPTIONAL AND TRANSLATIONAL CONTROL IN IMMUNOTOLERANCE**P.-Y. Tsai<sup>1</sup>, A. Beyer<sup>2</sup>, M. Dembinski<sup>1</sup>, S. Schallenberg<sup>1</sup>, C. Petzold<sup>1</sup>, T. Koenig<sup>1</sup>, K. Lahl<sup>3</sup>, T. Sparwasser<sup>3</sup>, S. Yerramilli<sup>4</sup>, E. Lader<sup>4</sup>, K. Kretschmer<sup>1</sup><sup>1</sup>CRDT/DFG-Centre for Regenerative Therapies Dresden, Immunotolerance in Regeneration, Dresden, Germany, <sup>2</sup>Biotechnology Center, TU Dresden, Cellular Networks & Systems Biology, Dresden, Germany, <sup>3</sup>Centre of Experimental and Clinical Infection Research, Twincore, Institute for Infection Immunology, Hanover, Germany, <sup>4</sup>QIAGEN Sciences, Germantown, United States

While the non-redundant role of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells (Treg) expressing the forkhead family transcription factor Foxp3 in immune homeostasis has been firmly established, the molecular pathways involved in the Foxp3-specified developmental and suppressor program remain poorly understood. Combined genome-wide location (ChIP-on-Chip) and global mRNA expression analysis indicated to us that the predominant effect of Foxp3 promoter occupancy is to modulate NFAT-dependent activation of protein-coding target genes upon T cell stimulation. Interestingly, Treg-specific ablation of Drosha or Dicer was recently shown to result in a phenotype that is reminiscent of the highly aggressive autoimmune disease observed in the absence of functional Treg due to lack of functional Foxp3 protein. Additionally, recent reports suggested specific functions of individual miRNAs in Treg physiology. Thus, while insights into the indispensable role of miRNAs in functional Treg development and maintenance is beginning to emerge, much less is known about the regulation of miRNA expression in Treg.

Here, we show that, in addition to transcription regulation of protein-coding genes, Foxp3 substantially contributes to the Treg-specific miRNA signature by directly regulating expression of a remarkably high number of miRNAs. Initially, we performed a transcriptome-wide miRNA quantification employing a novel real-time PCR approach recently developed by some of us (S.-Y. & E.L.). This led to the identification of approximately 180 miRNAs as being expressed in conventional and Foxp3-expressing CD4<sup>+</sup> T cells. Combining a comprehensive bioinformatics approach to predict T cell-relevant transcription factor binding sites in miRNA promoter regions with ChIP-on-Chip and conventional ChIP assays helped identifying a core set of approximately 30 miRNAs whose transcription is directly regulated by Foxp3, often through cooperation with NFAT. We further show that, in addition to T cell receptor signaling, the distinct miRNA profiles implemented by Foxp3 in thymus and peripheral lymphoid organs is fine-tuned by integrating TGF- $\beta$  and IL-2 receptor-emanating signals during Treg differentiation and activation. Taken together, these studies provide detailed insight into complex networks of transcription and translation regulation of Treg-mediated immune homeostasis.

**PB06/28 HUMAN PLASMACYTOID DENDRITIC CELLS EXPOSED TO AN AUTOIMMUNE ENVIRONMENT INDUCE POTENT REGULATORY T CELLS VIA INDOLEAMINE 2,3-DIOXYGENASE**M. Kavousanaki<sup>1</sup>, P. Sidiropoulos<sup>1</sup>, E. Choustoulaki<sup>1</sup>, H. Kritikos<sup>1</sup>, D. Boumpas<sup>1</sup>, P. Verginis<sup>1</sup><sup>1</sup>University of Crete, Medical School, Iraklion, Greece

Dendritic cells (DCs) play a crucial role in the regulation of immune responses via expansion and/or induction of regulatory T cells (Tregs). However, their contribution to the development of Tregs in an autoimmune disease setting in humans remains unclear. Here, we demonstrate that plasmacytoid (pDCs), but not myeloid (mDCs), induce potent Tregs in patients with rheumatoid arthritis (RA) in remission. In active RA patients, CD303<sup>+</sup> pDCs and CD1c<sup>+</sup> mDCs are undetectable in the periphery but re-appear in patients responding to therapy. Mature pDCs from inactive RA patients, but not healthy controls, express high levels of indoleamine 2,3-dioxygenase (IDO) and promote the differentiation of allogeneic naïve CD4<sup>+</sup>CD25<sup>+</sup> T cells into Tregs that show poor proliferation *in vitro* and secrete significant amounts of IL-10 in an IDO-dependent fashion. Most importantly, these pDC-primed Tregs, potently suppress the proliferation of autologous naïve CD4<sup>+</sup> T cells in a dose-dependent manner. Collectively, these data demonstrate for the first time that human pDCs, exposed to an autoimmune environment, acquire a tolerogenic phenotype that may be exploited for therapeutic purposes.

**PB06/29 NAD<sup>+</sup> MODULATES THE NUMBER, FUNCTION AND PHENOTYPE OF CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> REGULATORY T CELLS**S. Hubert<sup>1</sup>, S. Adriouch<sup>1</sup>, M. Vaillant<sup>1</sup>, S. Calbo<sup>1</sup>, L. Drouot<sup>1</sup>, C. Arnault<sup>1</sup>, L. Jean<sup>1</sup>, F. Haag<sup>2</sup>, F. Koch-Nolte<sup>2</sup>, O. Boyer<sup>1</sup>, M. Seman<sup>1</sup><sup>1</sup>University of Rouen, Faculty of Medicine and Pharmacy, Inserm Unit U905, Rouen, France, <sup>2</sup>University Medical Center of Hamburg, Institut of Immunology, Hamburg, Germany

Despite the considerable interest in the biology of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Regulatory T cells (Tregs), endogenous factors controlling their homeostasis and functions are still poorly understood. Purine nucleotides, such as ATP and NAD, can be released into the extracellular milieu following tissue damage, inflammation, cell stress or even during cell manipulation *in vitro*. We have previously shown that conventional T cells are induced to die from apoptosis following their encounter with micromolar NAD concentrations and that the molecular mechanism involves activation of the P2X7 receptor<sup>(1)</sup>. We show here that mouse Tregs from C57BL/6 mice express higher level of P2X7 than conventional T cells and are consequently more susceptible to this regulatory pathway. Contrastingly, Tregs from Balb/c express a higher level of P2X7 on their surface but a lower level of ART2.2. Consequently, treatment with NAD<sup>+</sup> in these mice leads to a relative enrichment into Tregs. Interestingly, while a natural P451L mutation in the sequence of P2X7 in C57BL/6 mice impairs the sensitivity to NAD induced cell death (NICD) of conventional T lymphocytes<sup>(2)</sup>, higher expression of the receptor on Tregs in these mice restores a high sensitivity to NAD induced cell death. As a consequence, a single injection of NAD into C57BL/6 Knock out for CD38 mice, the major endogenous NAD-glycohydrolase, induces the selective death of more than 80% of CD4<sup>+</sup>CD25<sup>+</sup> Tregs. Correlatively, NAD<sup>+</sup> treatment increases the antitumor response *in vivo* by eliminating Tregs in these mice and this effect is reverted by pre-treatment of mice with a llama single chain antibody that blocks ART2.2 activity. Moreover, we found higher number of Tregs in the P2X7<sup>-/-</sup> mice suggesting that ATP or NAD released from endogenous sources regulate their homeostasis *in vivo*. We also demonstrate that sufficient amount of NAD<sup>+</sup> is released during cell preparation *ex vivo* to affect their viability, their phenotype and their apparent functions. Collectively our data show that NAD<sup>+</sup> influences the survival, phenotype and function of Tregs and suggest that blocking P2X7 signalling may represent a new strategy to manipulate Tregs *in vivo*.

(1). Seman et al. Immunity 2003, 19:571

(2). Adriouch et al. J. Immunol. 2002, 169(8)

**PB06/30 ANTIGEN PRESENTATION BY PLASMACYTOID DENDRITIC CELLS CONTRIBUTE TO TOLERANCE IN EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS**S. Hugues<sup>1</sup>, W. Reith<sup>1</sup><sup>1</sup>University of Geneva, Pathology and Immunology, Genève 4, Switzerland

**Objectives:** In contrast to conventional DCs (cDC), which function as sentinels of the adaptive immune system and initiate T cell immunity and peripheral T cell tolerance, plasmacytoid DCs (pDC) were initially believed to be involved primarily in innate immune responses, particularly via the secretion of type I interferon and other cytokines in response to viral infections. However, like cDC, pDC express MHC class II (MHCII) molecules and recent evidence has suggested that they are also likely to be implicated in adaptive immune responses, particularly in the induction of T cell tolerance. The aim of our work is to study the contribution of MHCII-mediated Ag presentation by pDC in the induction of T cell tolerance during the development of Experimental autoimmune encephalomyelitis (EAE), a mouse model for Multiple Sclerosis.

**Methods:** We used mice characterized by the selective loss of MHCII expression on pDC. These mice therefore present a unique tool for distinguishing between the roles of pDC in innate and adaptive immune responses. EAE was induced in mice by active immunization with MOG peptide (MOG<sub>35-55</sub>).

**Results:** We have demonstrated that mice in which MHCII expression was selectively abrogated in the pDC population develop more severely EAE compared to WT mice. Accordingly, we observed a significant increase in the numbers of encephalitogenic Th1 and Th17 CD4<sup>+</sup> T cells in the peripheral lymphoid tissues and in the central nervous system of these mice. Furthermore, the numbers of Foxp3<sup>+</sup> regulatory T cells infiltrating the spinal cord of mice lacking MHCII expression by pDC was dramatically reduced compared to WT animals.

**Conclusions:** Our results suggest that MHCII-mediated Ag presentation by pDC attenuates the severity of EAE, and that this protective role may be mediated by the induction of regulatory T cells (Treg) capable of inhibiting EAE development.

**PB06/31 CHARACTERIZATION OF PROTECTIVE IL-10 PRODUCING CELL-TYPES AFTER *HELICOBACTER HEPATICUS* INFECTION**M.J. Barnes<sup>1</sup>, M. Asquith<sup>1</sup>, R. Flavell<sup>2</sup>, K. Maloy<sup>1</sup>, F. Powrie<sup>1</sup><sup>1</sup>University of Oxford, Sir William Dunn School of Pathology, Oxford, United Kingdom, <sup>2</sup>Yale University, New Haven, United States

Effective tolerance to intestinal microbes requires Interleukin-10, an immunosuppressive cytokine that can be expressed by lymphocytes, myeloid cells and epithelial cells. While CD4<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells are an important source of IL-10 in the colon, other cell populations may express IL-10 and contribute to intestinal homeostasis, particularly in infectious contexts. However, the identity and functional importance of these additional cell populations remains unclear. Under SPF conditions, oral infection with the gram(-) bacteria *Helicobacter hepaticus* is sufficient to induce colitis in mice treated with blocking anti-IL-10R antibody or in IL-10<sup>-/-</sup> mice. We have characterized intestinal IL-10 producing cells after *H. hepaticus* infection using a reporter mouse and noted an increase in caecal CD4<sup>+</sup>IL-10<sup>gfp</sup> T cells and GR1<sup>+</sup>IL-10<sup>gfp</sup> myeloid cells in the colon. In a colitis model driven solely by innate immune activation, in which 129.RAG<sup>-/-</sup> or 129.RAG<sup>-/-</sup>IL-10<sup>-/-</sup> mice are orally infected with *H. hepaticus*, we observed more severe colitis but not typhilitis in IL-10-deficient mice, suggesting a protective role for myeloid derived IL-10 in the colon. Both Foxp3<sup>+</sup> and Foxp3<sup>-</sup> CD4<sup>+</sup> T cells produced IL-10. To assess their protective capacity, we transferred T cells from *H. hepaticus* infected Foxp3-gfp reporter mice into infected RAG<sup>-/-</sup> mice. Among CD4<sup>+</sup>CD45RB<sup>low</sup> T cells, both the Foxp3<sup>+</sup> and Foxp3<sup>-</sup> cells protected against colitis and typhilitis, but only the Foxp3<sup>+</sup> cells prevented systemic pathology, including liver inflammation. Additional data on the kinetics, identity and induction of protective IL-10 producing T cell and myeloid cell populations will be presented. These studies should yield insights into the various sources of IL-10 in the intestine and may suggest ways to manipulate its production in order to maintain or restore intestinal homeostasis.

**PB06/32 ALLO-ANTIGEN INDUCED REGULATORY CD8<sup>+</sup>CD103<sup>+</sup> T CELLS**S.D. Koch<sup>1,2</sup>, E. Uss<sup>1,2</sup>, A. van Stijn<sup>1,2</sup>, R.A.W. van Lier<sup>1</sup>, I.J.M. ten Berge<sup>2</sup><sup>1</sup>Amsterdam Medical Center, Department of Experimental Immunology, Amsterdam, Netherlands, <sup>2</sup>Amsterdam Medical Center, Department of Internal Medicine, Renal Transplant Unit, Amsterdam, Netherlands

Both CD4<sup>+</sup> and CD8<sup>+</sup> T cell subpopulations have been described to function as regulatory T cells (Tregs) in autoimmune diseases, cancer and transplant immunology. Amongst the CD8<sup>+</sup> Tregs, we recently identified an alloantigen-induced subpopulation, which is characterized by expression of alphaE-beta7 integrin (CD103). Allo-stimulation directly induces CD103 expression on CD8<sup>+</sup> T cells. Allo-stimulated CD8<sup>+</sup>CD103<sup>+</sup> Tregs displayed an antigen-experienced effector phenotype, but showed reduced cytotoxicity, IFN- $\gamma$  production and proliferative capacity after re-stimulation. CD103<sup>+</sup> T cells were able to suppress allo-reactive effector T cell function in a cytokine independent, but cell-cell dependent way. Our aim is to identify the mechanism of induction and suppression of these CD8<sup>+</sup>CD103<sup>+</sup> Tregs.

CD8<sup>+</sup>CD103<sup>+</sup> Tregs were generated in PBMC- or DCs-based mixed lymphocyte cultures and were detected by CFSE dilution after 6 days of culture. After intensive functional and phenotypic characterization, we performed a DNA micro array with sorted CD8<sup>+</sup>CD103<sup>+</sup> and CD8<sup>+</sup>CD103<sup>-</sup> T cells. As an array confirmation we tested the protein expression of the selected gene products and their ligands on CD8<sup>+</sup>CD103<sup>+</sup> T cells and different PBMC lineages. Moreover, we investigated if these gene products are involved in the induction or contribute to the immune suppressive effects of CD8<sup>+</sup>CD103<sup>+</sup> T cells.

The results of the DNA micro array identified 55 differently regulated genes, which were not related with known Treg markers and immune regulatory molecules. Interestingly, a number of killer lectin-like receptors and associated molecules, were differently regulated in CD8<sup>+</sup>CD103<sup>+</sup> T cells. We selected two of those receptors and confirmed their up-regulation on allo-reactive CD8<sup>+</sup>CD103<sup>+</sup> T cells on protein level. Preliminary results indicate that the ligands of these molecules were strongly expressed on mDCs, which were identified as the most potent inducers of CD8<sup>+</sup>CD103<sup>+</sup> T cells.

These findings suggest that direct cellular interaction between ligand-expressing mDCs and killer lectin like receptors on CD8<sup>+</sup> T cells might be involved in the induction or regulating the suppressive activity of CD8<sup>+</sup>CD103<sup>+</sup> Tregs. Elucidation of the mechanisms of induction and suppression of CD8<sup>+</sup>CD103<sup>+</sup> Tregs will be followed by attempts to apply this knowledge to regulate the immune response in different diseases and especially in solid organ transplantation.

**PB06/33 SPECIFICITIES OF SPONTANEOUSLY INDUCED TUMOR-REACTIVE MEMORY T CELLS IN PANCREATIC CANCER PATIENTS AND THEIR DIFFERENTIAL SUPPRESSION BY REGULATORY T CELLS**D.-H.K. Pietsch<sup>1</sup>, A. Bonertz<sup>1</sup>, H.F. Schmitz-Winnenthal<sup>2</sup>, J. Weitz<sup>2</sup>, P. Beckhove<sup>1</sup><sup>1</sup>German Cancer Research Center, Translational Immunology, Heidelberg, Germany, <sup>2</sup>University Hospital of Heidelberg, Department of General Surgery, Heidelberg, Germany

**Objective:** Spontaneous tumor-specific T cell responses in pancreatic cancer patients have recently been described. We investigated the specificities of spontaneously induced memory T cells (TCs) against a broad variety of different pancreatic TAAs in the blood (PB) and bone marrow (BM) of pancreatic cancer patients and control by regulatory T cells (Tregs) in comparison to TCs from healthy donors (HDs).

**Methods:** BM and PB samples were taken from 101 patients with primary pancreatic cancer and 32 HDs. Presence of antigen-specific TCs was analyzed by IFN- $\gamma$  ELISPOT assay using autologous dendritic cells (DCs) pulsed with synthetic 40-100 amino acids long peptides derived from the defined TAAs p53, MUC1, EGFR, Her2/neu, heparanase, survivin, telomerase, mesothelin, MAGE-3 and CEA.

Additionally we co-cultured TAA-peptide pulsed DCs with autologous Tregs and evaluated their capacity to suppress proliferation of polyclonally activated TCs in an antigen-dependent manner by <sup>3</sup>H-incooperation.

**Results:** 78% of the patients showed reactivity against at least one tested TAA. The highest patient response rate in PB and BM were detected against p53 (29% and 25%), EGFR (25% and 14%) and heparanase (41% and 17%), which together were recognized by TCs from more than 76% of all tested patients. The combined reactivity of these three antigens did not exceed 18% in the HD group. The overall frequencies of TAA-specific TCs in PB and BM of cancer patients were significantly higher compared to those in HDs before and after Treg depletion. Depletion of Tregs did not result in an overall increase of the proportions of patients or HDs showing TAA-specific TC reactivity but in the case of some TAAs (e.g. EGFR) lead to an increase of TAA-specific TC frequencies. Treg induced suppression of proliferation was most frequently observed after Treg activation through CEA and EGFR.

**Discussion:** This study shows the regular occurrence of tumor-reactive memory TCs in the PB and BM of pancreatic cancer patients. Tregs significantly inhibited TAA-reactivity in some tested patients. However, Tregs differentially controlled T cell responses according to the chosen TAA, suggesting that the antigen specificities of Tregs differ substantially from the specificities of tumor reactive TCs.

**PB06/34 CHARACTERIZATION OF TRANSCRIPTIONAL PROFILES IN HUMAN NATURALLY OCCURRING REGULATORY T CELLS**K. Satoh<sup>1</sup>, S. Fondel<sup>1</sup>, J. Kubach<sup>1</sup>, C. Becker<sup>1</sup>, J. Joore<sup>2</sup>, H. Stahl<sup>3</sup>, W. Rist<sup>3</sup>, M. Lenter<sup>3</sup>, D. Mennerich<sup>3</sup>, F.-J. Schneider<sup>3</sup>, S. Gattenlöhner<sup>4</sup>, F. Berberich-Siebel<sup>4</sup>, H. Jonuleit<sup>1</sup>, A. Tuettenberg<sup>1</sup><sup>1</sup>Johannes Gutenberg-University, Dermatology, Mainz, Germany, <sup>2</sup>Pescan Presto B.V., Array Technologies, Lelystad, Netherlands, <sup>3</sup>Boehringer Ingelheim, Pharma GmbH & Co.KG, Biberach, Germany, <sup>4</sup>Julius-Maximilians-University, Institute of Pathology, Würzburg, Germany

Naturally occurring CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells (Tregs) are essential for T cell homeostasis and the maintenance of peripheral tolerance. They prevent the activation of autoaggressive T cells in the context of autoimmune diseases and suppress inadequate allergen specific T cells. On the opposite, Tregs inhibit also effective immune responses against tumors such as melanoma. A detailed understanding of molecular mechanisms that control functional properties of Tregs is mandatory for the development of novel immunotherapeutic strategies against allergy, autoimmunity and cancer. We initiated a general genomic, proteomic and kinome profiling of human Tregs to identify key molecules associated with their functional activation. Using large scale isolation of CD4<sup>+</sup> T helper cells and Tregs from leukapheresis products following polyclonal stimulation and analysis of transcriptional profiles at different time points after activation, we compared the molecular and proteomic activation pattern of human T helper cells and Tregs. Here we show that cytoplasmic expression and nuclear translocation of NFATc1, NFATc2, NFkB and



AP-1 family members is strongly upregulated in T helper cells after activation, but not observed in Tregs. In contrast, human Tregs show increased Foxp3 levels after activation and a constant Treg-specific expression of galectin-10 in cytoplasm and nucleus. Comparison of genomic, proteomic and kinome profiles are currently under investigation and additional data will be presented on the meeting. Impact of identified molecules will be tested in functional assays using specific inhibitors and siRNA mediated knockdown of these targets. The main objective of our analysis is the identification of novel targets for the immunotherapeutic intervention of dysregulated immune responses in the near future.

**PB06/35 TEMPORARY SELECTIVE DEPLETION OF REGULATORY T CELLS EXACERBATES MOG<sub>35-55</sub> PEPTIDE INDUCED EAE ONLY MODERATELY**

S. Berg<sup>1</sup>, S. Heink<sup>1</sup>, T. Sparwasser<sup>2</sup>, C. Stadelmann<sup>3</sup>, T. Kamradt<sup>1</sup>

<sup>1</sup>Medical School Friedrich-Schiller-University, Department of Immunology, Jena, Germany, <sup>2</sup>Centre for Experimental and Clinical Infection Research, Institute for Infection Immunology, Hanover, Germany, <sup>3</sup>Georg-August University, Department of Neuropathology, Göttingen, Germany

**Objectives:** To investigate the capacity of regulatory T cells (T<sub>reg</sub>) to modulate inflammatory attacks in the animal model experimental autoimmune encephalomyelitis, we performed comprehensive studies with systematic temporary T<sub>reg</sub> depletion at different EAE stages and monitored the activation of T effector cells and the clinical EAE course.

**Methods:** DEREg mice express a transgene encoding a fusion protein of EGFP and diphtheria toxin (DT) receptor under the control of *foxp3* promoter. Thus, T<sub>reg</sub> cells can specifically be identified *ex vivo* and depleted *in vivo*. We compared DEREg tg<sup>+</sup> mice and tg<sup>-</sup> littermates equally treated with DT at different stages during EAE, induced by immunization with MOG<sub>35-55</sub>/CFA. Furthermore, MOG-specific CD4<sup>+</sup>CD154<sup>+</sup> T cells isolated from lymph nodes (LN) and CNS, respectively, were quantified and analyzed for cytokine expression profiles by flow cytometry.

**Results:** Since prolonged DT treatment of immunized mice turned out to be fatal, we applied the toxin (500ng/mouse i.p.) only twice on consecutive days causing a complete, but short-term T<sub>reg</sub> depletion in tg<sup>+</sup> mice. Treatment before immunization (d-2 and d-1) resulted in increased frequencies of antigen-specific Th cells in draining LN from tg<sup>+</sup> mice compared to littermates (analyzed on d7). Significantly more T cells expressed IFN- $\gamma$ , IL-17 or TNF- $\alpha$ . In spite of the initially augmented T-cell response, we could observe little, if any exacerbation of EAE symptoms compared with tg<sup>-</sup> mice. This was accompanied by only a modestly altered cytokine expression profile of CNS residing Th cells in the acute EAE phase. Likewise, DT application during preclinical EAE (d5 and d6) amplified the T cell response in tg<sup>+</sup> (analyzed on d14), but failed to worsen significantly EAE. In contrast, T<sub>reg</sub> depletion at the EAE peak (d15 and d16) induced lethal disease. T<sub>reg</sub> depletion during the remission phase (> d50) could induce EAE relapses in tg<sup>+</sup> mice.

**Conclusion:** Surprisingly, T<sub>reg</sub> depletion prior to or shortly after immunization did not significantly worsen EAE symptoms, although an initially amplified T cell activation occurred in DEREg mice. Only during clinical manifest EAE phases, T<sub>reg</sub> depletion resulted in disease exacerbation.

**PB06/36 COMPARATIVE PROTEIN EXPRESSION PROFILING OF NATURAL REGULATORY T CELLS (NTR) FROM HUMAN THYMUS AND PERIPHERAL BLOOD**

C. Xufré<sup>1</sup>, E. Codina<sup>1</sup>, C. Roura-Mir<sup>1</sup>, M. Costa<sup>1</sup>, N. Colomé<sup>2</sup>, J.J. Bech<sup>2</sup>, F. Canals<sup>2</sup>, D. Jaraquemada<sup>1</sup>, M. Martí<sup>1</sup>

<sup>1</sup>Institut de Biociències i Biomedicina/Universitat Autònoma de Barcelona, Cerdanyola del Vallès, Spain, <sup>2</sup>Servei de Proteòmica/Hospital Universitari de la Vall Hebron, Barcelona, Spain

Natural CD4<sup>+</sup>CD25<sup>+</sup>T regulatory cells (nTR) are thymic-emigrants found in the periphery that can prevent immune-mediated diseases by suppressing activation of effector T cells. Human nTR are defined by high expression of CD25 and FOXP3, although these are not exclusive markers since they are also expressed by other regulatory cell types. The objective of our work was to analyze nTR cells to identify specific markers and molecules related to TR cell function.

Comparative proteomics of sorted CD4<sup>+</sup>CD25<sup>hi</sup> and CD4<sup>+</sup>CD25<sup>-</sup> fractions from human thymus (n=3) and peripheral blood (PBMCs, n=2) were performed by Isotope Code Protein Labeling (ICPL). Differentially expressed ICPL candidates were further tested by qPCR using a customized array (TaqMan<sup>®</sup>) on 4 sorted PBMCs samples. The array also included genes corresponding to chemokine receptors and molecules reported as TR markers.

The comparative expression analysis revealed a number of proteins differentially expressed by the TR cells that suggested a partially activated phenotype, preferentially including cytoskeleton, signalling and antiapoptotic proteins. In contrast, nuclear proteins and enzymes were preferentially increased in the non-TR population. RNA expression analysis confirmed some of the differentially expressed proteins. Sample quality was ascertained by the high RNA level of CD25 and FoxP3 (score >50), GITR and CTLA-4, and low CD127 in TR vs. non-TR cells. Chemokine receptor analysis demonstrated a high increase of CCR6 (score>13) and CCR10 (>6), both involved in T cell homing to epithelium. Other markers identified in human induced TR, such as TLR2 and TLR9, were significantly lower in nTR compared to controls.

Regarding to the candidate ICPL proteins, RNA analysis confirmed a number of them, such as Galectin-3 (LGALS3) and Macrophage Capping Protein (CAPG), both increased in TR. The lectin LGALS3 belongs to the same family as galectin-10, which has been involved in anergy and suppressor functions of TR. CAPG is an actin-binding protein which is related with WASP whose deficiency is related with impairment of TR function.

Most highly expressed proteins in periphery were not increased or even present in the thymic CD4<sup>+</sup>CD25<sup>+</sup> cells, as if the expression of many of these molecules, mostly antiapoptotic and activation-related, would be acquired in the periphery.

**PB06/37 ANTIGEN QUALITY DETERMINES THE EFFICIENCY OF ANTI-TUMOR IMMUNE RESPONSES GENERATED IN THE ABSENCE OF REGULATORY T CELLS**

A.-S. Bergot<sup>1,2,3</sup>, A. Durgeau<sup>1,2,3</sup>, B. Levacher<sup>1,2,3</sup>, B.M. Colombo<sup>2,3,4</sup>, J.L. Cohen<sup>1,2,3</sup>, D. Klatzmann<sup>1,2,3,5</sup>

<sup>1</sup>UPMC Univ Paris 06 UMR 7211, Paris, France, <sup>2</sup>CNRS, UMR 7211, Immunology-Immunopathology-Immunotherapy (I3), Paris, France, <sup>3</sup>INSERM, UMR-S 959, Immunology-Immunopathology-Immunotherapy (I3), Paris, France, <sup>4</sup>Département de Biologie, Université d'Evry-val d'Essonne, Evry, France, <sup>5</sup>AP-HP, Hôpital Pitié-Salpêtrière, Biotherapy, Paris, France

The observation that depletion or inhibition of regulatory T cells (T<sub>regs</sub>) unleashes efficient antitumor effector immune responses that can lead to tumor eradication in mice has opened new perspectives for the development of cancer immunotherapy. The quality and overall efficiency of the effector immune responses induced in the absence of T<sub>regs</sub> appear to depend on multiple factors that determine the result of a battle involving effectors T cells (T<sub>effs</sub>), T<sub>regs</sub> and tumor cells. Here we investigated the quality of the tumor-associated antigens (TAAs) as one such factor. We show that the presence of a strong dominant antigen is required for the induction of effector responses capable of tumor eradication in the absence of T<sub>regs</sub>. The sole addition of a dominant antigen on tumor cells does not change tumor growth in unmanipulated mice, but improve tumor eradication rate from a few to almost 100 percent in the absence of T<sub>regs</sub>. This eradication can be shown to result from the recruitment and activation of specific T<sub>effs</sub> recognizing this antigen. We also show that the presence of such dominant antigens has the side effect of restricting the breadth of the immune response to other TAAs, which could favor the generation of escape mutant by tumor editing. Altogether, our results highlight the potential, and some requirements for cancer immunotherapy based upon T<sub>reg</sub>-depletion. They also reveal that, ultimately, tumor fate depends on multiple factors that should be all taken into consideration for the design of more efficient immunotherapy.

**PB06/38 COMPARISON OF STABLE HUMAN TREG AND TH CLONES BY TRANSCRIPTIONAL PROFILING**

J. Stockis<sup>1</sup>, W. Fink<sup>1</sup>, V. François<sup>1,2</sup>, T. Connerotte<sup>1</sup>, C. de Smet<sup>1</sup>, L. Knoops<sup>1,3</sup>, P. van der Bruggen<sup>1,2</sup>, T. Boon<sup>1,2</sup>, P.G. Coulie<sup>1</sup>, S. Lucas<sup>1</sup>

<sup>1</sup>Université Catholique de Louvain, Institut de Duve, Brussels, Belgium, <sup>2</sup>Ludwig Institute for Cancer Research, Brussels Branch, Brussels, Belgium, <sup>3</sup>Cliniques Universitaires Saint-Luc, Department of Haematology, Brussels, Belgium

**Objectives:** Our long-term objective is to assess the contribution of regulatory T cells (Treg) in the immune suppressive environment that seems to prevail in human tumors, and notably in melanoma metastases. Because FOXP3 mRNA and protein are not specific markers of human Tregs, this question has remained difficult to address rigorously. We attempted to derive stable human Treg clones, and used them as a model to gain insight into human Treg biology.

**Methods:** From cancerous and non-cancerous patients, we derived stable clones of CD4<sup>+</sup> Treg, defined as clones that expressed high CD25 at rest, were anergic *in vitro*, and suppressed the proliferation of co-cultured CD4<sup>+</sup> cells. We analyzed the methylation status of a conserved regulatory region in FOXP3 intron 1 by methyl specific qPCR on bisulfite-treated gDNA in a panel of Treg and T helper (Th) clones. We also compared Treg and Th clones by expression microarrays.

**Results:** A conserved region of FOXP3 intron 1 was demethylated in all Treg clones, whereas it was methylated in non-regulatory Th and cytotoxic T cell (CTL) clones. In our panel of human clones, this stable epigenetic mark correlated better with suppressive activity than did FOXP3 mRNA or protein expression. We used expression microarrays to compare Treg and Th clones after activation, which is required for suppressive function. The transcriptional profile that is specific of activated Treg clones includes a TGF- $\beta$  signature. Both activated Treg and Th clones produced the latent form of TGF- $\beta$ . However, SMAD2 phosphorylation was observed after activation in the Treg but not in the Th clones, indicating that only activated Treg clones produced the bioactive form of TGF- $\beta$ . A TGF- $\beta$  signature was also displayed by a Th clone "suppressed" by a Treg clone.

**Conclusion:** The hallmark of our panel of activated human Treg clones is to produce bioactive TGF- $\beta$  which has autocrine actions on Tregs and can have paracrine actions on other T cells.

**PB06/39 REGULATORY T CELLS AND THE PD-L1/PD-1 PATHWAY MEDIATE IMMUNE SUPPRESSION IN MALIGNANT HUMAN BRAIN TUMORS**J.F.M. Jacobs<sup>1,2</sup>, A.J. Idema<sup>3</sup>, P. Wesseling<sup>4</sup>, I.J.M. de Vries<sup>2</sup>, G.J. Adema<sup>2</sup><sup>1</sup>Radboud University Nijmegen Medical Centre, Bloodtransfusion and Transplantation Immunology, Nijmegen, Netherlands, <sup>2</sup>Nijmegen Centre for Molecular Life Sciences, Tumor Immunology, Nijmegen, Netherlands, <sup>3</sup>Radboud University Nijmegen Medical Centre, Neurosurgery, Nijmegen, Netherlands, <sup>4</sup>Radboud University Nijmegen Medical Centre, Pathology, Nijmegen, Netherlands**Objectives:** The brain is a specialized immune site representing a unique tumor microenvironment. As large parts of many brain tumors are resected using ultrasonic aspiration, the availability of fresh brain tumor material for ex-vivo analysis is often limited. We analyzed ultrasonic tumor aspirates as a bio-source to study immune suppression in human brain tumors.**Methods:** Blood and ultrasonic aspirated brain tumor tissue from 78 patients with intracranial tumors were collected. Lymphocytes, including CD4<sup>+</sup>FOXP3<sup>+</sup>CD25<sup>high</sup>CD127<sup>low</sup> regulatory T cells (Tregs), were characterized using flow-cytometry, immunohistochemistry and immune suppression assays.**Results:** We detected massive, chemokine-mediated, Treg infiltration in glioblastomas (n=27) and metastatic brain tumors (n=19). No Treg accumulation was observed in benign tumors such as meningiomas (n=10) and pituitary adenomas (n=5). Only Tregs in high-grade tumors exhibited an activated phenotype as indicated by decreased proliferation and elevated CTLA-4 and FoxP3 expression relative to blood Tregs. Tumor derived Tregs efficiently suppressed cytokine secretion and proliferation of autologous intratumoral lymphocytes. Most tumor infiltrating Tregs localized in close proximity to effector T cells. Furthermore, 61% of the malignant brain tumors expressed programmed death ligand-1 (PD-L1). The inhibitory PD-1 receptor was selectively expressed on tumor infiltrating CD4<sup>+</sup> effector cells and not on intratumoral Tregs, providing a survival advantage to the Tregs in the tumor microenvironment.**Conclusion:** Using ultrasonic tumor aspirates as a bio-source we identify Tregs and the PD-L1/PD-1 pathway as immune suppressive mechanisms in malignant but not benign human brain tumors.**PB06/40 MYCOPHENOLIC ACID-INDUCED REGULATORY CD4<sup>+</sup> T CELLS CONFER TOLEROGENTIC PROPERTIES TO HUMAN DENDRITIC CELLS: CONTRIBUTION OF MULTIPLE MECHANISMS INCLUDING IL-10, TGF- $\beta$ , LFA-1 AND CTLA-4**R. Lemoine<sup>1</sup>, H. Nivet<sup>1,2</sup>, F. Velge-Roussel<sup>1</sup>, Y. Lebranchu<sup>1,2</sup>, C. Baron<sup>1,2</sup><sup>1</sup>EA 4245 'Cellules Dendritiques, Immunomodulation et Greffes', UFR de Médecine, Université François Rabelais, Tours, France, <sup>2</sup>Service de Néphrologie et d'Immunologie Clinique, CHRU, Tours, France**Introduction:** Regulatory T cells are able to suppress effector T lymphocyte responses. However, their effects on dendritic cells (DC) are not completely understood. In our laboratory, we have recently demonstrated that mycophenolic acid-treated DC induced human CD4<sup>+</sup> regulatory T cells. In this study, we further analyzed the effects of these regulatory T cells on the phenotype and the function of human DC.**Materials and methods:** The maturation of human monocyte-derived DC was induced by TNF- $\alpha$  (20ng/ml) with or without 100 $\mu$ M mycophenolic acid. Regulatory CD4<sup>+</sup> T cells (iTreg) and effector CD4<sup>+</sup> T cells (Teff) were obtained by repetitive allostimulation with either MPA-DC or TNF-DC respectively and then co-cultured for 2 days with immature or LPS-matured DC. Their T cell-priming ability was then analyzed in allogeneic MLR after negative selection of DC. Cytokine secretions were measured by ELISA and DC surface markers expressions were assessed by flow cytometry.**Results:****1/** We demonstrated that iTreg did not modify expression of CD80, CD83, CD86 and CD25 on immature DC contrary to Teff which induced a strong maturation markers expression (p< 0.05 ; n=5). Furthermore, CCR5 expression on immature DC was increased following incubation with iTreg (56%  $\pm$  10 versus 34%  $\pm$  12). Incubation with Teff led to a significant increase of IL-6, IL-12 and IFN- $\gamma$  secretions contrary to iTreg which only induced the production of IL-10 (p=0.008). These results taken together showed that iTreg and Teff had opposite effects on immature DC.**2/** iTreg increased CCR5 expression and decreased expression of CD80, CD83, CD86 and CD25 on LPS-DC (p< 0.03). Moreover, iTreg inhibit IL-6 (48pg/ml  $\pm$  20 versus 128pg/ml  $\pm$  30; p=0.031), IL-12 (209pg/ml  $\pm$  96 vs 667pg/ml  $\pm$  172; p=0.016) and also IFN- $\gamma$  secretions of LPS-DC while Teff had increased those secretions. Importantly, DC modified by iTreg did not support the proliferation of allogeneic CD4<sup>+</sup> T cells whereas DC modified by Teff did. These data suggested that Treg "reprogram" mature DC into semi-mature DC. Finally, multiple mechanisms contribute to iTreg-mediated modulation of DC such as IL-10, TGF- $\beta$ , LFA-1 and CTLA-4.**Conclusion:** CD4<sup>+</sup> T cells activated by MPA-DC conferred tolerogenic properties to human DC.**PB06/41 IDENTIFICATION AND CHARACTERIZATION OF TYPE 1 REGULATORY (TR1)-LIKE CELLS IN HUMAN BLOOD**B. Haerlinger<sup>1</sup>, L. Lozza<sup>1</sup>, B. Steckel<sup>1</sup>, J. Geginat<sup>1</sup><sup>1</sup>Charité Medical School and DRFZ, RCIS, Berlin, GermanyTwo subsets of natural and adaptive regulatory T cells have been described, but the identity of adaptive type1 regulatory-like (Tr1) cells in humans is unclear. Here we analyzed a human blood CD4<sup>+</sup> T cell subset – CD45RA-CD25-IL-7R- cells – that rapidly secreted high levels of IL-10 together with IFN- $\gamma$ , but produced little IL-2. These IL-7R- T cells were rare, anergic and largely Foxp3-. They expressed low levels of Bcl-2 but high levels of Ki-67 and ICOS, suggesting that they had been recently activated in vivo. Consistently, they responded selectively to persistent foreign and self-antigens under steady state conditions. Unlike natural CD25+ regulatory T cells (Tregs), IL-7R- cells suppressed T cell priming in an IL-10-dependent fashion, and they required strong TCR stimulation for suppression. In summary, we have identified Tr1-like cells in human blood that are distinct from CD25+ Tregs and have characteristics of chronically activated Th1 effector cells.**PB06/42 DYSREGULATION OF FOXP3 T REGULATORY CELL DEVELOPMENT IN NEONATAL MICE**S. Cuss<sup>1</sup>, A. Green<sup>1</sup><sup>1</sup>Cambridge University, Pathology, Cambridge, United Kingdom**Objectives:** Foxp3+ T regulatory (Treg) cells are central for the control of autoreactive T cells. Extensive investigations have determined diverse mechanisms by which Foxp3+ Treg cells control autoimmunity. In contrast, little is known about the molecular requirements for natural Foxp3 Treg cell development, expansion and survival in the thymus. Previously we showed that interaction of pre-Foxp3+ Treg cells expressing CD154 with thymic epithelium or dendritic cell expressed CD40 is a pre-requisite for continuation of the Foxp3+ Treg cell developmental pathway in adult mice. Here we show such signals are obsolete for efficient Foxp3+ Treg cell development in neonates.**Methods:** We performed time course flow cytometric and functional analysis of foetal thymic organ cultures, as well as thymi from newborn, neonatal and adult mice to quantify the immunological and molecular changes that are associated with changes in the commitment of Foxp3+ Treg cell development to the CD40-CD154 pathway.**Results:** We demonstrate that the dynamic changing thymic microenvironment dictates precisely the developmental time point when the immune system commits Foxp3+ Treg cell development to the CD154-CD40 pathway and the physiological significance of this transition.**Conclusion:** These unique findings provide novel insights into the complexities of Foxp3+ Treg cell development at the level of costimulation depending on the maturational status of the animal.**PB06/43 MAST CELLS DOWNREGULATE NUMBERS AND CD25 EXPRESSION OF TREGS WHILE MC-DEFICIENT MICE EXHIBIT HIGHER TREG NUMBERS AND IL-10 PRODUCTION**S. Mrabet-Dahbi<sup>1</sup>, A.-M. Latuske<sup>1</sup>, C. Loddenkemper<sup>2</sup>, M. Maurer<sup>1</sup><sup>1</sup>Allergie-Centrum-Charité, Charité – Universitätsmedizin Berlin, Department of Dermatology and Allergy, Berlin, Germany, <sup>2</sup>Charité – Universitätsmedizin Berlin, Department of Pathology, Berlin, GermanyRecent findings have demonstrated that regulatory T cell (Treg) numbers are markedly reduced in atopic dermatitis skin lesions and that TNF downregulates Treg function in asthma. Since mast cells (MCs) critically determine allergic reactions and are prominent TNF producers, we hypothesized that MCs can modulate Treg numbers and functions. Using the MACS separation technology CD4<sup>+</sup>CD25<sup>+</sup> T cells (Tregs) were positively selected from splenocytes of naïve C57BL/6 mice (purity: > 90%), subjected to anti-CD3/anti-CD28 activation and then cocultured with peritoneum-derived cultured MCs (PCMCs) at a 1:1 and 100:1 ratio for 3 days. FACS analyses revealed a pronounced decline in Treg numbers paralleled by a dramatic decrease of CD25 expression of Tregs within the 1:1 Treg-MC coculture. Both effects were not seen at 100:1 Treg-MC ratios (CD4<sup>+</sup> cells: "Treg" 8476 vs "Treg-MC 1:1" 1484; p=0.02, vs "Treg-MC 100:1" 6932; p=0.50 and FI CD25: "Treg" 4016 vs "Treg-MC 1:1" 450; p=0.01, vs "Treg-MC 100:1" 1935; p=0.15). Moreover, similar results were seen after incubation of Tregs with supernatants derived from non-activated MCs. Noteworthy, these downregulatory effects were restricted to the Treg compartment since PCMCs or their supernatants had no impact on T effector cells. Tregs cocultured with MCs at a 1:1, but not at a 100:1 ratio, failed to inhibit IL-6 production of activated T effector cells (IL-6: "activated control T effector cells" 625 pg/ml vs "T effector cells plus Treg-MC 1:1" 956 pg/ml; p=0.006 and vs "T effector cells plus Treg-MC 100:1" 288 pg/ml; p=0.007). In addition, immunohistochemistry revealed a predominance of larger, Foxp3-enriched T cell regions in the spleen of MC-deficient Kit<sup>W<sup>o</sup></sup>/Kit<sup>W<sup>v</sup></sup> mice (S Foxp3<sup>+</sup> cells Kit<sup>W<sup>o</sup></sup>/Kit<sup>W<sup>v</sup></sup>: 3545 vs S Foxp3<sup>+</sup> cells Kit<sup>+/+</sup>: 1795; n=5). Furthermore, Tregs isolated from Kit<sup>W<sup>o</sup></sup>/Kit<sup>W<sup>v</sup></sup> mice produced more IL-10 upon activation than wildtype Tregs (IL-10 Kit<sup>W<sup>o</sup></sup>/Kit<sup>W<sup>v</sup></sup>: 227 pg/ml vs IL-10 Kit<sup>+/+</sup>: 30 pg/ml; p=0.006, n=4). Taken together, MCs can – ex vivo – downregulate Treg numbers and functions which are – in vivo – upregulated in the absence of MCs. This novel MC function may be relevant for the pathogenesis of diseases with abnormal Treg numbers and/or activity and could be exploited for this prevention or treatment

**PB06/44 PLASTICITY OF NTREG LINEAGE COMMITMENT: LOSS OF FOXP3 EXPRESSION UPON CD122 SIGNALING INHIBITION**J. Goldstein<sup>1</sup>, L. Mercey<sup>1</sup>, G. Marodon<sup>1</sup><sup>1</sup>UPMC-P6/CNRS UMR 7211, INSERM U959, Groupe Hospitalier La Pitié Salpêtrière, Paris, France

Differentiation of natural regulatory T cells (Tregs) in the thymus involves three signaling pathways. Among those, CD122 (IL2Rbeta) signaling has been shown to play a crucial role via STAT5-mediated regulation of foxp3 promoter. Most of these studies used transgenic or deficient mice. The precise role of CD122 signaling in nTreg differentiation in the thymus of unmanipulated mice is however poorly described. We first used multi parameter flow cytometry to detect phosphorylation events in the thymus without further stimulation *ex vivo*. Increased STAT5 phosphorylation (pSTAT5) was detected specifically in thymic CD4<sup>+</sup>CD25<sup>+</sup>foxp3<sup>+</sup> cells (nTreg) compared to other subsets, showing that pSTAT5 is a specific marker of nTreg. Short *in vitro* stimulation of total thymocytes with IL-2 and IL-15 but not IL-7 reproduced preferential STAT5 activation in nTreg, suggesting that IL-2 and IL-15 together could play a role in foxp3 regulation via CD122 signaling. Indeed, inhibition of CD122 signaling induced a decrease in the proportions of thymic and peripheral Treg cells *in vitro* as soon as one hour after treatment. Co-culture experiment of CD25<sup>+</sup> with congenic CD25<sup>+</sup> cells upon inhibition of CD122 signaling showed that the loss of foxp3<sup>+</sup> cells was not due to their preferential death. The loss of foxp3 in CD4<sup>+</sup>CD25<sup>+</sup>foxp3<sup>+</sup> cells *in vitro* correlated with cessation of foxp3 mRNA transcription, diminished expression of GITR and CD152 and with a loss of their suppressive function. The loss of foxp3 expression was also observed *in vivo* after adoptive transfer of congenic Treg in normal mice and treatment with anti-CD122 monoclonal antibody. Taken together, these results demonstrate that foxp3 mRNA and protein expression is very unstable and needs to be constantly maintained in Treg at the steady-state by a mechanism implying CD122 and STAT5 phosphorylation. Our results unveil a previously under appreciated plasticity in the natural Treg lineage commitment.

**PB06/45 FOXP3+ CD25+ REGULATORY CD4 T CELLS EXPRESS HIGH LEVELS OF CD127 UPON ACTIVATION**F. Simonetta<sup>1</sup>, A. Chiali<sup>1</sup>, C. Bourgeois<sup>1</sup><sup>1</sup>INSERM U802, Le Kremlin-Bicêtre, France

**Objectives:** CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> regulatory T cells (Tregs) are critical players in maintaining peripheral immune tolerance. Low expression of CD127 (IL-7R) on these cells has been described and this criterion is at present widely employed to define and isolate viable Tregs cells. However Tregs are a heterogeneous population and we hypothesized CD127 expression may differ depending on the subsets considered.

**Methods:** Lymph-node and spleen cells were isolated from C57BL/6 mice. Using Foxp3 and CD25 staining, Tregs were analyzed based on markers previously used to distinguish Tregs subsets such as CD62L, CD69, ICOS and CD103. Reactivity to IL-7 was also assessed *in vitro* and *in vivo* (using lymphopenic models in which IL-7 availability is increased). Activation of sorted total or “non activated” (i.e. ICOS- CD103-) Tregs was also performed *in vitro* and *in vivo* using colitis and dermatitis models.

**Results:** Phenotypic characterization of CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> T cells revealed that the Tregs subpopulation expressing CD103 and ICOS presented high levels of surface CD127. We demonstrated that these two molecules were up-regulated on total or “non activated” Tregs after *in vitro* and *in vivo* activation, suggesting CD127 expression on Tregs during activation. Indeed, during *in vivo* activation after adoptive transfer into empty hosts, Tregs strongly up-regulated CD127 expression, while conventional CD4 T cells reduced its expression. To confirm these results in a non-lymphopenic environment, we used a DNFB-induced contact dermatitis model. We showed that CD127 was up-regulated on ICOS<sup>+</sup> activated Tregs while it was down-regulated on conventional ICOS<sup>+</sup> CD4 T cells. Moreover, activated Tregs exhibited a different reactivity to IL-7 both *in vitro* and *in vivo*: in the absence of IL-7, they exhibited massive up-regulation of surface CD127 expression, whereas they maintained stable expression upon IL-7 exposure.

**Conclusion:** Our results call for caution in the use of CD127 to identify and isolate Tregs as this approach would lead to the exclusion of the activated Tregs compartment. Moreover, the differential regulation of CD127 expression on conventional and regulatory CD4 T cells suggests a potential role for IL-7 in Tregs biology and function.

**PB06/46 THE NEUROPEPTIDE NPT1 IS SPECIFICALLY EXPRESSED IN HUMAN CD25+ REGULATORY T CELLS**I. Prots<sup>1</sup>, A. Skapenko<sup>1</sup>, P.E. Lipsky<sup>2</sup>, H. Schulze-Koops<sup>1</sup><sup>1</sup>University of Munich, Med. Poliklinik, Rheumatology Unit, Munich, Germany, <sup>2</sup>NIH, National Institute of Arthritis and Musculoskeletal and Skin Diseases, Bethesda, United States

**Objectives:** The mechanisms that regulate the generation of peripheral T regulatory cells (Tregs) are largely unknown. The aim of this study was to investigate molecular mechanisms driving differentiation of Tregs in the periphery.

**Methods:** Using microarray DNA technology, gene expression profiling of developing CD25<sup>+</sup> Tregs at different stages in a well characterized, recently described *in vitro* Treg differentiation system that allows the generation of peripheral Tregs under defined and standardized conditions was performed. Treg-specific genes were identified using the GeneSpringGX 10.0 software. mRNA expression of the genes of interest was confirmed by real-time PCR and their protein expression was analyzed by western blot and ELISA in Tregs generated in independent experiments.

**Results:** The neuropeptide npt1 was expressed at high levels in Tregs but not in effector T cells during late development. npt1 mRNA was more than 500-fold upregulated in Tregs at late developmental stage (e.g. mature Tregs) compared to CD25<sup>+</sup> T cells (p< 0.05). npt1 mRNA was also strongly expressed in resting naturally occurring Tregs compared to naive CD25<sup>+</sup> T cells. Moreover, the npt1 protein was expressed by *in vitro* generated Tregs and *ex vivo* activated naturally occurring Tregs and npt1 protein expression in Tregs was significantly higher compared to effector T cells.

**Conclusion:** npt1 might represent a new Treg-characteristic protein with a possible functional importance in late development and/or in functions of Tregs, providing an intriguing mechanism of neuroimmune regulation, and a potential target for novel immunomodulatory treatment strategies.

**PB06/47 IL-10-PRODUCING CD4<sup>+</sup>CD25<sup>+</sup> REGULATORY T CELLS ARE CRUCIAL FOR THE GENERATION OF CD8<sup>+</sup> SUPPRESSOR T CELLS DURING THE INDUCTION OF TOLERANCE TO CONTACT ALLERGENS**U.M. Frankenberg<sup>1</sup>, N. Lorenz<sup>1</sup>, S. Grabbe<sup>1</sup>, K. Steinbrink<sup>1</sup><sup>1</sup>Universitätsmedizin Mainz, Hautklinik, Mainz, Germany

The regulation and prevention of allergy is controlled by mechanisms of specific immune suppression and induction of tolerance. Using the murine low zone tolerance (LZT) model, where contact hypersensitivity (CHS) is prevented by repeated topical low dose applications of contact allergens, we showed that suppressor CD8<sup>+</sup> T cells of LZT inhibit the development of contact hypersensitivity (CHS). However, the precise mechanisms of LZT are not yet understood. In this study, we analyzed the role and function of naturally occurring CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> regulatory T cells (nTregs) in LZT. A significantly increased number of nTregs during the induction of LZT was observed. Depletion of CD4<sup>+</sup>CD25<sup>+</sup> Tregs during tolerization (by anti-CD25-Ab or cyclophosphamide) induced an elevated CHS response (significant ear swelling, hapten-specific T cell proliferation and Tc1 cytokine pattern). Adoptive transfer experiments of CD8<sup>+</sup> T cells purified from Treg-depleted and tolerized mice (by anti-CD25-Ab) into naïve mice revealed an abrogated development of CD8<sup>+</sup> suppressor T cells in the absence of CD4<sup>+</sup>CD25<sup>+</sup> T cells. Also, adoptive transfer of CD4<sup>+</sup>CD25<sup>+</sup> Tregs obtained from tolerized animals into naïve mice demonstrated that CD4<sup>+</sup>CD25<sup>+</sup> T cells exhibited regulatory functions for the transfer of LZT to allergens. In addition, ELISPOT analysis showed that CD4<sup>+</sup>CD25<sup>+</sup> nTregs produce IL-10 in significant amounts during induction of LZT. IL-10<sup>+</sup> CD4<sup>+</sup>CD25<sup>+</sup> nTregs derived from WT mice, but not IL-10<sup>+</sup> nTregs allowed for the induction of LZT in adoptively transferred T cell-deficient mice, indicating that IL-10-producing Tregs are required during the induction phase of LZT. Our data demonstrate that CD4<sup>+</sup>CD25<sup>+</sup> Tregs contribute to the control of hapten-specific CD8<sup>+</sup> T cell-mediated immune responses.

**PB06/48 POTENTIAL ROLE OF AIRE IN SELECTION OF CD8<sup>+</sup>CD28<sup>+</sup> REGULATORY T LYMPHOCYTES**C. Pomié<sup>1</sup>, P. Romagnoli<sup>1</sup>, H. Scott<sup>2</sup>, J.P.M. van Meerwijk<sup>1</sup><sup>1</sup>INSERM U563, Toulouse, France, <sup>2</sup>Institute of Medical and Veterinary Science, Adelaide, Australia

Immunological unresponsiveness to self and innocuous non-self antigens is in large part assured by regulatory T lymphocytes. While CD4<sup>+</sup> T lymphocytes have recently attracted most attention, several CD8<sup>+</sup> regulatory T cell populations are also thought to play an important role in immunoregulation. One of these CD8<sup>+</sup> populations is characterized by low-level expression of the co-stimulatory molecule CD28. We have previously shown that CD8<sup>+</sup>CD28<sup>+</sup> regulatory T lymphocytes could be isolated from unmanipulated wildtype mice and inhibited proliferation and interferon-gamma production by CD4<sup>+</sup> responder T-cells in allogeneic mixed lymphocyte cultures. CD8<sup>+</sup>CD28<sup>+</sup> regulatory T-cells freshly isolated from spleen efficiently prevented inflammatory bowel disease induced by transfer of CD4<sup>+</sup>CD45RBhigh T-cells into immunodeficient hosts. IL-10 and TGF-beta play a crucial and non-redundant role in prevention of experimentally induced colitis. In order to study the role of AIRE during differentiation of these cells, we have isolated CD8<sup>+</sup>CD28<sup>+</sup> T-cells from spleen of mice deficient for this transcription factor. AIRE<sup>-/-</sup> CD8<sup>+</sup>CD28<sup>+</sup> T-cells showed a normal activity in *in vitro* suppression assays: they inhibited proliferation and interferon-gamma production by CD4<sup>+</sup> responder T-cells in allogeneic mixed lymphocyte cultures as efficiently as wt CD8<sup>+</sup>CD28<sup>+</sup> T-cells. However AIRE<sup>-/-</sup> CD8<sup>+</sup>CD28<sup>+</sup> T-cells were unable to prevent inflammatory bowel disease induced by transfer of CD4<sup>+</sup>CD45RBhigh T-cells into immunodeficient hosts. AIRE may therefore be involved in shaping the CD8<sup>+</sup>CD28<sup>+</sup> regulatory T-cell repertoire.



**PB06/49 AN IMMEDIATE PRECURSOR TO FOXP3<sup>+</sup> TREG OUTSIDE THE THYMUS**S. Schallenberg<sup>1</sup>, T. Koenig<sup>1</sup>, K. Kretschmer<sup>1</sup><sup>1</sup>CRTD/DFG-Centre for Regenerative Therapies, Immunotolerance in Regeneration, Dresden, Germany

Accumulating evidence indicate that, in addition to thymic development, Foxp3-expressing CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells (Treg) can also be generated in peripheral lymphoid organs. However, extrathymic *de novo* generation of Treg has been unequivocally documented only for a limited number of T cell receptor (TCR) specificities. In addition, it is unclear to what extent this pathway of Treg generation described in TCR transgenic model systems contributes to the overall peripheral Treg pool in normal mice. Here, we ask whether DEC-205-mediated extrathymic Treg *de novo* generation by steady state dendritic cells can be achieved *in vivo* with a broad spectrum of transgenic TCR-expressing CD4<sup>+</sup> T cells recognizing foreign antigens as well as tissue-specific self-antigens. Tracking initially naïve antigen-specific T cells during the conversion process allowed us to delineate two mechanistically different developmental pathways of extrathymic Foxp3<sup>+</sup>CD25<sup>+</sup> Treg generation. These experiments employing adoptive transfers of TCR transgenic T cells helped identifying a phosphatidylinositol 3 kinase-dependent, and mTOR- and IL-6 receptor signaling-independent immediate precursor population to Foxp3<sup>+</sup> Treg, which is present in peripheral lymphoid organs of normal mice in the absence of deliberate antigen stimulation. Furthermore, we provide evidence that extrathymic conversion significantly contributes to the overall peripheral Treg pool in the steady state of nonmanipulated mice.

**PB06/50 ID3 REGULATES THE DEVELOPMENT OF INDUCED REGULATORY T CELLS BY BLOCKING IFN- $\gamma$  EXPRESSION**K. Beck<sup>1</sup>, C. Murre<sup>1</sup><sup>1</sup>University of California San Diego, Biology, La Jolla, United States

Foxp3<sup>+</sup> induced regulatory T (iT<sub>reg</sub>) cells act synergistically with thymus derived natural T<sub>reg</sub> cells in the maintenance of immunological tolerance. Foxp3<sup>+</sup> iT<sub>reg</sub> cells develop in the periphery from naïve CD4<sup>+</sup> T cells upon T cell receptor (TCR) stimulation in the presence of TGF- $\beta$ . However, the exact mechanisms as well as the transcription factors involved in the induction of peripheral T<sub>reg</sub> cells remain largely unknown. Here we show that the transcriptional inhibitor Id3 regulates the induction of iT<sub>reg</sub> cells from naïve CD4<sup>+</sup> T cells by regulating cytokine expression profiles. Examining Id3-GFP reporter mice we demonstrate that Id3 is highly expressed in peripheral T<sub>reg</sub> cells *in vivo*. *In vitro* development of iT<sub>reg</sub> cells is greatly diminished in Id3<sup>-/-</sup>CD4<sup>+</sup> T cells. In an *in vitro* mixed culture of wt and Id3<sup>-/-</sup> naïve CD4<sup>+</sup> T cells wt cells show diminished iT<sub>reg</sub> development after stimulation by TGF- $\beta$ , suggesting that Id3<sup>-/-</sup> T cells secrete an inhibitory factor blocking iT<sub>reg</sub> formation. Microarray profiling revealed that Id3<sup>-/-</sup>CD4<sup>+</sup> T cells express aberrantly high IFN- $\gamma$  upon TCR stimulation in the presence of TGF- $\beta$ . IFN- $\gamma$  as well as other cytokines have been shown to inhibit the development of iT<sub>reg</sub> cells. Consequently, inhibition of IFN- $\gamma$  by using a neutralizing antibody restores the ability of Id3<sup>-/-</sup>CD4<sup>+</sup> T cells to differentiate to iT<sub>reg</sub> cells. Id3 proteins act by inhibiting the E2A transcription factor and consequently *in vitro* stimulation of E2A<sup>-/-</sup>CD4<sup>+</sup> T cells leads to increased induction of Foxp3<sup>+</sup> cells, presumably through the lower expression of inhibitory cytokines. In accordance, over-expression of the E2A splice variant E47 in activated CD4<sup>+</sup> T cells *in vitro* partially inhibits the induction of iT<sub>reg</sub> cells upon TGF- $\beta$  treatment. These results provide the first evidence that Id3 represses IFN- $\gamma$  expression by CD4<sup>+</sup> T cells during TGF- $\beta$  driven differentiation into Foxp3<sup>+</sup> iT<sub>reg</sub> cells. Taken together, we propose that Id3 acts during T<sub>reg</sub> conversion by inhibiting E2A transcription factors, which activate the expression of cytokines expressed in alternative CD4<sup>+</sup> T cell lineages.

**PB06/51 ADDING TGF $\beta$  AND RETINOIC ACID TO AN ANTI-CD4 MAB BASED PROTOCOL OF EX VIVO GENERATION OF ALLOSPECIFIC TREGS ENHANCES THEIR STABILITY AND IN VIVO CAPACITY**U. Schliesser<sup>1</sup>, C. Appel<sup>1</sup>, S. Vogel<sup>1</sup>, K. Vogt<sup>1</sup>, I. Schmitt-Knosalla<sup>1</sup>, S. Schlickeiser<sup>1</sup>, H.-D. Volk<sup>1</sup>, B. Sawitzki<sup>1</sup>, Institute of Medical Immunology, Charité – Universitätsmedizin, Berlin, Germany<sup>1</sup>Charité – Campus Mitte, Institute of Medical Immunology, Charité – Universitätsmedizin, Berlin, Germany, Berlin, Germany

**Introduction:** Transfer of alloantigen specific Tregs into transplant recipients represents an attractive treatment option to improve long term graft acceptance. However, phenotype and functional stability upon *in vivo* transfer is of utmost importance. We recently described a protocol for the generation of allospecific Tregs using a non-depleting anti-CD4 antibody (Oliveira et al. *EJI* 2008). Here we investigated whether adding TGF $\beta$ /RA or Rapamycin to our established protocol can further enhance their stability and *in vivo* function.

**Methods:** CD4<sup>+</sup> T cells of C57BL/6 mice were cultured with allogeneic BALB/c B cells in the presence of 0.5  $\mu$ g/ml anti-CD4 mAb (YTS.177) alone, anti-CD4 mAb + 1 ng/ml TGF $\beta$ /0.5 mM RA or anti-CD4 mAb + 10 nM Rapamycin. 7 days later CD4<sup>+</sup>CD25<sup>+</sup> T cells were isolated by MACS, their Foxp3, t-bet, ROR $\gamma$ t, and CD62L expression determined (qPCR, FACS) and their cytokine production analyzed. Furthermore, harvested cells were re-stimulated with allogeneic B cells and the relative amount of Foxp3 producing cells determined. Finally, 5x10E5 generated Tregs were transferred together with 1x10E6 CFSE labeled CD4<sup>+</sup>Thy1.1+ effector T cells into Thy1.2+ congenic mice challenged with 2x10E6 bone marrow derived DCs from BALB/c. Percentage of proliferated CFSE-Thy1.1+ in spleen and MLNs were determined 5 days after cell application.

**Results:** Addition of TGF $\beta$ /RA or Rapamycin led to a relative and absolute increase of CD25<sup>+</sup>Foxp3<sup>+</sup> expressing T cells by 30% during the Treg generation culture. In contrast, production of inflammatory cytokines (IFN- $\gamma$ , TNF- $\alpha$ , IL-17) was completely abolished in cultures of aCD4mAb+TGF $\beta$ /RA treated Tregs. Restimulation of CD25<sup>+</sup> T cells harvested from the Tregs of anti-CD4 mAb only and Rapamycin cultures resulted in a reduction of Foxp3 frequency by 20% in comparison to TGF $\beta$ /RA treated cultures (1-5% decrease). Consequently, Tregs harvested from anti-CD4mAb+TGF $\beta$ /RA treated cultures could more effectively inhibit the *in vivo* proliferation of effector T cells (80%) in comparison to Tregs harvested from anti-CD4mAb only or anti-CD4mAb+Rapamycin cultures (40, 50% inhibition).

**Conclusions:** Thus addition of TGF $\beta$  in combination with retinoic acid is superior over Rapamycin in stabilizing the phenotype and functional capacity of allospecific Tregs.

**PB06/52 TREG HOMEOSTASIS IS DEPENDENT ON COMMENSAL MICROFLORA, BUT IS NOT NEGATIVELY AFFECTED BY DEFICIENCY OF BACTERIAL PAMP SENSING TOLL-LIKE RECEPTORS**S. Cording<sup>1</sup>, C. Siewert<sup>2</sup>, M.M. Heimesaat<sup>3</sup>, S. Bereswill<sup>3</sup>, O. Liesenfeld<sup>3</sup>, C. Loddenkemper<sup>4</sup>, M. Asquith<sup>5</sup>, S. Uematsu<sup>6</sup>, S. Akira<sup>6</sup>, F. Powrie<sup>5</sup>, A. Hamann<sup>2</sup>, J. Hühn<sup>1</sup>

<sup>1</sup>Helmholtz Centre for Infection Research, Department of Experimental Immunology, Braunschweig, Germany, <sup>2</sup>Charité, Campus Mitte, Department of Experimental Rheumatology, Berlin, Germany, <sup>3</sup>Charité, Campus Benjamin Franklin, Department of Microbiology and Hygienics, Berlin, Germany, <sup>4</sup>Charité, Campus Benjamin Franklin, Department of Pathology, Berlin, Germany, <sup>5</sup>University of Oxford, Sir William Dunn School of Pathology, Department of Mucosal Immunology, Oxford, United Kingdom, <sup>6</sup>Osaka University, Research Institute for Microbial Diseases, Department of Host Defense, Osaka, Japan

Colitis models have provided compelling evidence for a protective role of Foxp3<sup>+</sup>CD25<sup>+</sup>CD4<sup>+</sup> regulatory T cells (Tregs) in intestinal homeostasis. Foxp3<sup>+</sup> Tregs have been described as thymus-derived cells, however, more recent studies demonstrate a significant peripheral turnover. We questioned whether the gut-associated lymphoid tissue (GALT) is necessary for Treg homeostasis and whether this process is driven by stimuli originating from the commensal microflora. Here we investigated the proliferation of Foxp3<sup>+</sup> Tregs in GALT in the presence or absence of commensal microflora. Thereby, mice were treated with a cocktail of antibiotics to reduce the commensal microflora. This treatment led to a significant reduction of CD4<sup>+</sup>Foxp3<sup>+</sup> Treg numbers not only in the GALT, but also in spleens and peripheral lymph nodes. Analysis of the *in vivo* proliferation of Foxp3<sup>+</sup> Tregs by BrdU-incorporation revealed a significantly reduced frequency of cycling BrdU<sup>+</sup>Foxp3<sup>+</sup> Tregs solely in the GALT, but not in spleens and peripheral lymph nodes. These findings indicate that the commensal microflora contributes to the local proliferation of Foxp3<sup>+</sup> Tregs, which influences Treg numbers systemically. Analysis of frequencies, absolute cell numbers and homeostatic proliferation of Foxp3<sup>+</sup> Tregs in TLR2<sup>-/-</sup>, TLR4<sup>-/-</sup>, TLR2/4<sup>-/-</sup>, TLR5<sup>-/-</sup> and TLR9<sup>-/-</sup> mice showed that sensing of bacterial stimuli via these TLRs is not the major mechanism controlling intestinal homeostasis of Foxp3<sup>+</sup> Tregs.

Together, our data show that microbial stimuli critically influence the homeostasis of Foxp3<sup>+</sup> Tregs and suggest that these cells, which protect against intestinal inflammation, might not exclusively consist of self-reactive T cells.

**PB06/53 INTERLEUKIN-10 INFLUENCES THE ABUNDANCE OF CD4<sup>+</sup>FOXP3<sup>+</sup> T REGULATORY CELLS DURING PREGNANCY**L.R. Guerin<sup>1</sup>, J.R. Prins<sup>2</sup>, J.D. Hayball<sup>1</sup>, S.A. Robertson<sup>1</sup>

<sup>1</sup>University of Adelaide, Obstetrics and Gynaecology, Adelaide, Australia, <sup>2</sup>University Medical Center Groningen, Obstetrics and Gynaecology, Groningen, Netherlands, <sup>3</sup>Hason Institute and Sansom Institute, Adelaide, Australia

**Objective:** The alloantigenic nature of the mammalian reproduction results in the requirement for an active state of maternal immune tolerance toward the fetus. It has been shown that T regulatory (Treg) cells are critical in eliciting this state of immune tolerance. However that factors that determine T regulatory cell abundance and function during pregnancy still remain poorly understood. The aim of this study was to analyse the role that interleukin-10 has in controlling Treg cells in the uterus and lymph nodes throughout murine pregnancy.

**Methods:** Female IL-10 deficient (IL-10<sup>-/-</sup>) or wild type (WT) C57BL/6 were mated with either Balb/c or C57BL/6 males. Treg cells were analysed using anti-Foxp3 antibodies and FACS analysis in the iliac and inguinal lymph nodes or in the uterus via immunohistochemistry throughout gestation.

**Results:** In non-pregnant IL-10<sup>-/-</sup> females there was approximately a 30% increase in the percentage of CD4<sup>+</sup>foxp3<sup>+</sup> cells and a 2 fold increase in absolute cell numbers in both the iliac and inguinal lymph nodes compared to WT animals. Throughout pregnancy in IL-10<sup>-/-</sup> mice there were further increases in the abundance of CD4<sup>+</sup>Foxp3<sup>+</sup> cells with a peak at gd 10 with approximately a 50% increase in the percentage of CD4<sup>+</sup>Foxp3<sup>+</sup> cells and nearly a 10 fold increase in absolute Treg cell numbers. The increase in the percentage of CD4<sup>+</sup>Foxp3<sup>+</sup> cells was less evident in syngeneic pregnancies.

**Conclusion:** These results show that maternal IL-10 deficiency results in an increased abundance of Treg cells throughout gestation. As a result we conclude that IL-10<sup>-/-</sup> may influence the proliferation or lineage commitment of Treg cells. Additionally the decreased percentage of Treg cells in IL-10<sup>-/-</sup> mothers carrying syngeneic fetuses in comparison to those gestating allogeneic fetuses evokes a role of alloantigen in driving this response.

**PB06/54 A XENOGENEIC MOUSE MODEL TO EXPLORE THE MECHANISMS OF HUMAN T EFFECTOR CELL SUPPRESSION BY NATURALLY OCCURRING HUMAN REGULATORY T CELLS *IN VIVO***B. Becker<sup>1</sup>, J. Kubach<sup>1</sup>, C. Taube<sup>2</sup>, T. Bopp<sup>3</sup>, E. Schmitt<sup>3</sup>, C. Becker<sup>4</sup>, K. Reifenberg<sup>5</sup>, H. Jonuleit<sup>1</sup><sup>1</sup>Johannes Gutenberg-University Mainz, Dermatology, Mainz, Germany, <sup>2</sup>Johannes Gutenberg-University Mainz, Department of Internal Medicine, Mainz, Germany, <sup>3</sup>Johannes Gutenberg-University Mainz, Institute of Immunology, Mainz, Germany, <sup>4</sup>Johannes Gutenberg-University Mainz, Institute of Molecular Medicine, Mainz, Germany, <sup>5</sup>Johannes Gutenberg-University Mainz, Central Animal Facility, Mainz, Germany

Functional studies on human CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells (Tregs) are restricted so far to *in vitro* investigations. To overcome this limitation we established a simple and robust humanized mouse model to study the complex functional properties of human Tregs *in vivo*. Transfer of human peripheral blood cells (PBMC) into newborn immunodeficient NOD-Scid or Rag2gc<sup>-/-</sup> mice resulted in a lethal graft-versus-host disease (GvHD) characterized by decelerated growth, reduced mobility, and mortality of treated animals within 2 months. The development of GvHD was accompanied by massive cellular infiltration of human immune cells into multiple organs resulting in chronic hepatitis, pneumonia, colitis and inflamed skin. However, a single transfer of additional human Tregs inhibited GvHD induction in a dose-dependent manner. Mice that received increased numbers of human Tregs showed strongly reduced signs of cellular infiltration and inflammation and lacked disease symptoms and mortality up to 100 days after engraftment. Protection from GvHD by Tregs was associated with decreased early expansion and cytokine production of human CD4<sup>+</sup> T cells in lymphoid tissues, indicating preferential effects on CD4<sup>+</sup> T helper cell function. These data demonstrate the potent capacity of human Tregs to suppress GvHD by autologous human T cells and provide a simple and robust *in vivo* model to analyze the functional properties of human Tregs and novel biologics that modulate Treg functions.

**PB06/55 LAG-3 EXPRESSION IDENTIFIES A SUBPOPULATION OF CD4+CD25+ REGULATORY T CELL ENDOWED WITH SUPPRESSIVE FUNCTIONS AND SELECTIVELY EXPANDED IN MELANOMA PATIENTS**C. Camisaschi<sup>1</sup>, C. Casati<sup>1</sup>, F. Rini<sup>1</sup>, M. Perego<sup>1</sup>, A. De Filippo<sup>1</sup>, F. Triebel<sup>2</sup>, G. Parmiani<sup>3</sup>, L. Rivoltini<sup>1</sup>, C. Castelli<sup>1</sup><sup>1</sup>Fondazione IRCCS Istituto Nazionale dei Tumori, Unit of Immunotherapy of Human Tumors, Milano, Italy, <sup>2</sup>Immutep S.A., Faculté de Pharmacie, Châtenay-Malabry, France, <sup>3</sup>San Raffaele Scientific Institute, Unit of Immuno-Biotherapy of Melanoma and Solid Tumors, Milano, Italy

Studies in mice indicated that LAG-3 has a complex role in T cell homeostasis and marks CD4+CD25+ T regulatory cells. In order to explore the LAG-3 expression inside human CD4+ T cells, phenotypic studies have been performed on healthy donors' PBMC. We found that LAG-3 is expressed by CD4+CD25+ T cells and *ex vivo* analysis showed that LAG-3 is often associated to the expression of other activation markers, such as CD45RO, HLA-DR, CD71, CD122, CD62L, CD39 and of Foxp3 and that it is preferentially confined to CD4+CD25<sup>high</sup> T cells. Comparison of healthy donors' PBMC with those of stage III-IV metastatic melanoma patients indicated that CD4+CD25+LAG-3+ T cells are expanded in patients' PBMC. The role of tumor on affecting the percentage of CD4+CD25+LAG-3+ T cells was further documented in melanoma-invaded lymph nodes that exhibit an increased frequency of CD4+CD25+LAG-3+ T cells as compared to tumor-free counterpart. Multiparametric FACS analysis for CCR7 and CD45RO expression evidenced that, at tumor site or in patients' PBMC, CD4+CD25+LAG-3+ T cells display an effector/memory phenotype. Interestingly, inside the melanoma regulatory T cells subset, LAG-3 expression defines a functionally active subpopulation. In fact, without the need of further TCR triggering, CD4+CD25+LAG-3+ cells produce the suppressive cytokines IL-10 and TGF-β1 but not IL-2, indicating that they are not recently activated conventional effector cells. Contrarily to the LAG-3- subset, CD4+CD25+LAG-3+ T cells are also positive for Ki67 expression. Increased percentage of CD4+CD25+LAG-3+ cells, actively releasing suppressive cytokines and displaying effector/memory phenotype, can be achieved in PBMC of healthy donors upon *in vitro* activation. *In vitro* suppression assay using sorted CD4+CD25+LAG-3+ T showed that this cell subset has potent suppressor activity mediated by mechanism involving cell-cell contact.

All together our data show that LAG-3 defines CD4+CD25+ regulatory T cells with an activated effector/memory phenotype, endowed with enhanced suppressor activity, preferentially expanded *in vivo* in tumor bearing patients.

**PB06/56 IMMUNOREGULATORY T CELLS IN THE TARGET TISSUE OF ORGAN-SPECIFIC AUTOIMMUNITY**L. Usero<sup>1</sup>, E. Codina<sup>1</sup>, M. Boshuizen<sup>1</sup>, R. Planas<sup>2</sup>, M. Vives-Pi<sup>2</sup>, D. Jaraquemada<sup>1</sup>, M. Martí<sup>1</sup>, C. Roura-Mir<sup>1</sup><sup>1</sup>Univesitat Autònoma de Barcelona, Cell Biology, Physiology and Immunology, Bellaterra, Spain, <sup>2</sup>Research Institute Germans Trias i Pujol, Badalona, Spain

T cells with regulatory functions, including CD4+CD25+Foxp3+ regulatory T cells (nTregs) and invariant natural killer T cells (iNKT) are important in controlling pathogenic autoreactivity. Both populations are reduced in numbers or have compromised functions in several autoimmune disorders in humans including T1D. Recent studies have suggested that nTregs and iNKT cells can reciprocally influence each other, in addition to modulating several other immune-cell populations. We hypothesize that the cross-talk between these two populations of immunoregulatory cells influences the outcome of the autoimmune process. Therefore we determined the coexistence of these two populations of regulatory T cells in the pancreas of a diabetic patient.

We have analyzed the presence of nTreg cells and iNKT cells by quantifying the expression of FoxP3 and TCRVa24Ja18 by Real-Time PCR. Data were normalized both with the expression of the CD3g chain and GAPDH. The studied samples are from pancreatic whole tissue (TD), purified pancreatic islets (ILL) and spleen from a diabetic patient at disease onset. As a control, we analyzed the pancreatic tissue (cILL, cTD) from non diabetic donors.

The data show that iNKT cells are present at the diabetic pancreas. Interestingly, they concentrate mainly outside the pancreatic islets as there is a 5 fold increase in iNKT cell numbers in the TD compared to the islet's cell fraction. Despite this, iNKT cells are more abundant in ILL of the diabetic pancreas compared to ILL of a normal tissue. Furthermore, Foxp3 expressing T cells are 48 times more abundant in the TD than in the ILL of the diabetic patient were Foxp3 was almost undetectable. Moreover, Foxp3 was not seen on the control tissues. These differences are not reflected on the periphery where frequency of Foxp3+ cells was similar in diabetic patients and healthy controls.

Therefore we can find both populations of immunoregulatory cells, nTreg and iNKT cells, in the diabetic pancreas at disease onset although they are not found commonly in the inflamed islets. This suggests that at disease onset both populations are expanded in the affected tissue although their presence is not sufficient to suppress the effector T cell action.

**PB06/57 BREAST MILK-MEDIATED TRANSFER OF AN ANTIGEN INDUCES TOLERANCE AND PROTECTION FROM ALLERGIC ASTHMA : ALLERGIC MOTHERS DO BETTER**E. Mosconi<sup>1</sup>, B. Seitz<sup>1</sup>, D. Dombrowicz<sup>2</sup>, A. Kanda<sup>2</sup>, S. Fleury<sup>2</sup>, V. Verhasselt<sup>1</sup>, N. Glaichenhaus<sup>1</sup><sup>1</sup>Université de Nice-Sophia Antipolis, Valbonne, France, <sup>2</sup>Université de Lille 2, Lille, France

Allergic asthma is a chronic disease characterized by airway obstruction in response to allergen exposure. It results from an inappropriate T helper (Th)-2 response to environmental airborne antigens. Although some controversy exists, many epidemiological studies have shown a protective effect of breastfeeding on asthma. However, breast milk factors that are responsible for this protective effect have not yet been clearly identified. We formulated the hypothesis that breastfeeding could afford protection against asthma through immune tolerance induction. Our data obtained in a mouse model confirmed that hypothesis and showed that for tolerance induction, the mother mice needed to be exposed to the allergen by aerosol or oral route during the lactation period. This resulted in the transfer of the allergen to breast milk. The presence of the allergen together with TGF-β in breast milk were necessary and sufficient to induce the development of regulatory T lymphocytes in the progeny and their protection from asthma development. (1). We next investigated whether the immune status of the mother has an effect on the development of tolerance in the breast-fed mouse. We found that mice breastfed by allergen exposed allergic mothers were more resistant to allergic airway inflammation as compared to those breastfed on non allergic mothers. This phenomenon is likely to be dependent on the presence of allergen-specific Immunoglobulins in the breastmilk of allergic mothers and on the transfer of immunocomplexes across the intestinal barrier of the newborn through the neonatal Fc receptor.

**PB06/58 ROS MEDIATED ACTIVATION OF PI3K/AKT PATHWAY IMPAIRS SUPPRESSIVE FUNCTION OF HUMAN CD4+CD25+ T REGULATORY CELLS**C. Scotta<sup>1,2</sup>, G. Fanelli<sup>1</sup>, C. Camperio<sup>1</sup>, M. Soligo<sup>1</sup>, E. Piccolella<sup>1</sup><sup>1</sup>Sapienza University of Rome, Department of Cellular and Developmental Biology, Rome, Italy, <sup>2</sup>MRC Centre for Transplantation, King's College London, Department of Nephrology and Transplantation, London, United Kingdom

During the immune response, at sites of inflammation, cells of innate immunity and inflammatory mediators contribute in creating the microenvironment which can direct the differentiation of effector T cell responses. Now it is clear that the cross-talk between neutrophils, macrophages and T cells favours the production of reactive oxygen species (ROS) that can be involved in T cell activation and immune system homeostasis. An essential role to maintain the right balance between immune responses is played by regulatory T cells (Treg). Alterations in effector functions of Treg have been emphasized to promote a chronic inflammatory state and the development of autoimmune responses. To gain a better understanding of the ROS influence on Treg, we examined the effect of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), the main physiological metabolite of reactive oxygen, on phenotypic and effector functions of these cells. Our results show that ROS-exposed Treg presents a reduced ability to suppress CD4<sup>+</sup>CD25<sup>+</sup> T cell proliferation and this deficiency was H<sub>2</sub>O<sub>2</sub> dose-dependent. Moreover, in order to determine which signal transduction pathway could be target of oxidative status, PI3K/AKT phosphorylation was investigated. In addition, we also evaluated the expression of FOXP3 in H<sub>2</sub>O<sub>2</sub>-treated Treg by FACS analysis and real time PCR. The results show an increase of both PI3K/AKT phosphorylation and FOXP3 expression, suggesting a correlation between the activation of AKT and FOXP3 synthesis. Treg are characterized by anergic state and, due to the effect of FOXP3 on IL-2 and CTLA4 promoters, by lack of IL-2 synthesis and increase of CTLA4 expression. Therefore to give account of the phenotypic and effector functions modifications of Treg, we investigated the ability of ROS to modify these key molecules relevant for their suppressive function. We observed that exposure to H<sub>2</sub>O<sub>2</sub> results in the activation of IL-2 secretion and in a dose-dependent decrease of CTLA4 expression. In conclusion, our data suggest that ROS-mediated activation of AKT pathways may act as a deregulator of the suppressive functions of Treg allowing us to speculate that the production of ROS during inflammatory process and stressful conditions can amplify the activation of conventional effector T cells by decreasing suppressor T cell function.

**PB06/59 SUPPRESSION MECHANISMS OF CYTOKINE TRANSCRIPTION IN HUMAN CD4<sup>+</sup>CD25<sup>+</sup> T CELLS UPON INTERACTION WITH REGULATORY T CELLS**N. Oberle<sup>1</sup>, A. Schmidt<sup>1</sup>, E.-M. Weiß<sup>1</sup>, E. Suri-Payer<sup>1</sup>, P.H. Krammer<sup>1</sup><sup>1</sup>German Cancer Research Center, Immunogenetics, Heidelberg, Germany

CD4<sup>+</sup>CD25<sup>high</sup>Foxp3<sup>+</sup> regulatory T cells (Treg) are critical mediators of peripheral self tolerance and immune homeostasis. Treg are able to suppress proliferation and cytokine production of conventional T cells (Tcon). The exact mechanism of suppression, however, is still unknown.

To gain a better understanding of Treg function, we investigated cytokine suppression in Tcon re-isolated from cocultures with pre-activated human Treg. Treg inhibit induction of Th1 cytokine mRNA as early as one hour after stimulation, and this immediate mRNA suppression was neither dependent on TGF- $\beta$  or IL-10, nor on IL-2 consumption. There was no induction of the transcriptional repressor FOXP3 or other anergy-related genes (GRAIL, TOB, FOXP1, ROG, ICER) in suppressed Tcon, while a gene not previously described in T cells – which we call Treg induced signalling suppressor (TRISS) – is highly induced. The role of the gene is currently under investigation.

In addition, we were able to rule out cell death, the involvement of IL-35 and the change of the reactive oxygen species (ROS) in suppressed Tcon. Previously, induction of cAMP in murine Tcon upon contact with Treg was shown to lead to Tcon suppression. Interestingly, we could only observe an involvement of cAMP in the inhibition of IL-2 but not of IFN- $\gamma$ . Therefore, cAMP induction in suppressed Tcon cannot be the only mechanism.

The identification of a fast inhibitory mechanism in Tcon induced by Treg constitutes an important step for future efforts to unravel the entire suppressive mechanism. Due to the crucial role of Treg in immune homeostasis, this knowledge could help to improve the treatment of autoimmunity or tumors in future.

**PB06/60 REGULATORY T CELL RATIOS ARE INCREASED IN PERIPHERAL BLOOD OF PROGRESSING MELANOMA PATIENTS AND CORRELATE WITH A GENERAL IMPAIRED T CELL RESPONSIVENESS**A. Correll<sup>1</sup>, A. Tüttenberg<sup>1</sup>, C. Becker<sup>1</sup>, H. Jonuleit<sup>1</sup><sup>1</sup>Universitätsmedizin Mainz, Hautklinik, Mainz, Germany

Naturally occurring CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells (Tregs) are key players in maintenance of peripheral tolerance. While Treg deficiency is associated with autoimmunity and type-I allergy, increased Treg numbers and activity are assumed to facilitate tumor development and progression. In this study we used a panel of different Treg associated markers including CD25, Foxp3, HLA-DR and CD127 to quantify Treg frequencies in peripheral blood of stage I-IV melanoma patients. We observed increased Treg frequencies during melanoma progression. These elevated Treg numbers correlated with a general reduction of T cell reactivity, also detectable to recall antigens such as tetanus toxoid. However, DC-based immunotherapy induced tumor antigen-specific immune responses and restored recall antigen-specific immunity. Additionally, our preliminary data indicate that the re-induced T cell reactivity correlates with decreased Treg numbers in these patients. These findings point out that tumor progression in melanoma patients consequences in a general immunosuppression associated with enhanced Treg ratios in the periphery. However, DC-based vaccination restored at least transiently the immunological balance between tolerance and immunity in cancer patients.

**PB06/61 HBSAG-SPECIFIC REGULATORY T CELLS IN PATIENTS WITH DIFFERENT HBV INFECTION STATUS**T. Bauer<sup>1</sup>, J. Wenzel<sup>1</sup>, S. Schimanski<sup>1</sup>, A. Knoell<sup>2</sup>, W. Jilg<sup>1</sup><sup>1</sup>University of Regensburg, Medical Microbiology, Regensburg, Germany, <sup>2</sup>University of Erlangen-Nuremberg, Erlangen, Germany

Our previous work demonstrated HBsAg-specific regulatory T cells (HBsAg-T<sub>reg</sub>) present after hepatitis B vaccination of liver transplant recipients with history of HBV infection.

Here, we analysed the frequency and phenotypic characteristics of HBsAg-T<sub>reg</sub> in patients with different HBV infection status, and investigated the effect of those T<sub>reg</sub> on cellular immune responses as well as the mechanisms underlying their regulatory function. In addition we assessed, whether HBsAg-T<sub>reg</sub> have an inhibitory effect on anti-HBs production by suppressing corresponding T helper cell functions.

A total of 45 subjects including patients with chronic (n=16) and resolved acute hepatitis (n=10), solely anti-HBc-positive carriers (n=9) and vaccinated individuals (n=10), were enrolled in the study. Immune responses were determined by *ex vivo* Elispot analysis of HBsAg-specific T cells and anti-HBs-producing B cells. HBsAg-T<sub>reg</sub> were detectable in 7/16 chronic carriers and 4/9 solely anti-HBc-positive carriers, but neither in patients with resolved HBV infection nor in vaccinated individuals. Circulating HBsAg-T<sub>reg</sub> have a CD4<sup>+</sup>/CD25<sup>+</sup> phenotype, produce exclusively IL-10 and adversely affect function of HBsAg-specific CD4<sup>+</sup> effector T cells *ex vivo*. Suppression, as determined in depletion and IL-10 neutralization experiments, is dose-dependent, requires direct cell-cell contact, and is independent of IL-10. T cell epitope mapping revealed the same epitope specificity for HBsAg-Treg and CD4<sup>+</sup> effector T cells, respectively. Depletion of CD25<sup>+</sup>/CD4<sup>+</sup> T<sub>reg</sub> enhances HBsAg-specific effector T cell responses, however it has no impact on the number of detectable anti-HBs-secreting B cells in all tested patient groups.

Our data suggest that HBsAg-specific T<sub>reg</sub> are exclusively induced in chronic HBV patients and contribute to an inadequate cellular immune response against HBsAg by dampening effector T cell responses. The inability of chronic carriers to develop anti-HBs did not seem to be solely determined by the presence of HBsAg-T<sub>reg</sub>.

**PB06/62 THE EFFECT OF BOOSTING NATURAL REGULATORY T CELLS IN RESPIRATORY VIRAL INFECTION**J. Loebermann<sup>1</sup>, C. Johansson<sup>1</sup>, K. Webster<sup>2</sup>, J. Sprent<sup>2</sup>, P. Openshaw<sup>1</sup><sup>1</sup>Imperial College London, Respiratory Medicine, London, United Kingdom, <sup>2</sup>Garvan Institute of Medical Research, Darlinghurst, Australia

Regulatory T cells (Tregs) play a key role in chronic infections and inflammatory disorders, but their role in acute viral infections is not clear. Human respiratory syncytial virus (RSV) is the main cause of serious lower respiratory tract infection in infants. Over-exuberant and inappropriate immune responses play a major role in RSV disease. Although, much is known about the role of effector T cells in RSV infections, other T cell populations (e.g. Tregs) have been little studied. While Treg have established functions in attenuation of responses, it has recently been shown that they can have antiviral effects by enhancing cell recruitment into infected tissues. We boosted nTregs *in vivo* by intraperitoneal injection of pre-formed complexes of IL-2 with anti-IL-2 antibody. This lead to a dramatic increase in the number of nTregs in the spleen, the lung and in the bronchoalveolar lavage fluid. Boosting of nTregs during RSV infection lead to an increase in the percentage of nTregs in draining lymph nodes (LNs), lung and BAL at day 4 and 8 of infection. It also caused an increase of total cells in the lung on day 4, mainly due to elevated numbers of NK cells and nTregs. Interestingly, it markedly decreased total cell numbers in the draining LNs at day 4 and 8 and the total number of CD8<sup>+</sup> T cells in the lung at day 8. Although IL-2 complexes greatly boosted Treg, the lung's viral load was paradoxically reduced on day 4, and treated mice recovered earlier from RSV-induced weight loss. These experiments show that that boosting nTregs has potent antiviral effects, apparently due to enhanced NK responses and redistribution of antiviral cells to the site of infection. The mechanisms that regulate innate and adaptive immune responses to acute viral respiratory infections are being explored.

**PB06/63 REGULATORY ROLE OF CD4<sup>+</sup>FOXP3<sup>+</sup>CD25<sup>+</sup> T CELLS IN THE LUNGS OF MICE INFECTED WITH BORDETELLA PERTUSSIS**M. Coleman<sup>1,2</sup>, B. Moran<sup>1</sup>, J. Keane<sup>2</sup>, K.H.G. Mills<sup>1</sup>, P.J. Dunne<sup>1</sup><sup>1</sup>Trinity College Dublin, Immune Regulation Research Group, School of Biochemistry and Immunology, Dublin, Ireland, <sup>2</sup>Trinity College Dublin, Institute of Molecular Medicine, St James' Hospital, Dublin, Ireland

We have identified a novel subset of CD4<sup>+</sup>FoxP3<sup>+</sup> regulatory T (Treg) cells in the lungs of naive mice which lack CD25, distinguishing them from CD4<sup>+</sup>FoxP3<sup>+</sup>CD25<sup>+</sup> natural Treg cells (nTreg). CD4<sup>+</sup>FoxP3<sup>+</sup>CD25<sup>+</sup> Treg predominate over CD4<sup>+</sup>FoxP3<sup>+</sup>CD25<sup>+</sup> nTreg cells in the lung, liver and colon. Lung CD4<sup>+</sup>FoxP3<sup>+</sup>CD25<sup>+</sup> Treg cells express CD44 and CD69 but not CD45RB, CD28 or CD154, suggesting an activated, memory phenotype. In this study we investigated the functional role of CD4<sup>+</sup>FoxP3<sup>+</sup>CD25<sup>+</sup> Treg cells in mice.

*In vitro* function was assessed by pre-activating freshly isolated CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>+</sup> Treg cells before co-culture with CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>+</sup> effector T cells in a suppression assay. *In vivo* function was investigated using a murine model of lung infection with *Bordetella pertussis*. Bacterial clearance, cellular infiltration into the lung and intracellular cytokine production by CD4<sup>+</sup>FoxP3<sup>+</sup>CD25<sup>+</sup> Treg cells were determined at fixed time points post challenge. CD25<sup>+</sup> cells were temporarily depleted from C57/BL6 wild-type and IL-10<sup>-/-</sup> mice with a single injection of anti-CD25 depleting antibody 24 hours prior to challenge with *B. pertussis*.

Pre-activated lung CD4<sup>+</sup>FoxP3<sup>+</sup>CD25<sup>+</sup> Treg cells suppressed effector T cell responses *in vitro*, but enhanced IFN- $\gamma$  production. This suppression was IL-10-mediated and did not require cell contact. We found a high frequency of antigen-specific IL-10-secreting CD4<sup>+</sup>FoxP3<sup>+</sup>CD25<sup>+</sup> Treg cells in the lung, which peaked 7 days post-challenge with *B. pertussis*. Depletion of CD25<sup>+</sup> cells prior to *B. pertussis* infection did not significantly impair the immunoregulatory response or alter the course of infection. However, depletion of CD25<sup>+</sup> cells in IL-10<sup>-/-</sup> mice significantly enhanced bacterial clearance.

We propose that two immunomodulatory arms are involved in regulating immunity to *B. pertussis* in the lung, namely CD25<sup>+</sup> nTreg and IL-10-secreting Treg cells, which include CD4<sup>+</sup>FoxP3<sup>+</sup>CD25<sup>+</sup> Treg cells. Temporary depletion of nTreg prior to infection does not alter the course of infection as IL-10-secreting CD4<sup>+</sup>FoxP3<sup>+</sup>CD25<sup>+</sup> Treg cells compensate for their absence. However, depleting nTreg cells in IL-10-deficient mice results in accelerated bacterial clearance. We believe that inhibiting both nTreg and IL-10-secreting Treg cells completely impairs immunoregulation in *B. pertussis* infection in the lung. In short, CD4<sup>+</sup>FoxP3<sup>+</sup>CD25<sup>+</sup> Treg cells represent an additional immunoregulatory subset involved in modulating immunity to *B. pertussis* infection in the lung.



**PB06/64 THE COMPLEX INTERPLAY BETWEEN HELPER T CELLS AND REGULATORY T CELLS IN RESPONSE TO IMMUNOSUPPRESSIVE FRIEND RETROVIRUS**S. Nair<sup>1</sup>, G. Zelinsky<sup>1</sup>, G. Kassiotis<sup>2</sup>, T. Sparwasser<sup>3</sup>, U. Dittmer<sup>1</sup><sup>1</sup>University Hospital Essen, Institute for Virology, Essen, Germany, <sup>2</sup>National Institute for Medical Research, Division of Immunoregulation, Medical Research Council, London, United Kingdom, <sup>3</sup>Technische Universität München, Institut für Medizinische Mikrobiologie, Immunologie und Hygiene, Munich, Germany

Immune evasion is the most successful tool used by many viruses such as herpes viruses, hepatitis viruses and retroviruses to reside within the host undetected and cause persistent infections for life. One such escape mechanism is the heightened expansion of Regulatory T cells (Treg) which down-regulate effector immune responses affecting control of viral spread. The Friend retrovirus (FV) murine infection model helps in studying basic mechanisms of immunological control and escape in both acute and persistent retroviral infections. The aim of this work was to investigate the effect of Treg on the functional aspects of Helper T cells (Th) during acute FV infection. *In vivo* depletion of Th cells in acutely infected mice demonstrated that Th cells were not only vital in controlling viral spread and onset of erythroleukemia but also in the maintenance of FV-specific CD8<sup>+</sup> T cell and neutralizing antibody responses. Tetramer-II kinetic analysis of FV-specific Th cell responses showed that magnitude of protective immune responses in FV resistant mice was much higher than in susceptible mice owing to different MHC backgrounds. FV-specific TCR transgenic CD4<sup>+</sup> T cells were adoptively transferred into FV-resistant mice infected for different time periods (1wpi, 2wpi, 3wpi) to study the anti-viral effect of the transferred cells after recognition of FV antigen *in vivo*. Results indicated that FV-specific TCR transgenic CD4<sup>+</sup> T cells could effectively recognize FV antigen *in vivo* at all the time points but were functionally impaired starting from 3 wpi. Furthermore, ameliorating the suppressive activity of regulatory T cells by *in vivo* depletion of regulatory T cells in 3wpi DEREGE mice allowed adoptively transferred CD4<sup>+</sup> T cells to produce increased amounts of IFN- $\gamma$  and significantly reduced viral load. Current study demonstrates that helper T cells were critical for recovery from acute Friend retroviral infections and that regulatory T cells can cause immunosuppression of helper T cell effector functions thereby contributing towards viral persistence.

**PB06/65 IL-4R $\alpha$  RESPONSIVE CD4<sup>+</sup>CD25<sup>+</sup>CD103<sup>+</sup>FOXP3<sup>+</sup> CELLS CONTROL SCHISTOSOMA MANSONI EGG-INDUCED INFLAMMATION BY SECRETED IL-10**B. Dewals<sup>1</sup>, J. C. Hoving<sup>2</sup>, M. Leeto<sup>2</sup>, R. G. Marillier<sup>2</sup>, A. J. Cutler<sup>3</sup>, W. G. Horsnell<sup>2</sup>, F. Brombacher<sup>2</sup><sup>1</sup>University of Liège, Belgium, <sup>2</sup>University of Cape Town, Cape Town, South Africa, <sup>3</sup>Histocompatibility and Immunogenetics, London, United Kingdom

IL-4R $\alpha$  signalling drives Th2-type responses that mediate resistance to parasitic helminth infections. We generated a novel mouse model lacking IL-4R $\alpha$  expression specifically on all T cells (*iLck<sup>cre</sup>IL4ra<sup>-lox</sup>*) to investigate IL-4R $\alpha$ -dependent T cell responses during *Schistosoma mansoni* egg-driven inflammation. These mice showed higher mortality during acute schistosomiasis compared with *IL4ra<sup>-lox</sup>* controls and previously established CD4<sup>+</sup> T cell specific IL-4R $\alpha$  deficient mice (*Lck<sup>cre</sup>IL4ra<sup>-lox</sup>*). *iLck<sup>cre</sup>IL4ra<sup>-lox</sup>* mice developed a liver restricted pathology associated with drastic reductions of both Th2/type 2 responses and alternative macrophage activation within the granulomas. Additionally, *iLck<sup>cre</sup>IL4ra<sup>-lox</sup>* mice had (i) increased Foxp3<sup>+</sup> Treg cell responses in the granulomas, which was explained by IL-4 mediated inhibition of Foxp3 induction, and (ii) reduction of antigen-specific production of IL-10 by CD4<sup>+</sup>CD103<sup>+</sup>Foxp3<sup>+</sup> cells. In a footpad model of *S. mansoni* egg-induced inflammation with subsequent IL-10 neutralization and adoptive cell transfer experiments we found evidence that the increased inflammation in *iLck<sup>cre</sup>IL4ra<sup>-lox</sup>* mice was due to the impaired development of IL-10-secreting CD4<sup>+</sup>CD25<sup>+</sup>CD103<sup>+</sup>Foxp3<sup>+</sup> cells. Together, these data demonstrate that IL-4R $\alpha$  responsiveness by T cells promote IL-10-secreting CD4<sup>+</sup>CD25<sup>+</sup>CD103<sup>+</sup>Foxp3<sup>+</sup> cells and alternatively activated macrophages, which act in concert to control egg-induced inflammation.

**PB06/66 DIFFERENT CYTOLYTIC PATHWAYS OF CD8<sup>+</sup> T CELL MEDIATE CONTROL OF VIRAL INFECTION AND ARE COUNTER-REGULATION BY REGULATORY T CELLS**G. Zelinsky<sup>1</sup>, K. K. Dietze<sup>1</sup>, T. Sparwasser<sup>2</sup>, M. M. Simon<sup>3</sup>, U. Dittmer<sup>1</sup><sup>1</sup>University Clinics in Essen, Institute for Virology, Essen, Germany, <sup>2</sup>TWINCORE, Centre for Experimental and Clinical Infection Research; a Joint Venture between the Medical School Hannover (MHH) and the Helmholtz Centre for Infection Research, Institute for Infection Immunology, Hannover, Germany, <sup>3</sup>Max-Planck Institute of Immunobiology, Freiburg, Germany

Cytotoxic T-cells (CTL) control of viral infections is mediated by at least two cytotoxic pathways namely the granule exocytosis pathway, involving perforin and granzymes, and the Fas-FasL pathway. However, the factor(s) that influence the selection of one or the other pathway for pathogen control are elusive. We investigated the role of viral replication levels and regulatory T cells on the activation of CTL during acute Friend Murine Leukemia Virus (F-MuLV) infection. Both low- and high-level F-MuLV infection generated CD8<sup>+</sup> effector T cells that were essential for the control of viral replication. However, the low-level infection induced CD8<sup>+</sup> T cells expressing solely FasL but not the cytotoxic molecules granzyme A and B, whereas the high-level infection resulted in induction of CD8<sup>+</sup> effector T cells secreting molecules of the granule exocytosis pathway. During the acute infection, both types of cytotoxic T cells were counter-regulated by Foxp3 expressing regulatory T cells. Interestingly, in case of low level replicating virus the ablation of regulatory T cells resulted in expression of granzymes by effector CD8<sup>+</sup> T cells. However, induction of the exocytosis pathway did not enable the CD8<sup>+</sup> T cells to eliminate the low level virus infection. The current results show that T-reg control the pathway of CD8<sup>+</sup> T cell mediated killing during an acute viral infection.

**PB06/67 SELECTIVE INDUCTION OF ADAPTIVE REGULATORY T-CELLS IN CHRONIC HEPATITIS C**B. Langhans<sup>1</sup>, I. Braunschweiger<sup>1</sup>, S. Arndt<sup>1</sup>, W. Schulte<sup>1</sup>, N. Vidovic<sup>2</sup>, L. Layland<sup>3</sup>, A. Hoerauf<sup>2</sup>, J. Oldenburg<sup>3</sup>, T. Sauerbruch<sup>1</sup>, U. Spengler<sup>1</sup><sup>1</sup>University of Bonn, Department of Internal Medicine I, Bonn, Germany, <sup>2</sup>University of Bonn, Institute of Medical Microbiology, Immunology and Parasitology, Bonn, Germany, <sup>3</sup>University of Bonn, Institute for Experimental Hematology and Transfusion Medicine, Bonn, Germany

**Objectives:** Weak T-cell responses to hepatitis C virus (HCV) antigens are a hallmark of chronic hepatitis C. Regulatory CD4<sup>+</sup> T-cell (Tr-cell) subpopulations have been proposed to contribute to this T-cell dysfunction and may determine the outcome of HCV infection.

**Methods:** We studied phenotypic and functional characteristics of CD4<sup>+</sup> Tr-cell populations in PBMC from patients with chronic (n = 31) and self-limited HCV infection (n = 12) ex vivo and after in vitro stimulation with HCV core protein. Tr-cells in bulk cultures were compared with individual Tr-cell clones (Tr-TCC) obtained from both patient groups.

**Results:** Ex vivo phenotypic analysis revealed that CD4<sup>+</sup> T-cells expressing Foxp3 were markedly less frequent in both CD25<sup>high</sup> and CD25<sup>int</sup> CD4<sup>+</sup> Tr-cell subsets from patients with self-limited HCV infection than in the corresponding subsets of patients with chronic HCV infection and healthy controls. In contrast to self-limited HCV infection, in vitro stimulation of PBMC from patients with chronic hepatitis C using HCV core protein resulted in a significant expansion of Foxp3- and CTLA-4-expressing CD25<sup>+</sup> Tr-cells and revealed a relationship between IL-10 production and number of CD25<sup>int</sup>CD4<sup>+</sup> Tr-cells.

Characterisation of CD4<sup>+</sup> T-cells from patients with chronic hepatitis C revealed 14 putative Tr-TCC which were hypoproliferative and produced significant amount of IL-10. Among TCC which did not suppress reporter TCC, 13 Tr-TCC significantly inhibited proliferation of both TH1 and TH2 reporter T-cells; 2 Tr-TCC also produced TGF- $\beta$ 1 and suppressed IFN- $\gamma$  secretion. 11 Tr-TCC were activated in a HCV core-specific, HLA-DR restricted fashion. All Tr-TCC were Foxp3<sup>+</sup> and – with the exception of one CD25<sup>high</sup> clone – were CD25<sup>int</sup>. In contrast, cloning of CD4<sup>+</sup> T-cells from patients with self-limited hepatitis C resulted in 8 hypoproliferative IL-10-producing TCC, which resembled the phenotype of Tr-TCC obtained in chronic hepatitis C. However, none of the TCC from self-limited hepatitis C inhibited proliferation of autologous reporter cells and responded to HCV core protein.

**Conclusion:** Our data demonstrate that functionally active HCV core-specific CD4<sup>+</sup> Tr-cells exist exclusively in patients with chronic hepatitis C but not in self-limited HCV infection. Such HCV-specific Tr-cells may prevent the immune system from ultimately eliminating HCV.

**PB06/68 DE NOVO GENERATION AND ENHANCED SUPPRESSION OF HUMAN CD4<sup>+</sup>CD25<sup>+</sup> REGULATORY T CELLS BY RETINOIC ACID**J. Wang<sup>1</sup>, T. W. J. Huizinga<sup>1</sup>, R. E. M. Toes<sup>1</sup><sup>1</sup>Leiden University Medical Center, Rheumatology, Leiden, Netherlands

**Objectives:** Therapies based on CD4<sup>+</sup>FOXP3<sup>+</sup> T regulatory cells (Tregs) offer promise for the restoration of tolerance in many immune-mediated disorders. However, it has been proven difficult to obtain large numbers of Tregs with potent and stable suppressive ability from human peripheral blood due to the lack of specific markers, compromised function of isolated CD4<sup>+</sup>CD25<sup>+</sup> T cell populations and the difficulty to convert conventional T cells, for example by TGF- $\beta$ , into Tregs in a consistent manner. Therefore, the goal of this study was to develop an efficient approach to generate large numbers of CD4<sup>+</sup> Tregs with potent and stable suppressive ability from adult human peripheral blood mononuclear cells (PBMCs).

**Methods:** To investigate the effect of All-Trans Retinoic Acid (ATRA) on the de novo generation of Tregs as well as on the function of expanded CD4<sup>+</sup>CD25<sup>+</sup> T cells isolated from PBMCs, we purified total CD4<sup>+</sup>CD25<sup>+</sup>, CD4<sup>+</sup>CD25<sup>+</sup>CD45RA<sup>+</sup> naive/memory effector cells as well as CD4<sup>+</sup>CD25<sup>+</sup> T cells from adult PBMCs by FACS-sorting. Subsequently, these cells were stimulated in the absence/presence of TGF- $\beta$  and/or ATRA. After resting for differentiation, the phenotype and function of these cells were determined by FACS staining and standard in vitro suppression assay, respectively.

**Results:** Although addition of ATRA had no effect on the initial proliferation of peripheral CD4<sup>+</sup> T cells, these cells displayed significantly reduced proliferative ability upon re-stimulation. In the presence of exogenous TGF- $\beta$ , ATRA consistently generates CD4<sup>+</sup> Tregs from naive CD4<sup>+</sup>CD25<sup>+</sup>CD45RA<sup>+</sup> conventional cells isolated from PBMCs. These ATRA-induced Tregs displayed high and stable levels of FOXP3 expression, and more importantly, potent and stable suppressive capacity as they inhibited the proliferation of autologous effector cells to a comparable extent as did ex vivo isolated CD4<sup>+</sup>CD25<sup>+</sup> T cells. However, memory CD4<sup>+</sup>CD25<sup>+</sup>CD45RA<sup>+</sup> T cells are resistant to FOXP3 induction and, moreover, inhibit TGF- $\beta$ /ATRA-induced Treg-conversion of co-cultured naive T cells. Furthermore, treatment of isolated CD4<sup>+</sup>CD25<sup>+</sup> T cells with ATRA/TGF- $\beta$  preserves/enhances their FOXP3 expression as well as suppressive potential during the in vitro expansion.

**Conclusions:** Our results indicate that ATRA/TGF- $\beta$  can be employed to generate highly suppressive CD4<sup>+</sup>FOXP3<sup>+</sup> Tregs from adult human peripheral blood, and are relevant for the development of Treg-based therapies.

**PB06/69 ROLE OF REGULATORY T CELLS IN EXPERIMENTAL CEREBRAL MALARIA**C. Steeg<sup>1</sup>, G. Adler<sup>1</sup>, B. Fleischer<sup>1</sup>, T. Sparwasser<sup>2</sup>, T. Jacobs<sup>1</sup><sup>1</sup>Bernhard Nocht Institute for Tropical Medicine, Immunology, Hamburg, Germany, <sup>2</sup>Zentrum für Experimentelle und Klinische Infektionsforschung, Institut für Infektionsimmunologie, Hannover, Germany

**Objective:** Cerebral malaria (CM) associated with *Plasmodium berghei* ANKA (PbA) infection of mice is an accepted model of CM in humans and critically depends on sequestration of T cells into the brain. Downmodulation of T cell activation leads to an attenuation of the symptoms. Several studies aimed to address the role of regulatory T cells ( $T_{reg}$ ) in downmodulating this pathogenic T cell response by using CD25 as a marker for  $T_{reg}$  and employing anti CD25 antibodies for depletion. These studies are hampered by the fact that activated T cells also express CD25. With the recent generation of DERE mice (depletion of regulatory T cells), which express a diphtheria toxin receptor eGFP fusion molecule under the control of the *foxp3* locus, we could detect and also deplete  $T_{reg}$  without affecting other CD25 expressing T cells.

**Methods:** We infected DERE mice with PbA merozoites and quantified the T cell subsets in different organs during infection. Depletion of  $T_{reg}$  by applying diphtheria toxin led to the disappearance of  $T_{reg}$  during the course of infection. We also isolated  $T_{reg}$  from uninfected and infected mice and analyzed their inhibitory capacity.

**Results:** We found only a small increase in the absolute numbers of Foxp3<sup>+</sup>  $T_{reg}$  during PbA-infection and consequently the ratio of  $T_{reg}$  to T effector cells ( $T_{eff}$ ) decreased due to the rapid expansion of  $T_{eff}$ . Whereas the latter sequester in the brains of infected mice, almost no  $T_{reg}$  were found in the brain of infected mice. Furthermore, we demonstrate that depletion of  $T_{reg}$  had a minor influence on T cell activation, but no influence on sequestration of  $T_{eff}$  and did not alter the clinical outcome. Using *ex vivo* analysis of purified  $T_{reg}$  from either naive or PbA-infected mice we found that both exhibit similar inhibitory capacity on  $T_{eff}$ .

**Conclusion:** Our data suggest that experimental CM is triggered by a rapid expansion of potentially pathogenic T cells that cannot be adequately controlled by  $T_{reg}$ .

**PB06/70 INDUCED CD8<sup>+</sup>FOXP3<sup>+</sup> REGULATORY T CELLS CAN BE GENERATED IN THE ABSENCE OF DENDRITIC CELLS AND ARE SUPPRESSIVE *IN VITRO* AND *IN VIVO***C. T. Mayer<sup>1</sup>, K. Lahl<sup>2</sup>, C. Lodenkemper<sup>3</sup>, T. Sparwasser<sup>1</sup><sup>1</sup>TWINCORE, Centre for Experimental and Clinical Infection Research, Institute for Infection Immunology, Hannover, Germany, <sup>2</sup>Technical University Munich, Institute for Medical Microbiology, Immunology and Hygiene, Munich, Germany, <sup>3</sup>Charité, Campus Benjamin Franklin, Institute for Pathology, Berlin, Germany

CD4<sup>+</sup>Foxp3<sup>+</sup> natural and adaptive regulatory T cells (Tregs) play pivotal roles both in the maintenance of peripheral self tolerance and the limitation of pathologic immune responses (e.g. against pathogens or fetal allo-antigens) across different mammal species. In gross contrast, although suppressive T cell functions have been historically attributed to CD8<sup>+</sup> T cells, the relevance of mouse CD8<sup>+</sup>Foxp3<sup>+</sup> Tregs remains unclear. Importantly, the lack of specific surface markers has hampered the isolation and characterization of pure CD8<sup>+</sup>Foxp3<sup>+</sup> Treg populations. Although during the last decade several reports have highlighted natural or induced CD8<sup>+</sup> T cell populations with suppressive properties, their dependence on Foxp3 remains elusive and therefore their consideration as “relatives” of classical CD4<sup>+</sup>Foxp3<sup>+</sup> Tregs is questionable. By using CD8<sup>+</sup> T cells purified from Rag1<sup>-/-</sup> × DERE × OTI bacterial artificial chromosome (BAC) transgenic mice, we were able to generate and isolate OVA<sub>257-264</sub> peptide-specific induced CD8<sup>+</sup>Foxp3<sup>+</sup> (iCD8<sup>+</sup>) Tregs based on DTR-eGFP reporter gene expression. Carrying out phenotypic and functional studies, we show that iCD8<sup>+</sup> Tregs express classical Treg markers including CD25, CD73, CD103, CTLA4 and GITR, suppress polyclonal proliferation of CD4<sup>+</sup> and CD8<sup>+</sup> T cells *in vitro* and limit autoimmune pathology of Foxp3-deficient natural *scurfy* mutant mice upon adoptive transfer *in vivo*. Interestingly, iCD8<sup>+</sup> Tregs displayed cytotoxic effector function towards antigen-pulsed target cells *in vitro*. Surprisingly, induction of CD8<sup>+</sup>Foxp3<sup>+</sup> Tregs did not require antigen presenting cells and was strongly impaired in the presence of bone marrow-derived dendritic cells (DCs) by a cell contact-dependent mechanism. In contrast, B cells only slightly impaired iCD8<sup>+</sup> Treg induction. As the degree of inhibition among different APC subsets correlated with expression of co-stimulatory molecules (CD80, CD86), and as agonistic anti-CD28 antibody treatment induced similar inhibition, we propose that DCs inhibit iCD8<sup>+</sup> Treg generation by co-stimulation. These findings discriminate iCD8<sup>+</sup> Tregs from induced CD4<sup>+</sup>Foxp3<sup>+</sup> Tregs and might have implications for the action of iCD8<sup>+</sup> Tregs *in vivo*. Taken together, iCD8<sup>+</sup> Tregs can be generated independently of antigen presenting cells, express common Treg markers and are suppressive *in vitro* and *in vivo*.

**PB06/71 REGULATORY T CELLS LIMIT ACUTE BUT NOT MEMORY T CELL RESPONSES TO ACUTE VIRAL INFECTION**G. Gasteiger<sup>1</sup>, W. Kastenmüller<sup>2</sup>, L. Stross<sup>1</sup>, T. Sparwasser<sup>3</sup>, D. Busch<sup>4</sup>, I. Drexler<sup>1</sup><sup>1</sup>Helmholtz-Zentrum und Technische Universität, Institut für Virologie, München, Germany, <sup>2</sup>NIAID, NIH, Lymphocyte Biology Section, Laboratory of Immunology, Bethesda, United States, <sup>3</sup>Twincore Zentrum für Experimentelle und Klinische Infektionsforschung, Hannover, Germany, <sup>4</sup>Technische Universität, Institut für Medizinische Mikrobiologie, Immunologie und Hygiene, München, Germany

CD4<sup>+</sup> Regulatory T-cells (Tregs) are a crucial component of the adaptive immune system. Recent work using antibody depletion suggests that removal of Tregs during acute infection or vaccination can enhance both the peak acute and the recall memory response of antigen-specific CD8<sup>+</sup> T-cells. In our work, we used a mouse model that allows for specific depletion of Foxp3-positive Tregs and vaccinia virus MVA as a viral vector vaccine to specifically address this question. We found that in the absence of Tregs, primary immune responses against recombinant MVA expressing OVA were significantly increased (2-3 fold) and the phenotype of CD8 T-cells shifted towards more differentiated effector T-cells (CD62L<sub>low</sub>/CD127<sub>low</sub>). Interestingly, absolute numbers of central memory T-cells (CD62L<sub>high</sub>/CD127<sub>high</sub>) were equal in the absence or presence of Tregs. Priming under Treg-depletion led to enhanced numbers of monofunctional T-cells (IFN $\gamma$ +IL-2-TNF $\alpha$ -), while polyfunctional T-cells (IFN $\gamma$ +IL-2+TNF $\alpha$ +) considered as crucial for protective immunity were induced to the same level as in control animals. The higher number of primed T-cells generated in the absence of Tregs was reflected in the early memory phase (d30), but was not observed in the long-term memory phase (d70). There was no difference in the quality of induced T-cells based on *in vivo* cytotoxicity assays and their ability to expand upon booster vaccination.

In summary, we conclude that Tregs regulate the number of fully differentiated T-cells during the acute immune response but do not influence the long-term memory T cell pool. Therefore, removal of Tregs may be of little value in the case of prophylactic vaccinations but might be of great importance in therapeutic settings.

**PB06/72 CTLA-4 REGULATES PERIPHERAL FOXP3 INDUCTION**A. Izcue<sup>1,2</sup>, F. Powrie<sup>1</sup><sup>1</sup>University of Oxford, Oxford, United Kingdom, <sup>2</sup>Max-Planck Institute of Immunobiology, Freiburg, Germany

The inhibitory molecule CTLA-4 is an important factor in immune homeostasis. In humans, CTLA-4 variants have been associated with autoimmune diseases, whereas mice deficient for CTLA-4 die at an early age from an extensive lymphoproliferative syndrome. It is now generally accepted that CTLA-4 exerts its anti-inflammatory functions via different pathways. On one hand, it can reduce T cell activation by dampening TCR/CD28 signals. On another hand, it plays an important role in the function of CD4<sup>+</sup> Foxp3<sup>+</sup> regulatory T cells. In addition to these two mechanisms, CTLA-4 has been suggested to control Foxp3 induction on naïve T cells. We have used *in vitro* cell cultures and *in vivo* mouse models to characterize this effect. *In vitro* assays of Foxp3 induction using *ex vivo* mouse naïve T cells show a positive effect of CTLA-4 signals on *de novo* Foxp3 expression. This effect was independent of antigen-presenting cells as it was also observed when the cells were directly stimulated by plate-bound antibodies. *In vivo*, CTLA-4 was not essential for the induction of Foxp3<sup>+</sup> regulatory T cells in healthy mixed bone marrow chimeras. However, older chimeras showed a specific deficit in Foxp3<sup>+</sup> cells derived from CTLA-4-deficient bone marrow. This deficit was observed in the intestine, a preferential site for peripheral Foxp3 induction *in vivo*, but not in the spleen or lymph nodes. When CTLA-4 KO naïve T cells isolated from a non-inflamed environment were transferred into immunodeficient hosts, the frequency of Foxp3<sup>+</sup> cells was much lower than for wild-type T cells. This effect was due to both an increased accumulation of CTLA-4-deficient Foxp3<sup>+</sup> T cells and a reduction in the induction of Foxp3<sup>+</sup> cells. In contrast, lack of CTLA-4 did not affect the expansion or survival of naturally arising Foxp3<sup>+</sup> T cells. Our results argue for a specific role of CTLA-4 in peripheral, but not thymic, Foxp3 induction.

**PB06/73 PERIPHERALLY-INDUCED REGULATORY T CELLS DOWN-MODULATE ABETA-SPECIFIC CD4<sup>+</sup> T CELL RESPONSES IN A MOUSE MODEL OF ALZHEIMER'S DISEASE**C. Toly Ndour<sup>1,2</sup>, G. Lui<sup>1,2</sup>, T. Chaigneau<sup>1,2</sup>, M. Bruley-Rosset<sup>1,2</sup>, P. Aucouturier<sup>1,2</sup>, G. Dorothee<sup>1,2</sup><sup>1</sup>INSERM UMR S-938, Immune System and Conformational Diseases Laboratory, Paris, France, <sup>2</sup>Université Pierre et Marie Curie, Paris, France

Alzheimer's Disease (AD) is a severe neurodegenerative disorder characterized by progressive mental deterioration resulting in loss of memory and cognitive functions. According to the “amyloid hypothesis”, formation and accumulation of Abeta amyloid peptide is the initiating cause of pathogenic lesions. Among new therapeutic strategies in AD, vaccines targeting Abeta represent promising therapeutic options. Active immunization against Abeta provided encouraging results in experimental mouse models and, to a lesser extent, in a subsequent clinical trial (AN1792). However, the occurrence of aseptic meningoencephalitis in 6% of the treated patients underlined the need for better understanding the adaptive immune responses to Abeta. While these severe side effects were attributed to pro-inflammatory T cell responses, several reports suggest that Abeta-specific CD4<sup>+</sup> T cells may be implicated in the natural course of AD and could have a strong therapeutic potential as well. Since Abeta is processed from the self-protein APP, better understanding the tolerance mechanisms regulating natural and/or vaccine-induced Abeta-specific T cell responses is of fundamental interest for new vaccine development. Based on regulatory T cell (Treg) depletion experiments in a transgenic APPS1 mouse model of AD, we demonstrate that AD mice display an enhanced Treg activity that down-modulates Abeta-specific CD4<sup>+</sup> T cell responses. Further experiments in APP-deficient mice suggest that Abeta-specific Treg arise in the periphery of AD mice, both spontaneously and following Abeta immunization. Hence, peripheral induction of Abeta-specific Treg should be taken into account for the future design of efficient and safe T cell-based vaccination strategies in AD.

**PB06/74 THE ROLE OF THE MICRORNA PATHWAY IN THE FUNCTION OF EFFECTER AND REGULATORY T CELLS**A. Liston<sup>1,2</sup><sup>1</sup>University of Leuven, Leuven, Belgium, <sup>2</sup>Flanders Institute of Biotechnology, Gent, Belgium

MicroRNA modify the properties of cells by altering the transcriptional profile to produce a proteome with unique functional properties. We have found that in the absence of microRNA, the unmodified transcriptional profile of T cells produces a cell with limited functional capacity. Naïve T cells deficient in Dicer have a reduced capacity to progress through the differentiation pathway and to commit to both regulatory and effector lineages. Regulatory T cells deficient in Dicer have reduced IL2-dependent proliferation, reduced function as regulatory effectors and a reduced capacity to maintain lineage identity. Effector T cells, in the absence of microRNA, have a greatly reduced capacity to proliferate and perform, even in an autoimmune-prone environment with reduced regulatory T cells and an absence of tolerogenic TGFβ signalling.

**PB06/75 ARE HLA-E RESTRICTED CD8<sup>+</sup> T CELLS FUNCTIONALLY ALTERED IN MULTIPLE SCLEROSIS?**K. Pannemans<sup>1</sup>, R. Dobosi<sup>2</sup>, A. Goris<sup>2</sup>, B. Dubois<sup>2</sup>, B. Van Wijmeersch<sup>1</sup>, P. Stinissen<sup>1</sup>, N. Hellings<sup>1</sup><sup>1</sup>Hasselt University, Biomedical Research Institute, Diepenbeek, Belgium, <sup>2</sup>University Hospital Gasthuisberg, Experimental Neurology, Leuven, Belgium

Qa-1 restricted CD8<sup>+</sup> regulatory T cells suppress autoreactive CD4<sup>+</sup> T cells in the animal model for multiple sclerosis (MS). This study aims at elucidating the involvement of CD8<sup>+</sup> T cells restricted by HLA-E, the human variant of Qa-1, in immunoregulatory alterations in MS. In a first part, an MS association study of HLA-E polymorphisms was performed. The HLA-E gene harbors two polymorphic sites, codon 107 (HLA-E\*0101 versus HLA-E\*0103) and codon 77 (HLA-E\*010301 versus HLA-E\*010302). These polymorphisms were studied in 1078 HC and 832 MS patients. Plink option analysis showed a significant higher presence of HLA-E\*010301 and lower frequency of HLA-E\*0101 in MS patients ( $p=0.04$ ). Conditioning on DRB1\*1501 revealed that this HLA-E association actually reflected the effect of DRB1\*1501 on the development of MS. Although no genetic association was found, functional alterations in HLA-E restricted CD8<sup>+</sup> T cells could still be involved in the dysbalanced autoimmune responses observed in MS patients. Therefore, HLA-E restricted CD8<sup>+</sup> T cells are characterised at the level of their phenotype and regulatory potential. First, the phenotype of both NKG2C<sup>+</sup> and NKG2A<sup>+</sup> CD8<sup>+</sup> T cells was studied using flow cytometry. The frequency of NKG2A<sup>+</sup> CD8<sup>+</sup> T cells was not significantly different between HC ( $n=25$ ,  $6.9 \pm 3.2\%$ ) and MS patients ( $n=13$ ,  $8.7 \pm 3.2\%$ ). The same could be concluded for NKG2C<sup>+</sup> CD8<sup>+</sup> T cells (HC:  $3.82 \pm 2.1\%$ , MS:  $3.32 \pm 1.0\%$ ). In HC the NKG2C<sup>+</sup> CD8<sup>+</sup> T cells showed a significant lower expression of CD28 ( $p=0.003$ ) and CD25 ( $p=0.02$ ) and higher expression of CD45RA ( $p=0.01$ ) compared to the NKG2A<sup>+</sup> CD8<sup>+</sup> population. Further experiments need to clarify if the same phenotypic characteristics can be found in MS patients. We further hypothesize that the low CD25 and high CD45RA expression on NKG2C<sup>+</sup> CD8<sup>+</sup> T cells implies that this subpopulation needs to be induced by activated (autoimmune) T cells before acquiring a possible suppressive capacity. This will be investigated in co-culture experiments with autoreactive T cells and these CD8<sup>+</sup> subsets. This study may lead to the generation of new insights into the pathogenesis of MS.

**PB06/76 TCR/CD3 COMPLEXES IN CD4<sup>+</sup> TREG CELLS: DIFFERENCES IN COMPLEX LEVELS AND IN THE QUALITY OF CD3ε CHAINS**J.M. Rojo<sup>1</sup>, G. Ojeda<sup>2</sup>, P. Portolés<sup>2</sup><sup>1</sup>Centro de Investigaciones Biológicas, CSIC, Cellular and Molecular Physiopathology, Madrid, Spain, <sup>2</sup>Centro Nacional de Microbiología, Instituto de Salud Carlos III, Unidad de Inmunología Celular, Majadahonda, Spain

Regulatory thymus-derived T cells (Treg cells) are CD4<sup>+</sup> T lymphocytes that exert active suppression to control normal immune responses and prevent autoimmune diseases. In mice, Treg cells characteristically express the Foxp3 (Scurfin) transcription factor and the CD25 (IL-2R β chain) surface molecule. Whereas Foxp3 expression is both necessary and sufficient to the generation of Treg cells, available data show that TCR is essential to normal Treg differentiation in two respects: Firstly, induction of Foxp3 expression during thymic development is strictly dependent on TCR/self MHC interactions. Furthermore, one function of Foxp3 is probably the stabilization of signals imparted by chronic TCR stimulation by self antigens. We have previously observed that different functional subsets of T lymphocytes, or T lymphocytes at different developmental stages, have distinct characteristics concerning the level of TCR/CD3 complexes, but also in the relative abundance of CD3ε chain isoforms as determined by their pI (Criado et al., Eur J Immunol 2000; 30:1469-1479; Bello et al., J Biol Chem 2007; 282:22324-22334). Using flow cytometry analysis of spleen and thymus populations, and one- and two-dimension immunoblot of CD3ε chains, we have studied whether TCR/CD3 complexes in CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> Treg cells differ quantitatively and qualitatively from the rest of the CD4<sup>+</sup> population, a fact that might explain some of Treg activation properties.

Our results show that, indeed, Treg cells express TCR/CD3 levels significantly lower (30-40%) than their CD25<sup>+</sup> Foxp3<sup>+</sup> CD4<sup>+</sup> counterparts, a phenomenon that is also found in the expression of the CD4 coreceptor. Furthermore, they express CD3ε chains that are enriched for undegraded N-terminal sequence isoforms, as determined by the lower avidity of the YCD3-1 anti-CD3 antibody, or by immunoblot with anti-CD3ε antibody after IEF and SDS-PAGE of CD3 immunoprecipitates. Taken together, our results suggest that Treg cells have a hypo-responsive phenotype concerning TCR/CD3-mediated activation. This phenotype could contribute to Treg differentiation in the thymus or to Treg function in the periphery.

**PB06/77 THE ROLE OF IL-4 IN REGULATION OF NATURAL AND INDUCED TREG DEVELOPMENT AND MAINTENANCE**J. Prochazkova<sup>1,2</sup>, J. Fric<sup>1</sup>, K. Pokorna<sup>1,2</sup>, A. Neuwirth<sup>1</sup>, A. Zajicova<sup>1,2</sup>, V. Holan<sup>1,2</sup><sup>1</sup>Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague, Czech Republic, <sup>2</sup>Faculty of Natural Science, Charles University, Prague, Czech Republic

**Objectives:** CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> regulatory T cells (Tregs) can be classified as naturally occurring (nTregs) and induced (iTregs) according to the developmental criteria. The development and function of Tregs are strictly regulated by cytokines. In this study we analyzed the effect of different cytokines on the development and function of nTregs and iTregs.

**Methods:** Purified and CFSE-labelled mouse T cell subpopulations of CD4<sup>+</sup> CD25<sup>-</sup> and CD4<sup>+</sup> CD25<sup>+</sup> T cells were cultured in the presence of alloantigen and different cytokines. The proliferation of these cells was tracked together with expression of CD4, CD25 and Foxp3 molecules. Finally, the suppression capacity of different Treg subtypes was determined in mixed lymphocyte cultures.

**Results:** We have previously demonstrated that CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> Tregs arise from CD4<sup>+</sup> CD25<sup>-</sup> T cells upon stimulation with alloantigen in the presence of TGFβ. The development of these Tregs and their proliferation was inhibited by IL-4 and IL-12. We have not observed alloantigen-induced cell proliferation within a population of nTregs. Interestingly, when IL-4 was added to the nTregs cultures, it sustained their CD25 expression and greatly supported nTreg viability. Addition of IL-4 to the iTreg cultures decreased the number of Foxp3<sup>+</sup> cells. These results demonstrated distinct responses of natural and induced Tregs to the regulatory action of IL-4 and an opposite role of IL-4 in maintenance of nTregs and iTregs phenotype. In spite of these differences, nTregs and Tregs induced by alloantigen in the absence or presence of IL-4 display comparable immunosuppressive effects when added to the mixed lymphocyte culture containing fresh effector cells and irradiated allogeneic cells.

**Conclusion:** Altogether these results showed that TGFβ and IL-4 differentially regulate the development of Tregs and distinctly sustain Foxp3 expression and the number of nTregs and iTregs, but have no influence on the suppressive activity of Tregs on a per cell basis.

**PB06/78 HUMAN ADIPOSE-DERIVED STEM CELLS (hASC) THERAPY INDUCE THE GENERATION OF REGULATORY T CELLS**O. DelaRosa<sup>1</sup>, E. Lombardo<sup>1</sup>, C. Ramirez<sup>1</sup>, P. Mancheño<sup>1</sup>, B. del Río<sup>1</sup>, R. Menta<sup>1</sup>, A. Beraza<sup>1</sup>, L. Rico<sup>1</sup>, D. Buscher<sup>1</sup><sup>1</sup>CELLERIX, R&D, Madrid, Spain

Human adipose-derived stem cells (hASCs) are mesenchymal stem cells with reduced immunogenicity and the capability to modulate immune responses. These properties make hASCs of special interest as therapeutic agents in the settings of both chronic inflammatory and autoimmune diseases. Given the significant role of regulatory T cells in most of immune mediated diseases we studied whether the immunomodulatory and antiproliferative role of hASC could be correlated with the presence of regulatory/suppressor T cells.

The use of hASCs in cell therapy for the treatment of inflammatory/autoimmune diseases deserved further investigation regarding the potential effects of hASCs therapy, over the biology of regulatory T cells, which are of special relevance in terms of therapeutic potency. Here we confirmed that hASCs inhibit T cell proliferation in both cell-cell contact and transwell conditions. The inhibitory effect of hASCs was accompanied by a reduction of proinflammatory cytokines (IFN-γ, TNF-α, IL-5 and IL-2).

To further study the functional role of T cells that have been in contact with hASC, CD3 cells were isolated and used in a proliferation assay in the presence of autologous PBMCs. Results demonstrate that CD3 cells isolated after coculture of PBMC with hASCs were able to inhibit PBMC proliferation in a dose dependent manner. Therefore, the presence of allogeneic hASC in PBMC cultures generated T cells with a regulatory/suppressive function. After phenotypic study we found that, regulatory/suppressive T cells generated in the presence of hASC, included a significant increase of CD4<sup>+</sup> CD25<sup>bright</sup> cells. The CD4<sup>+</sup> CD25<sup>bright</sup> gated population was also positive for the forkhead box P3 (FOXP3) marker.

Taken together, these results indicate that in an allogeneic system, hASC are able to trigger the generation of highly effective regulatory/suppressive cells that include a population with Treg phenotype. This finding may be important for the therapeutic use of allogeneic hASC to modulate immune responses, especially in autoimmune diseases.



**PB06/79 REDUCED SUSCEPTIBILITY OF T CELLS FROM SYNOVIAL FLUID OF PATIENTS WITH JUVENILE IDIOPATHIC ARTHRITIS TO IMMUNOREGULATION BY NATURAL REGULATORY T CELLS**S. Haufe<sup>1</sup>, A. Hospack<sup>2</sup>, G. Dannecker<sup>2</sup>, N. Tzaribachev<sup>1</sup>, J. Kümmerle-Deschner<sup>1</sup>, H.-G. Rammensee<sup>3</sup>, U. Holzer<sup>1</sup><sup>1</sup>University of Tuebingen, Children's Hospital, Tuebingen, Germany, <sup>2</sup>Olgahospital, Department of Pediatrics and Pediatric Rheumatology, Stuttgart, Germany,<sup>3</sup>University of Tuebingen, Department of Immunology, Tuebingen, Germany

**Objectives:** Natural CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells (nTregs) play a key role in maintaining immune homeostasis and controlling autoimmunity. Although patients with juvenile idiopathic arthritis (JIA) typically show an increase in the total number of nTregs with regulatory phenotype in the synovial fluid (SF) compared to peripheral blood (PB), inflammation occurs. Understanding the mechanism of Tregs could give further insight into the pathogenesis of JIA and potential therapies.

**Methods:** CD4<sup>+</sup>CD25<sup>+</sup> nTregs and autologous CD4<sup>+</sup>CD25<sup>+</sup> effector T cells (Teff) were isolated from PB and SF from healthy donors and patients with JIA by MACS sorting. The cells were co-cultured with antigen-presenting cells (APC) in the presence of anti-CD3. Suppressive activity was examined by [<sup>3</sup>H] thymidine incorporation.

Furthermore, the ability of induced CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> regulatory T cells (iTregs) to suppress Teff cells of healthy controls was examined. To induce CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> cells, APC and CD4<sup>+</sup>CD25<sup>+</sup> cells from healthy controls were cultured with IL-2 and stimulated with anti-CD3 in the presence or absence of TGF-beta1 and/or TNF-alpha antagonist Etanercept. Induced Tregs were sorted by MACS and added to allogeneic Teff cells. Suppression was analyzed by measuring [<sup>3</sup>H] thymidine incorporation.

**Results:** The suppressive effect of nTregs of PB and SF is comparable using Teff cells of peripheral blood. However Teff cells from SF were less susceptible to nTregs from both, PB and SF. Furthermore we analyzed the induction of regulatory T cells (CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup>) in the presence of TGF-beta1 and/or Etanercept. The percentage of iTregs was higher in the presence of TGF-beta1 or Etanercept as compared to the control group stimulated with anti-CD3 alone. The suppressive activity of iTregs with different induction protocols was comparable using Teff cells of PB.

**Conclusion:** Taken together our data demonstrate that although functional Tregs are present in the inflamed joints, Teff cells in the SF are less prone to cellular immunoregulation. This might be caused by the activated state of SF Teff cells in contrast to PB Teff. However, blocking TNF-alpha increases the number of Tregs resulting in a higher Treg:Teff ratio which might be part of the clinical effect of these substances.

**PB06/80 REGULATORY T CELLS WITH POTENT SUPPRESSOR FUNCTION CAN BE EFFECTIVELY EXPANDED FROM UMBILICAL CORD BLOOD**A. Theil<sup>1</sup>, A. Platz<sup>2</sup>, P. Monti<sup>1</sup>, L. Rank<sup>3</sup>, E. Bonifacio<sup>1</sup><sup>1</sup>DFG-Center for Regenerative Therapies Dresden (CRTD), TU Dresden, Dresden, Germany, <sup>2</sup>DKMS Cord Blood Bank, Dresden, Germany, <sup>3</sup>Forscherguppe Diabetes der Technischen Universität München, Munich, Germany

Regulatory T cells (Tregs) have a fundamental role in the control of autoimmunity in the periphery. There is an increasing interest in the possibility of using Treg-based cellular therapies to restore or maintain tolerance. The current challenges that need to be overcome include the isolation of pure Treg populations and the development of robust and highly efficient expansion protocols.

Human umbilical cord blood (CB) contains a significant population of naive highly functional CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>low</sup>FOXP3<sup>+</sup> Tregs and very few antigen experienced memory T-cells, potentially making it an ideal source for Tregs. This study investigated the potential of isolation and expansion of CB derived FOXP3<sup>+</sup> Tregs. CD4<sup>+</sup> T cells were isolated from cord blood mononuclear cells (CBMCs) by negative magnetic bead activated cell sorting (MACS) selection. The CD25<sup>+</sup> subpopulation was isolated by positive bead selection using a depletion plus a second selection MACS column. Cells were expanded using αCD3αCD28 beads and high dose IL-2. Expanded cells were examined for phenotype, suppressive function, cytokine production and mRNA expression.

The isolation procedure yielded approximately 2x10<sup>4</sup> cells per ml of cord blood. Isolated Tregs were 90% pure as assessed by CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>low</sup>FOXP3<sup>+</sup> phenotype. CB derived Tregs expanded up to 10000 fold within 14 days with a purity of >95% CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>low</sup>FOXP3<sup>+</sup>. Expanded cells could be stored frozen and thawed cells were shown to suppress (>90%) CD4<sup>+</sup>CD25<sup>+</sup> cell proliferation at suppressor:effector ratios of 1:1 until 1:32 in a bead stimulated suppression assay. FOXP3 expression could also be detected on the mRNA level by qRT-PCR. Only minor contamination with T<sub>H</sub>1 effector cells could be detected by cytokine production and T-bet mRNA expression. Addition of rapamycin to the culture medium reduced the expansion yield but had only minor effects on the phenotype of these cells.

These results support the feasibility of isolating high purity FOXP3<sup>+</sup> Tregs by MACS from human CB. Based on expansion capacity, phenotype and functional properties, these expanded Tregs represent a cell population with potential towards future cellular therapies.

**PB06/81 ANTIGEN-DRIVEN BYSTANDER SUPPRESSION AND TRUE ANTIGEN-SPECIFIC SUPPRESSION IS MEDIATED BY DISTINCT REGULATORY T CELL SUBPOPULATIONS**Y.F. Fuchs<sup>1</sup>, T. König<sup>1</sup>, S. Schallenberg<sup>1</sup>, K. Kretschmer<sup>1</sup><sup>1</sup>CRTD/DFG-Centre for Regenerative Therapies Dresden, Immunotolerance in Regeneration, Dresden, Germany

Regulatory T cells (Treg) play a pivotal role in maintaining immune homeostasis under physiological conditions. Exploiting antigen-specific Treg in settings of unwanted or exacerbated immune responses offers the opportunity for targeted intervention and specific correction towards immune homeostasis, thereby avoiding disadvantages of non-specific immunosuppressive drugs. The mechanisms underlying immunosuppressive Treg functions described so far include secretion of inhibitory cytokines, cell-contact-dependent suppression, induction of cytolysis, and functional modification of antigen presenting cells. The variety of inhibitory mechanisms ascribed to Treg suggests that these cells take a multi-pronged approach to achieve immune regulation, although the relative importance of individual mechanisms remains elusive. It is reasonable to speculate that the contribution of different suppression mechanisms is context-dependent and modulated by the inflammatory milieu and the magnitude of the immune response. However, how tasks are distributed among different Treg subsets is poorly understood. With regard to antigen specificity, it appears commonly accepted that once activated by the respective antigen, Treg-mediated immunosuppression occurs in an antigen non-specific manner.

In this study we present evidence for two phenotypically distinct Foxp3-expressing CD4<sup>+</sup>CD25<sup>+</sup> Treg subpopulations. Both initially require antigen-specific TCR stimulation to activate suppressor function. Once activated, inhibition of CD4<sup>+</sup> and CD8<sup>+</sup> T effector cells can either be antigen non-specific (antigen-driven bystander suppression), or Treg-mediated suppression can be restricted to CD4<sup>+</sup> effector T cells with the same antigen specificity (antigen-specific suppression). Furthermore, our molecular and functional studies comparing both Foxp3<sup>+</sup> Treg populations with Tr1-like cells that lack Foxp3 expression provide insight into mechanisms underlying antigen-specific and bystander suppression.

**PB06/82 HOMEOSTASIS AND DE NOVO INDUCTION OF REGULATORY T CELLS DURING TUMOR DEVELOPMENT**K. Klages<sup>1</sup>, U. Lauer<sup>2</sup>, A. Hamann<sup>2</sup>, N. Garbi<sup>3</sup>, C. Mayer<sup>4</sup>, T. Sparwasser<sup>4</sup>, J. Huehn<sup>1</sup><sup>1</sup>Helholtz Centre for Infection Research, Experimental Immunology, Braunschweig, Germany, <sup>2</sup>Charite Medical School and DRFZ, Berlin, Germany, <sup>3</sup>DKFZ, Heidelberg, Germany, <sup>4</sup>TWINCORE – Centre for Experimental and Clinical Infection Research, Hannover, Germany

Tumors establish an immunosuppressive environment to efficiently escape anti-tumor immunity. Tregs can suppress tumor-specific CD8<sup>+</sup> T cells and impair the efficiency of tumor vaccination therapies. An increase of Foxp3<sup>+</sup> regulatory T cells (Tregs) has been described in cancer patients. To characterize the influence of Tregs on tumor escape mechanisms, we have established murine tumor models using B16-Ova or RMA-Ova cells for subcutaneous tumor formation. In vitro studies showed an elevation of Treg induction from naïve CD4<sup>+</sup>CD25<sup>+</sup> T cells induced by tumor-cell-derived supernatant, indicating that tumors might induce Tregs from tumor infiltrating conventional T cells. In transgenic DERE mice (Depletion of regulatory T cells) all Foxp3<sup>+</sup> Tregs express a fusion protein composed of GFP and the human Diphtheria Toxin Receptor (DTR). Therefore, all Foxp3<sup>+</sup> Tregs can be eliminated via application of Diphtheria Toxin (DT). Using this transgenic mouse model, we depleted Foxp3<sup>+</sup> Tregs at various time points during tumor development without touching tumor-specific effector T cells. Upon Treg depletion the tumor growth rate decreased compared to untreated controls. The reduction of tumor growth was more effective when Treg depletion was induced early during tumor formation. The homeostasis and de novo induction of Tregs during the formation of solid tumors will be further analysed in this model.

**PB06/83 CORD BLOOD ANTIGEN PRESENTING CELLS FACILITATE THE INDUCTION OF ANTIGEN SPECIFIC REGULATORY T CELLS**S. Hoeks<sup>1,2</sup>, A. Steur<sup>1</sup>, M. Hoekstra<sup>1</sup>, B. Prakken<sup>2</sup>, I. de Kleer<sup>1,2</sup><sup>1</sup>University Medical Center Utrecht, Center of Pediatric Allergic Diseases, Utrecht, Netherlands, <sup>2</sup>University Medical Center Utrecht, Center of Molecular and Cellular Intervention, Utrecht, Netherlands

**Introduction:** The neonatal immune system is characterized by a defective interleukine-12 (IL12) production. This condition may facilitate Th2 differentiation but also induce FOXP3<sup>+</sup>CD4<sup>+</sup> regulatory T cells (Tregs). We propose that to prevent early sensitization to food- and airborne allergens and consolidation of potentially dangerous memory Th2 cells, antigen specific induced Tregs are important especially in the neonatal period. Therefore, we investigated if cord blood (CB) derived naive CD4<sup>+</sup> T cells are more prone to differentiate into FOXP3<sup>+</sup> regulatory T cells than adult blood (AB) derived naive CD4<sup>+</sup> T cells.

**Methods:** CB or AB derived, MACS sorted CD4<sup>+</sup>CD25<sup>+</sup>CD45RO<sup>+</sup> T cells were stimulated with plate bound (pb) anti-CD3 and soluble anti-CD28 or with pb anti-CD3 and antigen-presenting cells (APCs). After 6 days of culture FOXP3 levels were evaluated by FACS. For suppression assays, proliferative responses were calculated as the mean [<sup>3</sup>H] thymidine incorporation of triplicate wells. Cytokine production was measured with Luminex and Flow cytometry.

**Results:** When naive CD4<sup>+</sup>CD25<sup>+</sup>CD45RO<sup>+</sup> T cells were stimulated with anti-CD3 and anti-CD28 without APCs, we observed no difference in induction of FOXP3<sup>+</sup> Tregs in CB or HC (mean 7.1, ±2.5 SD versus 4.4 ±2.8 SD). Cultured with APCs, a significantly higher proportion of CB derived T cells expressed FOXP3 compared

to HC (mean 11.6,  $\pm 6.1$  SD versus 5.4,  $\pm 4.0$  SD,  $p < 0.01$ ). These FOXP3+CD4+ T cells express all phenotypic markers determined for Tregs [GITR, CTLA-4, CD39 positive and CD127low] and induce a dose dependent suppression of T cell proliferation in classical in vitro suppression assays. Neonatal monocytes were identified as one of the responsible APC subtype to facilitate this in vitro induction of FOXP3.

**Conclusion:** Neonatal monocytes direct naive neonatal T cells in CB into CD4+CD25<sup>high</sup>FOXP3+ Tregs. Interpretation: An increased sensitivity to Th2 and Tregs polarizing conditions might counterbalance potential harmful Th1 responses in neonates. We are investigating the importance of cytokine production and activation status of neonatal monocytes and dendritic cells.

**PB06/84 REGULATORY T CELLS CONTROL CNS-INFILTRATION OF AUTOACTIVE T CELLS DURING VIRAL INFECTION WITHOUT AFFECTING THE ANTIVIRAL IMMUNE RESPONSE**

L. Cervantes-Barragan<sup>1</sup>, S. Firner<sup>1</sup>, C. Prodigier<sup>2</sup>, T. Sparwasser<sup>3</sup>, A. Waisman<sup>4</sup>, I. Bechmann<sup>2</sup>, V. Thiel<sup>1</sup>, B. Ludewig<sup>1</sup>

<sup>1</sup>Institute of Immunobiology, St Gallen, Switzerland, <sup>2</sup>Institute of Clinical Neuroanatomy, Johann Wolfgang Goethe-University, Frankfurt am Main, Germany, <sup>3</sup>Institute for Medical Microbiology, Immunology, and Hygiene Technische Universität München, Munich, Germany, <sup>4</sup>Medical Department, Johannes Gutenberg-University Mainz, Mainz, Germany

Regulatory T cells are essential for suppressing immune responses to autoantigens and therefore help to prevent autoimmunity. During viral infections in the CNS, an indiscriminate regulation of T cells can prevent autoimmune diseases but could also impair the control of viral replication. We analyzed here the impact of regulatory T cells in the control of T cell infiltration to the CNS, virus-induced CNS pathology, and viral clearance using the mouse hepatitis virus (MHV) A59 intranasal infection model; a virus infection that leads to encephalitis and demyelination. MHV infection of "depletion of regulatory T cell" (DEREG) mice, where regulatory T cells can be transiently depleted by diphtheria toxin injection, revealed that the lack of regulatory T cells during MHV infection leads to an increased T cell infiltration and pathology in the CNS. However, antiviral T cells response were not affected by the depletion of FoxP3+CD4+ T cells indicating that regulatory T cells control infiltration of T cells to the CNS without impairing or delaying the antiviral immune response. Moreover, in MHV infected mice, adoptively transferred-myelin oligodendrocyte glycoprotein (MOG<sub>35-50</sub>)-specific CD4+ T cells proliferated in cervical lymph nodes and migrated to the CNS, suggesting that MHV infection in the CNS induces the activation of self-reactive T cells which are controlled by regulatory T cells to reduce the risk of developing inflammatory CNS disease.

**PB06/85 GRANZYME B/PERFORIN DEPENDENT CYTOTOXIC ACTIVITY OF IL-10 PRODUCING TYPE 1 REGULATORY T CELLS**

C.F. Magnani<sup>1,2</sup>, M.G. Roncarolo<sup>1,3</sup>, S. Gregori<sup>1</sup>

<sup>1</sup>San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET), Division of Regenerative Medicine, Stem Cells and Gene Therapy, San Raffaele Scientific Institute, Milan, Italy, <sup>2</sup>Università di Milano-Bicocca, Milan, Italy, <sup>3</sup>Vita-Salute San Raffaele University, Milan, Italy

**Objectives:** CD4+ type 1 regulatory T (Tr1) cells are important players in modulating immune responses to antigens (Ags), including self-Ags, and allo-Ags. Tr1 cells are characterized by high production of IL-10, and the ability to suppress T-cell mediated responses mainly via IL-10 and TGF- $\beta$ .

Recent reports show that perforin and granzyme B are involved in the immunosuppressive mechanism of regulatory T cells generated by activation of naive CD4+ T cells with CD3/CD46 cross-linking (Grossman W.J. et al., Immunity 2004).

To explore whether *bona fide* Tr1 cells utilize granzyme B to suppress immune responses, we examined expression, release, and function of granzyme B in Tr1 cell lines and cell clones.

**Methods:** Tr1 cell lines were differentiated in the presence of IL-10 and IFN- $\alpha$  using artificial APC consisting in murine L-cells co-transfected with hCD32, hCD80, and hCD58 (Levings M.K. et al., JI 2001). Tr1 cell clones were isolated from peripheral blood of normal donors using limiting dilution and polyclonal stimulation.

**Results:** Tr1 cell lines and cell clones express higher levels of granzyme B compared to Th0 cell lines and cell clones. Granzyme B expression correlates with the presence of IL-10 in the culture. Tr1 cell lines, enriched in IL-10 producing T cells, spontaneously secrete granzyme B. Both Tr1 cell lines and cell clones specifically lyse unprimed target cells of monocytic origin. The cytotoxic activity correlates with the expression of the degranulation marker CD107a and is perforin- and granzyme B-dependent.

**Conclusion:** Overall, our results demonstrate that granzyme B together with perforin play an important role in the mode of action of Tr1 cells. Further studies are ongoing to dissect the receptor-ligand recognition involved in this mechanism.

**PB06/86 EFFECTOR T CELLS LIMIT THEIR OWN-PATHOGENICITY BY ACTIVATING REGULATORY T CELLS**

E. Grinberg<sup>1</sup>, D. Saadoun<sup>1</sup>, A. Baeyens<sup>1</sup>, F. Billiard<sup>1</sup>, S. Gregoire<sup>1</sup>, N. Derian<sup>1</sup>, J. Goldstein<sup>1</sup>, D. Klatzmann<sup>1</sup>, G. Marodon<sup>1</sup>, E. Piaggio<sup>1</sup>, B.L. Salomon<sup>1</sup>

<sup>1</sup>UMR7211 (UPMC/CNRS) U959/INSERM), Paris, France

The development of an autoimmune process depends on the balance between autoreactive effector T cells (Teffs) and CD4+CD25+FOXP3+ regulatory T cell (Treg) responses. Here, we identify a novel helper function of CD4+ Teffs, exerted on Tregs, with an impact on this balance. We show that Teff activation boosts the expansion and suppressive activity of Tregs. This novel function of helper CD4+ T cell was observed when both Teffs and Tregs were stimulated in the same lymph nodes. The physiological impact of this Treg boost was assessed in autoimmune diabetes. As opposed to mice transferred with islet-specific Tregs alone, mice co-transferred with islet-specific Teffs and Tregs were protected from a subsequent challenge with diabetogenic Teffs. Thus, diabetogenic Teffs had paradoxical protective effects on diabetes when combined with Tregs. Consequently, Treg-mediated suppression is strengthened when most needed, as under risk of autoimmunity due to strong autoreactive Teff activation. This newly described feedback regulatory loop between Teffs and Tregs, which is interleukin-2 independent, may be crucial to limit the development of autoimmune diseases.

**PB06/87 ASSOCIATION OF T REGULATORY CELLS IN ADENOTONSILS WITH PNEUMOCOCCAL CARRIAGE AND CELLULAR RESPONSES TO A CANDIDATE PNEUMOCOCCAL WHOLE CELL VACCINE IN CHILDREN**

Q. Zhang<sup>1</sup>, R. Malley<sup>2</sup>, A. Finn<sup>3</sup>

<sup>1</sup>University of Liverpool, Division of Immunology, Liverpool, United Kingdom, <sup>2</sup>Boston Children's Hospital, Boston, United States, <sup>3</sup>University of Bristol, Bristol, United Kingdom

**Background:** T regulatory cells (Tregs) regulate immune tolerance and possibly the control of infectious diseases. Little is known about their role in the regulation of immune responses to pneumococcus. We have investigated the effect of Tregs on CD4+ T cell responses to a candidate ethanol-killed unencapsulated pneumococcal whole cell vaccine (WCV) and associated cytokine production in adenotonsils, part of nasal-associated lymphoid tissue (NALT) in children.

**Methods:** Intracellular Foxp3 and surface CD4/CD25 expression were analysed by flow cytometry in mononuclear cells (MNC) isolated from adenotonsils and peripheral blood (PBMC) from children (n=16, 2-8y) undergoing adenotonsillectomy. Nasopharyngeal swabs were cultured for pneumococcus. MNC or PBMC depleted of Treg were co-cultured with WCV. Cellular proliferation was measured using CFSE and flow cytometry. Production of Th1/Th2 Cytokines was measured using a cytometric bead array and IL17 measured by ELISA.

**Results:** An age-dependent increase in numbers of Tregs (Foxp3+) in adenotonsillar MNC (range 3.2-12.5% of CD4+ T cells) was noted although no such trend was observed in PBMC (range 2.9-7.3%). CD25<sup>high</sup> expression correlated well with Foxp3 expression in CD4+ cells. Within the same age groups, higher numbers of Tregs in adenotonsillar MNC ( $p < 0.05$ ) were found in children colonised with pneumococcus. Depletion of Tregs from MNC significantly increased effector CD4+ T cell proliferation following WCV stimulation ( $p < 0.01$ ) with significant increases in IFN $\gamma$ , TNF $\alpha$  and IL17 concentrations ( $p < 0.01$ ) while replacement of Tregs inhibited such responses. Pre-incubation with anti-IL10 antibody reduced the Treg-mediated inhibitory effect. WCV induced proliferation of both effector and regulatory T cells.

**Conclusion:** Treg numbers in adenotonsils of children increase with age and are associated with pneumococcal carriage. Adenotonsillar Tregs are functional and suppress effector T cellular responses and proinflammatory cytokine production. Tregs may play an important role in the regulation of mucosal response in the upper respiratory tract and have significant effect on pneumococcal carriage.

**PB06/88 REGULATORY T CELLS, IMMUNE ACTIVATION AND APOPTOSIS ARE INVOLVED IN PERSISTENTLY REDUCED CD4 COUNTS IN HIV-INFECTED IMMUNOLOGICAL NON-RESPONDERS**

S. Parisotto<sup>1</sup>, S. Picconi<sup>2</sup>, M. Borelli<sup>1</sup>, C. Magni<sup>2</sup>, A. Capetti<sup>2</sup>, P. Meraviglia<sup>2</sup>, G. Dedivitis<sup>2</sup>, G. Rizzardini<sup>2</sup>, D. Trabattini<sup>1</sup>, M. Clerici<sup>1,3</sup>

<sup>1</sup>University of Milan, Milano, Italy, <sup>2</sup>Luigi Sacco Hospital, I e II Divisione di Malattie Infettive, Milano, Italy, <sup>3</sup>Don C. Gnocchi ONLUS Fndn IRCCS, Milano, Italy

CD4 counts are persistently reduced and CD4 T lymphocytes are functionally-defective in a percentage of HIV-infected, HAART-treated patients (immunological non-responders -INR-). T regulatory cells (Treg), immuneactivation and apoptosis are suggested to play a role in such CD4 count defects. The PD-1/PD-L1 and the Fas/FasL pathways elicit apoptosis of antigen-specific cells; Treg down-regulate T cell function and induce apoptosis by PD1/PD-L1 interaction.

To verify possible role for these mechanisms in INR we enrolled 38 HIV-infected patients with comparable CD4 nadirs and HIV RNA  $< 50$  copies/ml who had undergone HAART for  $> 1$  year. CD4 counts were  $> 500$  cells/ml in 15 patients (full responders -FR-); in 23 other patients (INR) CD4 were  $< 200$  cells/ml. PBMC were stimulated with gag+env or with CMV peptides. Flow-cytometry was used to analyze intra and extracellular PD1 in CD4+ T lymphocytes as well as the expression of PD-L1, TLR2 and TLR4, and of Fas and FasL on immune cells. Treg lymphocytes (CD4+/CD25<sup>high</sup>/Foxp3+), IL-10 production, activation of caspases 8 and 9, and plasmatic LPS concentration were also examined in all patients.

Results showed that in INR compared to FR patients:

- 1) intra- and extracellular PD1 positive CD4+ T lymphocytes,
- 2) Fas- and FasL-expressing CD4+ T lymphocytes,
- 3) Treg and IL-10 production, and
- 4) the percentage of apoptotic cells are increased.

Additionally, in INR patients CD14+ /TLR2+ TLR4+ cells are augmented, plasmatic LPS concentration is higher, and CD4+ T cells are hyperactivated. These increases are seen both in gag+env- and CMV-stimulated PBMC. Notably, PD-L1 expression was comparable in CD14+ and CD19+ cells of both groups of patients. The augmented plasmatic LPS concentration detected in INR could explain immune activation and presence of higher Treg cell function. PD1/PD-L1 interaction results in an increased apoptosis of antigen-specific CD4+ T lymphocytes. IL-10 is also increased in INR, this observation could explain the functional T helper impairments also seen in these patients. Apoptosis plays an important role in the defective immune reconstitution seen in INR.

#### PB06/89 IL-17 PRODUCING CELLS INCREASE IN CD4+ LYMPHOCYTES POPULATION IN BLOOD PRIOR TO OVERT MANIFESTATION OF AGVHD

D. Dlubek<sup>1,2</sup>, E. Jaskula<sup>1</sup>, M. Sedzimirski<sup>2</sup>, E. Turlej<sup>1</sup>, A. Lange<sup>1,2</sup>

<sup>1</sup>Institute of Immunology and Experimental Therapy, Polish Academy of Science, Clinical Immunology, Wrocław, Poland, <sup>2</sup>Lower Silesian Center for Cellular Transplantation with National Bone Marrow Donor Registry, Wrocław, Poland

**Objectives:** aGvHD is a major factor affecting the outcome of hematopoietic stem cells transplantation (HSCT). In this study we focused on the presence of IL-17 producing cells in patients post HSCT in the context of FoxP3 positive lymphocytes and those with IFN $\gamma$  production potential.

**Methods:** We studied the cytoplasmic expression of IL-17, FoxP3 and IFN- $\gamma$  in stimulated PBMC of 30 alloHSCT pts. Fifteen of them developed aGvHD including 9 pts with aGvHD seen at later stage post hematological reconstitution. Stimulated (brefeldin A, ionomycin and PMA) PBMC were stained with CD4, IL-17A, IFN- $\gamma$  and FoxP3 MoAbs and analyzed in CD4+ and CD4- lymphocytes subpopulations.

##### Results:

- (1) Percentage of IL-17 producing lymphocytes was higher in patients at the stage of hematological reconstitution post HSCT as compared to healthy controls,
- (2) CD4+ cells are more frequently IL-17 positive than CD4- lymphocytes, however, both having and lacking CD4 lymphocytes had a higher proportion of IL-17 + cells in patients post HSCT as compared to healthy controls
- (3) contribution of IL-17A+ producing cells to CD4+ lymphocytes increased from 1.16% $\pm$ 0.41 at the day of hematological recovery to 5.16% $\pm$ 2.47 shortly before aGvHD manifestation (p=0.027, Wilcoxon Test) and then decreased to 0.74% $\pm$ 0.27 (p=0.008, Wilcoxon Test) when patients had overt aGvHD, (4) aGvHD manifestation was associated with an increase of FoxP3+CD4+ cells (p=0.01, Wilcoxon Test).

##### Conclusions:

1. Stimulated CD4+ cells had a higher proportion of IL-17 producing cells before but not at the time of overt manifestation of aGvHD likely being marginalized in the inflamed tissues,
2. FoxP3+CD4+ cells increase at the manifestation of aGvHD likely to counter-balance alloreactive stimulation of the immune system.

Supported by Grant 2P05E 037 30 from the Polish Ministry of Science & Higher Education.

#### PB06/90 INDUCTION OF ALLOANTIGEN-SPECIFIC HUMAN T REGULATORY CELLS WITH VASOACTIVE INTESTINAL PEPTIDE

E. Gonzalez-Rey<sup>1</sup>, D. Pozo<sup>2</sup>, P. O. Anderson<sup>3</sup>, M. Delgado<sup>3</sup>

<sup>1</sup>University of Seville, Seville, Spain, <sup>2</sup>CABIMER, Seville, Spain, <sup>3</sup>Institute of Parasitology and Biomedicine Lopez-Neyra, CSIC, Granada, Spain

**Objectives:** Regulatory T (Treg) cells are instrumental in the maintenance of immunological tolerance and Treg cell base immunotherapy is a potential treatment for several immune disorders. Although this approach proved successful in preclinical animal autoimmunity and transplantation, the factors involved in the generation of human antigen-specific Treg cells ex vivo are poorly known. Vasoactive intestinal peptide (VIP) is a potent immunosuppressive neuropeptide that affects both innate and adaptive immunity and is therapeutically effective in various experimental models of inflammatory and autoimmune diseases. Here, we investigate the potential of VIP to promote immune tolerance toward alloantigens

**Methods:** We assessed the inhibitory potential of VIP on the proliferation and cytokine production of PBMCs in an alloantigen-driven system. We next characterized the molecular mechanisms involve in the hyporesponsiveness induced by VIP. Finally, we investigated the potential therapeutic effect of VIP-treated T cells generated in an alloantigen-driven system in a model of acute graft-versus-host disease (GVHD) following allogeneic bone marrow transplantation (BMT).

**Results:** VIP treatment of human CD4+CD25- T cells during in vitro stimulation induces an anergic FoxP3+CD4+CD25high T-cell subset that shows potent regulatory activities against allo-specific effector T cells. VIP seems to directly program the CD4+CD25- T-cell repertoire toward a regulatory phenotype independent on the presence of naturally occurring CD4+CD25+ Tr cells. VIP-tolerant T cells are biochemically characterized by an incapability to progress through G1 to S phase of the cell cycle during stimulation with HLA class II disparate antigen-presenting cells due to defective synthesis of cyclin D3 and cyclin E, defective activation of cyclin-dependent kinase cdk2 and cdk4 and defective down-regulation of the cdk inhibitor p27kip1. VIP-interaction with the VPAC1 receptor and subsequent activation of cAMP/protein kinase A pathway play a major role in all these effects. Noteworthy from a therapeutic point of view, VIP-tolerant T cells protect against acute GVHD in a mouse model of allogeneic BMT.

**Conclusion:** Therefore, the inclusion of alloantigen-specific Tr cells generated with VIP ex vivo in future therapeutic regimens may be a valuable aid in the applicability of bone marrow transplantation and minimize the dependence on nonspecific immunosuppressive drugs.

#### PB06/91 ROLE OF THE STAT FAMILY OF TRANSCRIPTION FACTORS IN THE MODULATION OF THE RESPONSE TO SUPERANTIGENS

C. Juares<sup>1</sup>, J. Manils<sup>1</sup>, G. Civit<sup>1</sup>, E. F. Mateus<sup>1</sup>, M. Agusti<sup>1</sup>

<sup>1</sup>Hospital de la Santa Creu i Sant Pau, Universitat Autònoma de Barcelona, Immunology, Barcelona, Spain

The repeated injection of low doses of the bacterial superantigen staphylococcal enterotoxin B (SEB) is a model of induction of T cell tolerance that could be very useful in the study of the mechanisms responsible for the generation of regulatory T cells (Treg). To give insights into the implication of the JAK-STAT pathways in the generation of Treg in this superantigen model, we have treated mice with low doses of SEB and quantified IL-2, interferon- $\gamma$ , IL-4 and IL-10 in serum. We then analyzed the activation and/or expression of STAT1, STAT3, STAT5A, STAT5B, SOCS1, SOCS3, PIAS1 and PIAS3 in spleen cells. We show in this study that repeated injection of SEB changes the cytokine expression from a Th1 to a Th1 pattern while important changes in the isoform expression and activation of STAT1, STAT3, STAT5A and STAT5B take place. Expression of SOCS1, SOCS3, PIAS1 and PIAS3 increases as a consequence of repeated SEB injection. Collectively, these data demonstrate that changes in the JAK-STAT pathway occur as a consequence of the repeated injection of low doses of SEB and that the analysis of these changes could provide valuable clues to understand the molecular mechanisms involved in the generation of different subsets of regulatory T cells.

#### PB06/92 COMPARATIVE STUDIES ON THE GENE EXPRESSION OF REGULATORY T CELLS BY DNA MICROARRAY ANALYSIS

W.-J. Chiang<sup>1</sup>, B.-L. Chiang<sup>2</sup>

<sup>1</sup>Graduate Institute of Oral Biology, National Taiwan University College of Medicine, Taipei, Taiwan, Republic of China, <sup>2</sup>Graduate Institute of Clinical Medicine, National Taiwan University College of Medicine, Taipei, Taiwan, Republic of China

**Objectives:** Regulatory T cells play an important role in the immune regulation, and their principal function is to exert suppressive activity to maintain the self-tolerance and immune homeostasis. In recent years, in addition to the surface marker CD25, the researchers also found Foxp3 to be the key master factor in naturally occurring CD4<sup>+</sup>CD25<sup>+</sup> T cells. However, there still have many questions about development, differentiation and mechanisms in the Tregs.

**Methods:** We exploited lentivirus system carrying IL-10 gene to infect T cells. Making T cells over-express IL-10 gene and converted it into an IL-10-secreting Tr1-like T cell, the DNA microarray technique was used for gene analysis and compared with the data of Foxp3- and TGF- $\beta$ -transduced T cells derived in our group, then selected some target genes for examination of gene expression in resting Treg or activation after 12 and 24 hours.

**Results:** In our results, there were 37 genes expression in both of the microarray data, narrowed down to 11 genes which we interest and further investigate, such as Gsk3b, Ogt, Dub1, Ptptrj, Ccr2, Lck, Icos, Ptpkr, Slc2a1, Adm and Crem genes. Notably, we also found that the two genes were highly expressed in the data of Foxp3- and TGF- $\beta$ -transduced T cells, and have been considered as specific markers of natural Treg in several reports, such as Gzmb and Nkg7.

**Conclusion:** After real-time PCR analysis, we found that Gzmb and Nkg7 were not highly expressed in Treg as reported in other studies. Instead, Gsk3b, Slc2a1, Icos, Ptptrj and Ogt genes were up-regulated in Treg cells after activation, and the other genes were not much changed either in resting or activated regulatory T cells. In the future, more studies might be needed for further characterization of molecules expressed on regulatory T cells.

#### PB06/93 HLA-G EXPRESSION AND REGULATORY T LYMPHOCYTE-ASSOCIATED GENES EXPRESSION IN ORAL CANCER. CORRELATION WITH CLINICAL VARIABLES

I. Sukhoska<sup>1</sup>, R. Sirera<sup>1</sup>, E. Jantus Lewintre<sup>2</sup>, G. Sarrion<sup>2</sup>, J.V. Bagan<sup>2</sup>, C. Camps<sup>2</sup>

<sup>1</sup>Universidad Politécnica de Valencia, Valencia, Spain, <sup>2</sup>Hospital General Universitario de Valencia, Valencia, Spain

**Background:** HLA-G is a human non-classical MHC molecule, mainly expressed in the trophoblast whose main function is to suppress immunologic activity that allows maternal tolerance to phoetus. On the other hand, infiltrating regulatory T lymphocytes (Treg) could promote peripheral immunotolerance fo oncogenic transformation.

**Methods:** We performed real-time PCR in frozen oral cancer specimens from untreated patients who had undergone surgical resection (n=22) and in normal oral mucosa from healthy subjects (n=10). Samples were processed for mRNA extraction and quantification of gene expression was expressed as relative concentration by an endogenous gene and a cell line. To assess the presence of infiltrating Treg we analysed CTLA-4, Foxp-3, IL-10, TGF-beta, CD4, CD8, CXCR4, CD127 and CD25 and also we determined HLA-G levels. We correlate the expression of immunologic mediators with clinical variables.

**Results:** Patients presented squamous carcinomas of the tongue (n=12) or gum (n=10) and stages ranged from I to IV (stage I=4; II=9; III=4; IV=5). 10 patients presented well-differentiated lesions and the other 12 moderately-differentiated cells. Eight patients received post-surgery chemo and radiotherapy. Our results



show that tumor samples had significant higher expression of the CTLA-4, Foxp-3, TGF-beta and CD127 genes than normal tissue. However, those data showed no correlation between the levels of expression and clinico-pathologic variables. When patients were grouped according to tumor size, there was a trend in the way that bigger tumoral lesions expressed relative higher amounts of Treg. By contrast we could not observe an increase of the expression of HLA-G in patients.

**Conclusions:** Our results reveal that there is an increase in the expression of Treg in oral cancer patients. These Treg in tumoral tissues might contribute to the impairment of immunological rejection of the neoplastic transformation. Conversely we have not been able to demonstrate tumoral expression of HLA-G as a strategy to escape from immunosurveillance. Further analysis of this cells and their function is important in order to develop new therapeutic strategies.

#### PB06/94 CHARACTERIZATION OF CMV-SPECIFIC REGULATORY T CELLS AND THEIR INFLUENCE ON ADOPTIVE T CELL THERAPY

A.M. Fischer<sup>1</sup>, S. Zwinger<sup>1</sup>, P. Reinke<sup>2</sup>, H.-D. Volk<sup>1,2</sup>

<sup>1</sup>Charité – University Hospital Berlin, Medical Immunology, Berlin, Germany, <sup>2</sup>Berlin Brandenburg Center for Regenerative Therapies (BCRT), Berlin, Germany

Cytomegalovirus (CMV) is a major cause of mortality after transplantation. In haematopoietic stem cell transplantation adoptive immunotherapy with healthy donor-derived T cells directed against CMV has been proven to prevent clinical manifestation of the virus. To introduce this promising treatment also in the setting of solid organ transplantations, autologous T cell lines of organ recipients suffering from CMV disease are generated in our group. Phenotypical and functional analysis revealed that these CMV-specific T cell lines also contain regulatory T cells (Treg). Aim of this study is to delineate regulatory T cells that are induced upon cytomegalovirus infection, as these are thus far not characterized.

CD4<sup>+</sup> CD25<sup>high</sup> T cells that are obtained from *in vitro* expansion cultures of IFN $\gamma$ -producing T cells specific for immunodominant CMV proteins are able to inhibit IL-2 secretion of effector T cells of the same specificity after triggering, what substantiates their functional activity as Tregs. Multicolor flow cytometry is used to compare expression of receptors on CMV-specific Tregs and natural Tregs. In order to reveal potential unique markers, microarray analysis are performed to contrast expression of antigen-stimulated and -unstimulated specific CD25<sup>high</sup>, CD25<sup>-</sup> T cells and natural Tregs. First results showed the expected up-regulation of cell cycle and proliferation pathways upon stimulation, but also hints for proteins regulated more specifically in stimulated Tregs. As another approach to differentiate between the CD25<sup>high</sup> and -low subset, DNA methylation analysis of the FOXP3 and CAMTA1 locus were performed. It has been shown that natural Tregs can be distinguished from activated FOXP3<sup>+</sup> conventional T cells as the former are completely demethylated at these loci. So far no significant differences in the epigenetic pattern could be detected. Furthermore, we investigate if these specific Tregs suppress proliferation of effector cells via soluble factors like e.g. TGF $\beta$  or via contact-dependent mechanisms.

The precise delineation of this CD4<sup>+</sup> CD25<sup>high</sup> expanded Treg subset is of high interest in order to improve the *in vitro* and *in vivo* efficacy of CMV-specific T-cell lines, i.e. their potency to induce an anti-viral immune response in patients, by understanding the influence of viral-induced suppressive Tregs on transferred effector T cells.

#### PB06/95 TLR STIMULATION PREVENTS MODULATION OF B CELL RESPONSES BY CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> REGULATORY T CELLS

T. Adjobimey<sup>1</sup>, K. Arndts<sup>1</sup>, L. Layland<sup>1</sup>, A. Hörauf<sup>1</sup>

<sup>1</sup>Institute of Medical Microbiology, Immunology and Parasitology (IMMIP), Bonn, Germany

Regulatory T cells have been shown to modulate the adaptive immune system by inducing B-cells to secrete the non complement fixing immunoglobulin G4 (IgG4). Although Toll-like receptors (TLRs) are known to be implicated in the initiation of innate immune responses, their implication in the control of adaptive immunity by inducing co-stimulatory signals on T cells, B cells or dendritic cells is now well established. In addition, emerging data suggest that activation of certain tumor necrosis factor (TNF) receptor family members such as GITR, OX40 and 4-1BB or the triggering of TLRs can reverse suppression mediated by Treg. We investigate here the effects of triggering three different TLRs (TLR2, TLR4 and TLR9) on the ability of CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> CD127<sup>dim</sup> regulatory T cells to modulate the immunoglobulin production of activated B cells. Interestingly, activated Treg reduce the total IgG production by B cells but increased the ratio of IgG4/IgG and IgG4/IgE in favor of IgG4. Most interestingly, additional stimulations with LPS (TLR4) and CpG (TLR9) but not Pam3Cys (TLR2) reversed the ability of Treg to modulate B cell responses. This modulation of B cell activity by Treg was only observed when the B cells were pre-incubated with TLR ligands but not when the Treg were pre-stimulated. Furthermore, these effects of TLR ligands correlated with an up-regulation of IL-17 and other pro-inflammatory cytokines and an increase of ROR $\gamma$ T expression in the Treg populations.

#### PB06/96 INDUCTION OF THYMOCYTES WITH SUPPRESSIVE ACTIVITY BY THE RAT THYMIC NURSE CELL LINE IN VITRO

D. Vucevic<sup>1</sup>, S. Vasiljic<sup>1</sup>, D. Vucevic<sup>1</sup>, I. Majstorovic<sup>1</sup>, B. Draskovic-Pavlovic<sup>1</sup>, M. Colic<sup>1</sup>

<sup>1</sup>Institute for Medical Research, Military Medical Academy, Belgrade, Serbia

Regulatory cells (Treg) have been shown to develop in the thymus, although the cellular and molecular mechanisms responsible for their generation are incompletely defined. Accumulated findings indicate that the thymic epithelial cells (TEC) have been implicated in the induction and/or selection of Treg cells in the thymus. We have established a rat cortical TEC line R-TNC.1 with nursing activity. In our previous studies, we have demonstrated that cocultivation of syngeneic thymocytes (AO rats) with R-TNC.1 line (primary culture) induces the generation of thymocytes (E-Treg) which suppress ConA-induced proliferation of responder syngeneic thymocytes (secondary culture).

**Objectives:** This study describes further phenotypic and functional characterization of E-Treg cells.

**Methods:** Experiments were performed on inbred AO rats. Phenotypic analysis of thymocytes was performed by flow cytometry. Cell proliferation was measured by incorporation of <sup>3</sup>H thymidine. Cytokine production was measured by ELISA.

**Results:** In this study we assessed the expression of forkhead box protein p3 (Foxp3) transcription factor, as a critical factor for the development and function of Treg. We found that the frequencies of Treg and the expression of Foxp3 were higher in E-Treg in comparison with control (thymocytes cultivated alone). We did not find any significant differences in the level of expression of a number of surface markers tested (CD45RC, CD53, CD11a, CD54, CD18, MHC I, MHC II, CD80, CD86, CD90, CD71), except for CD28, which was down regulated, and CD62L which was up regulated in E-Treg, compared to controls. In the presence of ConA, E-Treg did not proliferate, IL-2 production was decreased, and they were arrested in G0/G1 phase of cell cycle. Using a transwell system we demonstrated that direct cell-to-cell contact is necessary for the E-Treg cell induction. The suppressive capacity of E-Treg cells could be dependent on IL-10, since we detected increased production of IL-10 in the secondary culture.

**Conclusion:** In conclusion, our results support a potential role of thymic nurse cells in the generation of T cells with regulatory activity.

#### PB06/97 EVIDENCE OF A ROLE OF T REGULATORY CELLS MEDIATING ATHEROPROTECTION BY APO B 100 PEPTIDE IMMUNIZATION

M. Wägren<sup>1</sup>, D. Bengtsson<sup>1</sup>, P. Dunér<sup>1</sup>, H. Björkbacka<sup>1</sup>, G.N. Fredrikson<sup>1</sup>, J. Nilsson<sup>1</sup>

<sup>1</sup>Lund University, Clinical Sciences, Malmö, Sweden

**Objective:** Immunization with apo B-100 derived peptides has been shown to inhibit atherosclerosis development in mice. The immune mechanism mediating the protection is however not yet fully understood. The aim of the present study was to elucidate the role of CD25 positive T cells in the atheroprotection.

**Methods:** Apo E deficient mice were immunized with an apo B-100 peptide at 6, 9, 11 and 24 weeks of age. Mice were fed a high fat diet from 10 weeks and were given weekly injections of neutralizing CD25 antibodies from 6 to 11 weeks of age and one week before the sacrifice at 25 weeks of age. Atherosclerosis was determined by Oil Red O staining of the descending aorta. The T cell population in the spleen was analyzed with flow cytometry.

**Results:** Immunization with the apo B-100 peptide reduced atherosclerosis with 36% (P=0.05). Coadministration of CD25 antibody completely blocked the protective effect of the immunization (2.28 $\pm$ 1.03% vs. 1.27 $\pm$ 0.69%; P=0.015). Peptide immunization increased the regulatory T cell population expressing CD4CD25Foxp3 compared to control (13.51 $\pm$ 1.73% vs. 1.65 $\pm$ 0.39% of CD4 cells; P<0.0001), whereas CD25 antibody treatment of immunized mice reduced the population (0.70 $\pm$ 0.45% vs. 13.51 $\pm$ 1.73% of CD4 cells; P<0.0001).

**Conclusion:** Immunization with an apo B-100 peptide induced an atheroprotective immune response. By neutralizing CD25 in immunized mice the protection was blocked, indicating that regulatory T cells play an important role in the atheroprotection induced with apo B-100 peptide immunizations.

**Funding:** The Swedish Medical Research Council and the Swedish Heart-Lung foundation.

#### PB06/98 RAPID REGULATORY T-CELL RESPONSE PREVENTS CYTOKINE STORM IN CD28 SUPERAGONIST TREATED MICE

T. Gogishvili<sup>1</sup>, D. Langenhors<sup>1</sup>, F. Lühder<sup>2</sup>, F. Elias<sup>1</sup>, K. Elflein<sup>1</sup>, K.M. Dennehy<sup>3</sup>, R. Gold<sup>4</sup>, T. Hünig<sup>1</sup>

<sup>1</sup>University of Würzburg, Institute for Virology and Immunobiology, Würzburg, Germany, <sup>2</sup>University of Göttingen and Gemeinnützige Hertie-Stiftung, Institute for Multiple Sclerosis Research, Göttingen, Germany, <sup>3</sup>Eberhard Karls University, Institute for Cell Biology, Department of Immunology, Tübingen, Germany, <sup>4</sup>Ruhr University Bochum, Department of Neurology at St. Josef Hospital, Bochum, Germany

Superagonistic CD28-specific monoclonal antibodies (CD28SA) are highly effective activators of regulatory T-cells (Treg cells) in rats, but a first-in-man trial of the human CD28SA TGN1412 resulted in an unexpected cytokine release syndrome. Using a novel mouse anti-mouse CD28SA, we investigated the relationship between Treg activation and systemic cytokine release.

Our results using a mouse-anti mouse CD28SA confirm the preferential expansion and activation of Treg over conventional CD4 T-cells. Besides we found that Treg activation by CD28SA was depended on paracrine IL-2 from CD28SA-stimulated conventional T-cells. Additionally, Treg cells recovered from CD28SA-stimulated mice tested *in vitro*, show a more than fivefold higher suppressive activity on a per cell basis than control mice.

Administration of CD28SA to rodents revealed no significant cytokine release, but depletion of Treg cells prior to CD28SA stimulation led to systemic release of pro-inflammatory cytokines, indicating that in rodents, Treg cells effectively suppress the inflammatory response.

In the present study we also tested whether pharmacologic suppression with corticosteroid prophylaxis would be compatible with the desired effect of the mAb, i. e. transient polyclonal Treg activation. We show that neither the expansion nor the functional activation of Treg cells is affected by high-dose dexamethasone sufficient to control systemic cytokine release. Our findings warn that preclinical testing of activating biologicals in rodents may miss cytokine release syndromes due to the rapid and efficacious response of the rodent Treg compartment, and suggest that polyclonal Treg activation is feasible in the presence of antiphlogistic corticosteroid prophylaxis.

#### PB06/99 THE EFFECT OF TNF $\alpha$ ON THE DIFFERENTIATION OF T REGULATORY CELLS

L. Geirsdóttir<sup>1</sup>, I. Skaftadóttir<sup>2</sup>, B. Gunnlaugsdóttir<sup>3</sup>, B. R. Lúdvíksson<sup>2</sup>

<sup>1</sup>University of Iceland, Faculty of Medicine, Reykjavik, Iceland, <sup>2</sup>Landspítali University Hospital, Department of Immunology, Reykjavik, Iceland, <sup>3</sup>Landspítali University Hospital, Center for Rheumatology Research, Reykjavik, Iceland

**Introduction:** CD4+CD25+ regulatory T cells (Tregs) play a critical role in the maintenance of peripheral tolerance and prevention of autoimmunity. The forkhead/winged helix transcription factor FoxP3 has been shown to be essential for the development as well as for the suppressive function of CD4+CD25+ Treg cells. Tumor necrosis factor alpha (TNF $\alpha$ ) is a multifunctional cytokine that induces T cell growth and development. Although, TNF $\alpha$  inhibitors are commonly used as a treatment for many autoimmune diseases such as rheumatoid arthritis (RA) their effect on Treg differentiation has not been clarified.

**Aim:** Estimate the effects of TNF $\alpha$  upon the differentiation of CD4<sup>+</sup>CD25<sup>high</sup>CD127<sup>+</sup>FoxP3<sup>+</sup> Tregs.

**Methods:** PBMCs were isolated from peripheral blood of healthy adults. Isolated PBMCs were grown on a 96 well round bottom plate, and stimulated for three and seven days with immobilized anti-CD3 mAb (5  $\mu$ g/mL) +/- soluble anti-CD28 (10  $\mu$ g/mL) and +/- TGF $\beta$ 1, TNF $\alpha$ , IL-1 $\beta$  and a TNF $\alpha$  inhibitor (infliximab). Treg differentiation was evaluated through the expression of CD4, CD25, CD127, TGF $\beta$ RII and Foxp3 by flow cytometric analysis. Proliferation was assessed through CFSE fluorescence intensity.

**Results:** TNF $\alpha$  did not induce the differentiation of Tregs with or without TGF $\beta$ 1. However, the treatment with anti-TNF $\alpha$  (Infliximab) significantly induced their differentiation after long term cultures whereas this was not apparent following short term cultures regardless of the presence of TGF $\beta$ 1. On the other hand, IL-1 $\beta$  reduced expression of FoxP3 even in the presence of TGF $\beta$ 1 after optimal 7 days stimulatory conditions. Furthermore, TGF $\beta$ 1 completely prevented the proliferation of CD4+ T-cells. In contrast, the presence of TGF $\beta$ 1 significantly enhanced the number of cell cycles Tregs had undergone.

**Conclusion:** The clinical implications of our results suggest that biological agents aimed at TNF $\alpha$  such as Infliximab could be working by expanding the circulatory pool of Tregs. Furthermore, since our result demonstrated that IL-1 $\beta$  significantly prevented the differentiation of Tregs and it has been shown by others that TNF $\alpha$  inhibitors significantly inhibit IL-1 $\beta$  secretion from neutrophils this could be contributing to the differentiation of Tregs. It is tempting to speculate that defects in the above regulatory pathways could be accountable for the immunopathogenesis in some T-cell driven autoimmune diseases (e.g RA).

#### PB06/100 T<sub>REG</sub> CELL EXPANSION INDUCED BY PARTIAL MYELOABLATION AMELIORATES EXPERIMENTAL ARTHRITIS

L. Weng<sup>1</sup>, R. Williams<sup>2</sup>, P. Vieira<sup>3</sup>, G. Screaton<sup>3</sup>, M. Feldmann<sup>2</sup>, F. Dazzi<sup>1</sup>

<sup>1</sup>Imperial College, Hammersmith Hospital, Haematology, London, United Kingdom, <sup>2</sup>Imperial College, Kennedy Institute of Rheumatology Division, London, United Kingdom, <sup>3</sup>Imperial College, Hammersmith Hospital, Immunology, London, United Kingdom

**Objectives:** We have previously demonstrated that partial myeloablation favours the selective retention /expansion of regulatory T (T<sub>reg</sub>) cells. The aim of this study is to test the hypothesis that a similar approach could be exploited for the treatment of experimental arthritis.

**Methods:** Collagen induced arthritis (CIA) was induced in DBA/1 male mice by a single intradermal injection of bovine type II collagen emulsified in CFA. A group of mice was also sublethally irradiated (4Gy) to induce a partial myeloablation prior to CIA induction. Mice receiving or not receiving irradiation were monitored and compared for signs of arthritis. CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Tregs were enumerated in PB. The role of Tregs was assessed by selectively depleting CD25<sup>+</sup> cells.

**Results:** We demonstrated that mild myeloablation was sufficient to protect mice from the development of CIA, since the onset of the disease was much delayed and its severity reduced after the treatment. The procedure is associated with an increment in the proportion of T<sub>reg</sub> cells in the PB and induced suppressive activity against collagen-specific T cells in vitro. The removal of T<sub>reg</sub> cells in vitro abolished the inhibitory activity on T cell proliferation. Furthermore, the in vivo depletion of CD25<sup>+</sup> before irradiation eliminated the protective effect of myeloablation, thus indicating a causative effect of T<sub>reg</sub> cells in the prevention of CIA. Irradiation was equally efficacious when used when CIA has developed.

**Conclusion:** Partial myeloablation ameliorates CIA by inducing a retention/expansion of T<sub>reg</sub> cells. These findings have important implications for the therapeutic design of autoimmune disease.

#### PB06/101 ENDOGENOUS NAD<sup>+</sup> DERIVED FROM INJURED CELLS MEDIATES PS EXTERNALIZATION AND CD62L SHEDDING ON MURINE REGULATORY T CELLS

B. Rissiek<sup>1</sup>, S. Hubert<sup>2</sup>, N. Schwarz<sup>1</sup>, S. Adriouch<sup>1,2</sup>, F. Scheuplein<sup>1,3</sup>, F. Haag<sup>1</sup>, M. Seman<sup>2</sup>, F. Koch-Nolte<sup>1</sup>

<sup>1</sup>University Clinic Hamburg-Eppendorf, Hamburg, Germany, <sup>2</sup>INSERM, Rouen, France, <sup>3</sup>The Jackson Laboratory, Bar Harbor, United States

NAD and ATP are universal currencies of energy metabolism found in all kingdoms of life. These nucleotides play important roles also as signaling molecules in the extracellular environment. It has been proposed that NAD and ATP released from lysed cells alert cells of the immune system to tissue damage. Here we show that foxp3+ regulatory T cells (Tregs) are equipped with numerous sensors for extracellular NAD and ATP, including nucleotide-metabolizing ecto-enzymes and nucleotide receptors. Extracellular NAD and ATP trigger the shedding of L-selectin (CD62L) and the externalization of phosphatidylserine (PS) on murine Tregs. These events depend on the P2X7 ion channel. While ATP acts as a soluble ligand to activate P2X7, gating of P2X7 by NAD requires ecto-ADP-ribosyltransferase ART2-catalyzed transfer of the ADP-ribose moiety from NAD onto Arg125 of P2X7 (1). We find that the routine preparation of peripheral lymph node and spleen cells induces the release of NAD in sufficient concentrations for ART2 to ADP-ribosylate P2X7, even at 4°C (2). Gating of P2X7 occurs when Tregs are returned to 37°C, rapidly inducing CD62L shedding as well as PS-externalization by a substantial fraction of the cells. The "spontaneous" activation of P2X7 on Tregs during preparation of primary cells can be prevented by i. v. injection of ART2-inhibitory single domain antibodies (3) ten min prior to sacrificing mice. supported by DFG grants No310/6-3 and No310/7-1

1. Adriouch S, P Bannas, N Schwarz, R Fliegert, AH Guse, M Seman, F Haag, F Koch-Nolte. 2008. ADP-ribosylation at R125 gates the P2X7 ion channel by presenting a covalent ligand to its nucleotide binding site. *FASEB J* 22:861-869.

2. Scheuplein F, N Schwarz, S Adriouch, C Krebs, P Bannas, B Rissiek, M Seman, F Haag, F Koch-Nolte. 2009. NAD and ATP released from injured cells induce P2X7-dependent shedding of CD62L and externalization of phosphatidylserine by murine T cells. *J. Immunol.* 182(5):2898-9083.

3. Koch-Nolte F, J Reyelt, B Schössow, N Schwarz, F Scheuplein, S Rothenburg, F Haag, V Alzogaray, A Cauerhff, FA Goldbaum. 2007. Single domain antibodies from llama effectively and specifically block T cell ecto-ADP-ribosyltransferase ART2.2 in vivo. *FASEB J* 21:3490-8.

#### PB06/102 A MURINE MODEL OF POLYMICROBIAL SEPSIS: IS THERE A ROLE FOR TREGS?

M. Rath<sup>1</sup>, C. Pötschke<sup>2</sup>, K. Cziupka<sup>2</sup>, J. Hühn<sup>3</sup>, T. Sparwasser<sup>4</sup>, B.M. Bröker<sup>1</sup>

<sup>1</sup>E.-M. Arndt University Greifswald, Institute of Immunology and Transfusion Medicine, Greifswald, Germany, <sup>2</sup>E.-M. Arndt University Greifswald, Department of Surgery, Greifswald, Germany, <sup>3</sup>Helmholtz-Zentrum für Infektionsforschung, Braunschweig, Germany, <sup>4</sup>TWINCORE, Zentrum für Experimentelle und Klinische Infektionsforschung, Institut für Infektionsforschung, Hannover, Germany

Despite high therapy standards, postoperatively acquired abdominal sepsis due to intestinal leakage remains a life-threatening clinical complication that is associated with a mortality rate of about 30 – 50%.

Because adaptive immunity to the invading pathogens in generalized bacterial infection is poorly understood, we have characterized the T cell response in colon ascends stent peritonitis (CASP), a mouse model that closely mimics the clinical situation. We have recently shown that CD4<sup>+</sup> T cells have a negative effect on the outcome and bacterial clearance in CASP. So we hypothesized that CD4<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells (Treg) might be responsible for the dampening effect the T cells exert on innate defense against invading bacteria.

Using DREG mice with Foxp3-positive Tregs expressing GFP and diphtheria toxin receptors we were able to directly visualize Tregs *ex vivo* and to selectively deplete them before induction of sepsis. We investigated the activation of the T cell compartment in sepsis after depletion of Tregs by measuring expression of activation markers via fluorescence microscopy and flow cytometry. In a second experiment we compared the survival and bacterial dissemination between Treg-depleted and non-depleted animals.

Our data show that splenic Tregs upregulated CTLA-4 and other activation markers within hours after induction of sepsis indicating their rapid systemic activation. Therefore it was unexpected that their depletion had no influence on outcome, bacterial dissemination or the activation of the Foxp3-negative T cells, even though the Treg-depleted animals appeared more sick.

We conclude that in sepsis the immune system is maximally activated so that despite their rapid involvement, regulatory T cells have neither a significant impact on hyperinflammation nor on the early innate immune effector mechanisms.

**PB06/103 CHARACTERIZATION OF T REGULATORY CELLS IN HUMAN PERIAPICAL LESIONS**D. Gazivoda<sup>1</sup>, D. Vucevic<sup>2</sup>, I. Majstorovic<sup>2</sup>, S. Vasiljic<sup>2</sup>, R. Rudolf<sup>3</sup>, Z. Brkic<sup>4</sup>, P. Milosavljevic<sup>2</sup>, M. Colic<sup>2</sup><sup>1</sup>Military Medical Academy, Clinic for Maxillofacial and Oral Surgery, Belgrade, Serbia, <sup>2</sup>Military Medical Academy, Institute for Medical Research, Belgrade, Serbia, <sup>3</sup>University of Maribor, Faculty of Mechanical Engineering, Maribor, Slovenia, <sup>4</sup>Military Medical Academy, Clinic for Stomatology, Belgrade, Serbia**Objectives:** CD4<sup>+</sup>CD25<sup>hi</sup>Foxp3<sup>+</sup> regulatory T cells (Tregs) are of crucial importance in regulating the immune response, including the control of any defense against infection. Their presence in periapical lesions has not been demonstrated, as yet. We hypothesized that Tregs infiltrate periapical lesions, where they inhibit T-cell proliferation. The aim of this study was to characterize Tregs in periapical lesions by confocal microscopy, flow cytometry, and functional assays.**Methods:** Cryostat sections of human periapical lesions were processed for confocal microscopy using antibodies to CD4, CD25, GITR and Foxp3. Periapical lesion mononuclear cells (PL-MNC) were cultivated for 24h with PMA/Ca<sup>2+</sup> ionophore and after that the levels of cytokines (TGF- $\beta$  and IL-10) were determined in supernatants. Peripheral blood (PB)-MNC were used as control cells. Flow cytometry was used for phenotypical analysis of Tregs within PL-MNC and for detection of intracellular TGF- $\beta$  and IL-10. Functional assays were performed to demonstrate the suppressive activity of Tregs *in vitro*.**Results:** We identified CD4<sup>+</sup>CD25<sup>hi</sup>Foxp3<sup>+</sup> cells in periapical lesions by both confocal microscopy and flow cytometry and showed that these cells expressed IL-10 and TGF- $\beta$ . Their frequency was significantly higher than in peripheral blood and correlated with the levels of TGF- $\beta$  and IL-10 in culture supernatants of PL-MNC. Tregs inhibited the proliferation of responder T cells *in vitro* by stimulating the production of IL-10.**Conclusion:** These findings suggest that CD4<sup>+</sup>CD25<sup>hi</sup>Foxp3<sup>+</sup> cells in periapical lesions may play regulatory roles in controlling local immune/inflammatory processes.**PB06/104 REGULATORY T CELLS IN LONG-TERM *BORDETELLA BRONCHISEPTICA* INFECTION**A. Jeron<sup>1</sup>, T. Yevsa<sup>2</sup>, M. Gereke<sup>3</sup>, R. Geffers<sup>3</sup>, D. Bruder<sup>1</sup><sup>1</sup>Helmholtz Center for Infection Research, Immune Regulation, Braunschweig, Germany, <sup>2</sup>Helmholtz Center for Infection Research, Vaccinology, Braunschweig, Germany, <sup>3</sup>Helmholtz Center for Infection Research, Cell and Immune Biology, Braunschweig, Germany**Objectives:** With their ability to counterbalance acute as well as long-lasting immune responses regulatory T cells (Tregs) account for prevention of excessive immune responses potentially leading to immune-pathology at the risk of favouring chronic infections. *Bordetella bronchiseptica* is a close relative of *B. pertussis* and typically infects the respiratory tract of smaller mammals. Interestingly, *B. bronchiseptica* was shown to interfere with the hosts Tregs by several direct and indirect mechanisms contributing to the pathogen's persistence. Therefore we studied alterations of the Tregs in *B. bronchiseptica* infected mice throughout the course of infection. By assessing Treg properties, functions and molecular patterns we aimed to define pathogen associated Treg responses accompanied with long-term infection.**Methods:** Infection of BALB/c mice was performed by intra nasal administration of a sublethal dose of  $5 \times 10^5$  CFU. The frequency of Tregs as well as changes within the Treg population in different compartments of diseased mice (e.g. cervical LN, bronchial LN, lung, spleen), was typically monitored over a period of 6 weeks by flow cytometry, CFSE and <sup>3</sup>H-based suppressor/proliferation assays. To better define changes of the molecular pattern of Tregs in infected animals microarray analysis of sorted CD4<sup>+</sup>CD25<sup>+</sup> Tregs from spleen was performed and expression of assorted genes was confirmed by real-time RT-PCR.**Results:** Flow cytometric analysis reveals only minor changes in expression of known Treg markers (e.g. FOXP3, CTLA4, PD-1 etc.) within the CD4<sup>+</sup>CD25<sup>+</sup> Tregs during infection. However, microarray analysis of Tregs, especially from late time-points of infection, clearly shows alterations on the transcriptional level, as compared to non-infected controls as well as earlier time-points.**Conclusion:** It is still unclear if pathogen induced Tregs can be distinguished from conventional steady-state Tregs. Nevertheless, specific targeting of pathogen-induced Tregs seems to be promising for re-strengthening the immune response against a persisting pathogen and to clear the pathogen from the host without affecting the entire Treg population. Therefore, the functional meaning of changes found in the expression profiles of Tregs in the late infection phase will be further investigated.**PB06/105 SCLUSTERIN IN TUMOR T CELL INFILTRATE: A NEW ROLE IN TUMOUR IMMUNE ESCAPING?**S. Pucci<sup>1</sup>, P. Mazzarelli<sup>1</sup>, L. G. Spagnoli<sup>1</sup><sup>1</sup>University of Rome Tor Vergata, Biopathology, Rome, Italy**Objectives:** Clusterin is an appealing pleiotropic protein with a broad range of functions. CLU appears to act as a tumor suppressor and can yet work as an oncogene, depending on the cell state. This diverse set of functions can be attributed to the existence of two alternatively-spliced forms of the CLU gene encoding secretory CLU (sCLU) or nuclear CLU. The induced sCLU form in cancer cells is cytoprotective and inhibiting its pro-survival function is the basis of current Phase I/II clinical trials in prostate and breast cancers. TGF $\beta$  and IL-6, that play an important role in the development and the prognosis of colorectal cancer, influence the transcription of this protein. We have already shown the influence of these cytokines in sCLU induction in cancer cells. We demonstrated that in colorectal cancer (CRC) the pro-apoptotic nCLU is lost, while there is an increasing production of pro-survival sCLU within the tumoral cells. Recent studies have highlighted the role of TGF $\beta$  in suppression of T cell mediated tumor immunity. In fact it has been indicated to play a role in the induction of peripheral Treg population. Clusterin promoter is influenced directly by TGF $\beta$ , through the activation of the transcription factor AP1. In the present study we characterized sCLU expression in T tumor cell infiltrate and the potential role of this tumor pro-survival factor in T cell population.**Methods:** In human CRC (n=50) at different stages of disease, we characterized the T cell infiltrate and the expression of clusterin by immunohistochemistry. Blood and tissues regulatory Foxp3<sup>+</sup> T cells from 10 CRC patients were isolated and sClusterin expression was evaluated.**Results:** In colon carcinoma we observed that the increased level of TGF $\beta$  in the tumor microenvironment, induce as previously demonstrated in colon cancer cells, the upregulation of sClusterin expression also in CD4<sup>+</sup> CD8<sup>+</sup> T cell infiltrate associated with a great suppressive regulatory cell function.**Conclusion:** Thus the secreted CLU in CRC may play an intriguing role as pro survival factor in cancer cells, suggesting its potential involvement in tumor immune evasion. Further study are in progress in order to evaluate the potential role of clusterin in CD4<sup>+</sup> and CD8<sup>+</sup> Foxp3 cells.**PB06/106 THE SYNERGY BETWEEN IDO METABOLITES AND IMMUNOSUPPRESSIVE FACTORS MEDIATES IMMUNOSUPPRESSION BY TISSUE STEM CELLS**A. L'Huillier<sup>1</sup>, G. Ren<sup>1</sup>, L. Zhang<sup>1</sup>, Y. Shi<sup>1</sup><sup>1</sup>University of Medicine and Dentistry of New Jersey, Molecular Genetics and Microbiology, Piscataway, United StatesIt has previously been shown that IDO has immunosuppressive effects *in vitro* as well as *in vivo*, though the mechanisms are not well defined. Traditionally this effect has been attributed to tryptophan starvation, though other models such as the bioactivity of tryptophan metabolites have been proposed. IDO is thought to play a role both in natural immune regulation as well as in immune escape by tumors. We have used a stem cell model to investigate the mechanisms of IDO mediated immunosuppression. Human CD4<sup>+</sup> T cells isolated from human PBMCs were co-cultured with human stem cells producing IDO, or IDO metabolites, and were supplemented with known immunosuppressive factors. To determine the effects of the interactions of stem cells and T cells via IDO, apoptosis and T regulatory cell induction were measured. Apoptosis was determined using PI staining, while T regulatory cell induction was measured with three color flow cytometric analysis.**Results:** We have found that tryptophan metabolites produced by IDO, in combination with immunosuppressive factors such as TGF- $\beta$ , Retinoic Acid, and PGE2, can induce T cell apoptosis, as well as induce CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> T regulatory cell differentiation. Through these mechanisms stem cells can use IDO to be both directly and indirectly immunosuppressive. These results suggest that immune reactions can be prevented, and immune tolerance generated through exposure of T cells to IDO metabolites and immunosuppressive factors, having implications of treating autoimmune diseases, organ transplants, and as a target for preventing immune evasion by tumors.**PB06/107 THE ROLE OF CD4<sup>+</sup> FOXP3<sup>+</sup> REGULATORY T CELLS WITHIN ACUTE MURINE INFLUENZA A VIRUS INFECTION**R.J. Betts<sup>1</sup>, D.M. Kemeny<sup>1</sup><sup>1</sup>National University of Singapore, Immunology Programme, Singapore, SingaporeInfluenza A virus is an inactivated, negative-sense single stranded RNA virus, resulting in an acute infection of the respiratory system which is responsible for considerable morbidity and mortality. In addition to being the subject of study due to the clinical syndrome induced by infection, influenza A virus is also studied as a prototypical respiratory virus for greater understanding of the immunology of the airways. While several aspects of the adaptive immune response to influenza A virus are well understood, the role of CD4<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells (Treg) in influenza A infection is poorly defined. This study sought to identify and define any Treg responses during primary influenza A infection and examine the physiological effects of this suppressive T cell subset. C57BL/6 mice were infected with 5pfu of Influenza A PR/8/34 virus and their responses to the virus was examined. Regulatory T cells are apparent within BAL fluid and lung tissue at day 5 post-inoculation in parallel with the induction of the CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses, with Treg numbers peaking at day 7 post-inoculation, coinciding with peak pulmonary IL-10 levels and when viral clearance mostly completed. BAL Treg numbers subsequently fell while lung Treg numbers remain static, despite the overall CD4<sup>+</sup>, CD8<sup>+</sup>, antigen-specific CD8<sup>+</sup> and activated CD8<sup>+</sup> T cell responses peaking at day 11 post-inoculation. These findings demonstrate infection with a prototype respiratory virus results in a robust induction of regulatory arms of the immune system in conjunction with a strong adaptive immune response. Intra-tracheal adoptive transfer of TGF-induced Foxp3<sup>+</sup> Tregs resulted in 7.5-fold increase in IL-10 levels within BAL fluid, although BAL levels of pro-inflammatory mediators IFN- $\gamma$  and TNF- $\alpha$  remain unchanged. These findings collectively suggest a potential anti-inflammatory and immunoprotective role of Foxp3<sup>+</sup> regulatory T cells within influenza A virus infection.



**PB06/108 RAPAMYCIN, UNLIKE CYCLOSPORINE A, ENHANCES SUPPRESSIVE FUNCTIONS OF *IN VITRO* INDUCED CD4<sup>+</sup>CD25<sup>+</sup> TREGS**

K. Bocian<sup>1</sup>, J. Borysowski<sup>2</sup>, P. Wierzbicki<sup>2</sup>, J. Wyzga<sup>3</sup>, D. Klosowska<sup>2</sup>, A. Białoszewska<sup>1</sup>, L. Pączek<sup>3</sup>, A. Górski<sup>2</sup>, G. Korczak-Kowalska<sup>1,2</sup>  
<sup>1</sup>University of Warsaw, Department of Biology, Faculty of Immunology, Warsaw, Poland, <sup>2</sup>Medical University of Warsaw, Department of Clinical Immunology, Transplantation Institute, Warsaw, Poland, <sup>3</sup>Medical University of Warsaw, Transplantation Institute, Warsaw, Poland, <sup>4</sup>Medical University of Warsaw, Department of Histology and Embryology, Warsaw, Poland

A growing body of data shows that CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells (Tregs) can induce transplantation tolerance by suppressing immune responses to allograft antigens. However, both the generation and the suppressive capacity of CD4<sup>+</sup>CD25<sup>+</sup> Tregs can be substantially affected by different immunosuppressive drugs used in clinical transplantation. The goal of this study was to compare the effects of cyclosporine A and rapamycin on the induction and suppressive functions of human CD4<sup>+</sup>CD25<sup>+</sup> Tregs *in vitro*.

CD4<sup>+</sup>CD25<sup>+</sup> Tregs were induced in MLR in the presence of rapamycin (Treg-Rapa) or cyclosporine A (Treg-CsA). Tregs were identified by flow cytometry using anti-CD4, anti-CD25, anti-CTLA-4, anti-CD122, anti-GITR, anti-FOXP3 mAbs. Suppressive capacity of induced Tregs was evaluated by their capability to inhibit anti-CD3 Ab-triggered proliferation of PBMCs, as measured by flow cytometry. The concentration of TGF-β1 in culture supernatants was measured by ELISA.

Although both rapamycin and cyclosporine A suppressed the induction of CD4<sup>+</sup>CD25<sup>+</sup> Tregs during MLRs, this effect was significantly more pronounced in cells cultured with cyclosporine. On the other hand, only rapamycin significantly decreased the percentage of CD4<sup>+</sup>CD25<sup>+</sup> Tregs which expressed GITR, a negative regulator of Treg's suppressive capacity. Importantly, Treg-Rapa, unlike Treg-CsA, displayed significant suppressive activity and were capable of inhibiting the proliferation of CD4<sup>+</sup> T cells. This activity was likely mediated by TGF-β1.

Rapamycin, unlike cyclosporine A, doesn't inhibit the generation of functional CD4<sup>+</sup>CD25<sup>+</sup> Tregs. This implies that rapamycin could contribute to the development of transplantation tolerance by promoting the induction of functional CD4<sup>+</sup>CD25<sup>+</sup> Tregs. Moreover, our results suggest beneficial effects of combining rapamycin with Tregs.

**PB06/109 REGULATORY T CELL-MEDIATED MODIFICATION OF LYMPH NODE CHEMOKINE MICROENVIRONMENT**

V. Dal Secco<sup>1</sup>, C. Soldani<sup>1</sup>, F. Asperti-Boursin<sup>2</sup>, E. Donnadieu<sup>2</sup>, A. Viola<sup>3</sup>, A. Sarukhan<sup>1</sup>

<sup>1</sup>Istituto Clinico Humanitas IRCCS, Laboratory of Adaptive Immunity, Rozzano, Italy, <sup>2</sup>Institut Cochin, Université Paris Descartes, Centre National de la Recherche Scientifique, Paris, France, <sup>3</sup>Laboratory of Adaptive Immunity, Department of Translational Medicine, University of Milan, IRCCS Istituto Clinico Humanitas, Rozzano, Italy

Although evidence exists that regulatory T cells (Tregs) can suppress the effector phase of immune responses, it is clear that their major role is in suppressing T cell priming in secondary lymphoid organs. Recent experiments using two photon laser microscopy indicate that dendritic cells are central to Treg cell function and that the *in vivo* mechanisms of T cell regulation are more complex than those described *in vitro*. Here we have addressed DC migration to and within the LN in the presence of low or high numbers of Tregs of known Ag specificity. We show that pro-inflammatory chemokines are secreted in the lymph node early (24h) after antigen-specific T cell activation, that this secretion is dependent on Ag-mediated DC-T cell interactions and that it is inversely related to the frequency of Tregs specific for the same antigen. This, in turn, inhibits recruitment of DCs into the lymph node thus affecting the overall magnitude of the early response. Interestingly, the altered LN microenvironment generated by high Treg frequency results in decreased colocalization of T cells and DCs in the draining LN. These results substantiate for the first time a major role of Tregs in LN patterning during inflammation.

**PB06/110 CYCLOPHOSPHAMIDE ADMINISTRATION REDUCES CD4<sup>+</sup> CD25<sup>+</sup> TREG CELL NUMBER AND PROMOTES CD4<sup>+</sup> T CELL EFFECTOR FUNCTION**

S. Vitale<sup>1</sup>, L. Pace<sup>1</sup>, S. Rizzo<sup>1</sup>, C. Calombi<sup>1</sup>, B. Dettori<sup>1</sup>, I. Troiani<sup>1</sup>, V. La Sorsa<sup>2</sup>, E. Proietti<sup>2</sup>, G. Doria<sup>1</sup>

<sup>1</sup>University of Rome Tor Vergata, Biology, Rome, Italy, <sup>2</sup>Istituto Superiore di Sanità, Cell Biology and Neurosciences, Rome, Italy

Recent findings assess a potential influence of chemotherapy on CD4<sup>+</sup>CD25<sup>+</sup> Treg cells. Cyclophosphamide (CTX) is an alkylating agent directly cytotoxic on various tumour cell types. The present research focuses on the effects of CTX treatment on CD4<sup>+</sup>CD25<sup>+</sup> Treg cell development, peripheral homeostasis and function before and after treatment. The effects of this drug has been analysed also in terms of naïve Th cell activation and effector function.

CTX administration decreases peripheral frequencies and total number of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells. Results demonstrate that CD4<sup>+</sup>CD25<sup>+</sup> Treg cell inhibition was associated with a decrease in total IL-2 production by CD4<sup>+</sup> T cells. We analysed the effect of CTX-induced CD4<sup>+</sup>CD25<sup>+</sup> Treg cell decrease, in terms of CD4<sup>+</sup> T cell activation and effector function. To this purpose, after CTX treatment Balb/c mice were adoptively transferred with DO11.10 CD4<sup>+</sup> T cells. CTX pre-treatment promotes CD4<sup>+</sup> T cell homing in secondary lymphoid organs. After OVA challenge, DO11.10 CD4<sup>+</sup> T cells adoptively transferred in CTX-treated mice share an increase in the recovered transgenic cells, as well as an increase in cell proliferation and cytokine production following OVA 323-339 peptide stimulation *in vitro*.

These results have been translated in the murine cancer model EG7-OVA, which constitutively expresses OVA. The effects of CTX have been evaluated in terms of frequency and activity of infiltrated CD4<sup>+</sup>CD25<sup>+</sup> Treg cells in tumour-bearing mice, and CD4<sup>+</sup> Th and CD8<sup>+</sup> Tc cell frequencies and activation. Following CTX administration, we observed a decrease in tumour infiltrating CD4<sup>+</sup>CD25<sup>+</sup> Treg cell number, and boosted tumour specific T cell response following OVA challenge, leading to a potent synergistic effect against pre-established tumours. Our data indicate that the CTX administration decreases CD4<sup>+</sup>CD25<sup>+</sup> Treg cell number and promotes CD4<sup>+</sup> T cell activation, indicating CTX as a valuable molecule for immunotherapy against cancer.

**PB06/111 MODULATION OF THE T-CELL RECEPTOR (TCR) STIMULATION STRENGTH CAN RENDER HUMAN ACTIVATED CD4<sup>+</sup> T CELLS SUPPRESSIVE**

G. Noël<sup>1</sup>, C. Brinster<sup>2</sup>, D. Bruniquel<sup>3</sup>

<sup>1</sup>UPRES 3889, Faculté de Médecine de Rennes, Laboratoire d'Immuno-Hématologie, Rennes, France, <sup>2</sup>Research Center Jean-Pierre Aubert, INSERM U837, Institut de Recherches sur le Cancer de Lille (IRCL), Lille, France, <sup>3</sup>UPRES 3889, Faculté de Médecine de Rennes, Etablissement Français du Sang, Bretagne, Rennes, France

In this study, we explored the potential of human naïve CD4<sup>+</sup> T cells to acquire regulatory properties upon stimulation. We demonstrated that *in vitro* pre-activated naïve CD4<sup>+</sup>CD25<sup>+</sup>CD45RA<sup>+</sup> T cells could become anergic and suppressive upon lower intensity TCR stimulation. These CD4<sup>+</sup>CD25<sup>+</sup> T cells generated *in vitro* potentially suppressed the proliferation of allogeneic CD4<sup>+</sup>CD25<sup>+</sup> T cells independently of cytokines and in a contact dependent manner. Our data indicated that expression of Foxp3 was not necessary to induce the suppressive T cell activity. We demonstrated that these CD4<sup>+</sup>CD25<sup>+</sup> T cells were unresponsive upon re-stimulation and that their suppressive activity was transient. However, we showed that the anergy and the suppressive function could be reversed by increasing the stimulus and their level of TCR activation. We concluded from our data that these anergic and suppressive activities are related to a fine tuning of TCR activation threshold.

**PB06/112 IL-10 PRODUCING REGULATORY T CELLS INDUCE IGG4 PRODUCTION BY B CELLS THROUGH GITR/GITR-L INTERACTION, IL-10 AND TGF-BETA**

T. Adjibimey<sup>1</sup>, K. Arndts<sup>1</sup>, J. Satoguina<sup>1</sup>, A. Hoerauf<sup>1</sup>, L. Layland<sup>1</sup>

<sup>1</sup>Institute of Medical Microbiology, Immunology and Parasitology (IMMIP), Bonn, Germany

Recent studies demonstrated that IL-10-producing Treg (Tr1) can induce B cells to secrete IgG4 in a cell-contact-dependent manner. The benefit of such non-inflammatory B-cell responses is apparent in the hyporesponsive state of patients with helminth infections such as Onchocerciasis. The present work aimed to investigate the mechanisms involved in the induction of IgG4 in B cell:Tr1-cell co-cultures, using IL-10-producing antigen specific regulatory T cell lines and clones generated from human PBMC. Interestingly, we could show that, increasing Foxp3 levels in regulatory T cell lines correlated with their ability to induce IgG4 in B cells. In addition, blocking glucocorticoid-induced tumour necrosis factor receptor-related protein (GITR) molecules selectively prevented IgG4 production as did neutralizing Ab to glucocorticoid-induced tumour necrosis factor receptor-related protein ligand (GITR-L), IL-10 and TGF-beta. Furthermore, the prevention of IgG4 induction by anti-GITR Ab could be reversed by addition of an excess of recombinant IL-10 but not rTGF-beta. In contrast, anti-ICOS and anti-CTLA-4 Abs had shown no effect. In addition, neutralizing antibodies to GITR could significantly reduce the IL-10 dependant phosphorylation of STAT3. Thus, the mechanism of IgG4 induction by regulatory cells involves GITR-GITR-L interactions, IL-10 and TGF-beta.

**PB06/113 ROLE OF PULMONARY FIBROBLASTS IN INDUCTION AND REGULATION OF CD4<sup>+</sup>CD25<sup>+</sup> REGULATORY T CELLS**

E. Conte<sup>1</sup>, M. Failla<sup>1</sup>, C. Mastruzzo<sup>1</sup>, M. Fruciano<sup>1</sup>, M. P. Pistorio<sup>1</sup>, E. Fagone<sup>1</sup>, C. La Rosa<sup>1</sup>, E. Trovato-Salinaro<sup>1</sup>, E. Gili<sup>1</sup>, N. Crimi<sup>1</sup>, C. Vancheri<sup>1</sup>

<sup>1</sup>University of Catania, Department of Internal Medicine and Specialistic Medicine, Section of Respiratory Diseases, Catania, Italy

Pulmonary fibroblasts are known to modulate the immunological response by regulating the expression of activation markers as well as some functional features of lymphocytes. Anergy and T lymphocytes deletion are considered classical mechanisms regulating immunological tolerance. Recently, regulatory T cells (Treg)-mediated active suppression renewed the old concept of immunological suppression. Treg naturally circulating in the blood could be induced also in periphery and there is evidence demonstrating the possibility of expanding this lymphocyte subpopulation also *in vitro* by using different experimental protocols. Aim of this study was to evaluate functional modifications induced by fibroblasts in CD4<sup>+</sup> T cells cultured *in vitro*. Fibroblasts from pulmonary biopsies and CD4<sup>+</sup> T lymphocytes immunomagnetically purified from peripheral blood were co-cultured separated by a semi-permeable membrane for 36h. We show here that fibroblasts negatively modulated the lymphocytes production of Th1 cytokines, such as IL-2, TNF-α and INF-γ, whereas Th2 cytokines, such as IL-4, IL-10 and TGF-β, levels resulted unaffected. Moreover, lymphocytes mitogenic response to concanavalin A or anti CD3/CD28-ab was attenuated by fibroblasts co-culturing. This effect was probably

mediated by PGE<sub>2</sub> because the fibroblasts pretreatment with indometacin abrogated the reduction of T lymphocytes proliferation. On the other hand, fibroblasts induced a significant raise of a CD4+CD25+ T lymphocytes subpopulation characterized also by intracellular expression of Foxp3 and CTLA-4 as well as by high levels of IL-10 and TGF- $\beta$ . These results confirm the immunomodulatory activity of fibroblasts. In particular the expansion of Treg population demonstrates that cross-talk between fibroblasts and lymphocytes could orientate the immune-inflammatory response. Further investigations on the interactions between pulmonary fibroblasts and T lymphocytes could gain new insight on the control of immune response in lung pathologies.

#### PB06/114 OVARIAN CANCER PRIMARY CYTOREDUCTION AS A UNIQUE CHANCE TO REDUCE TUMOR IMMUNOSUPPRESSION

V. Visconti<sup>1</sup>, C. Napoletano<sup>1</sup>, F. Bellati<sup>1</sup>, S. Pauselli<sup>1</sup>, C. Marchetti<sup>1</sup>, M. Antonilli<sup>1</sup>, A. Rugghetti<sup>1</sup>, L. Frati<sup>1</sup>, P. Benedetti Panici<sup>1</sup>, M. Nuti<sup>1</sup>

<sup>1</sup>University of Rome 'Sapienza', Rome, Italy

**Objective:** Surgery is the primary therapeutic strategy for most solid tumors, although modern oncology has established that neoplasms are frequently systemic diseases. Being however a local treatment, the mechanisms through which surgery plays its systemic role remain unknown. We have investigated the influence of cytoreduction on the immune system of primary and recurrent ovarian cancer.

**Methods:** Peripheral blood mononuclear cells were isolated from 25 primary ovarian cancer patients, 15 patients treated with interval debulking surgery and 15 patients affected by platinum sensitive recurrent ovarian cancer the day before, between two and four days and two weeks after surgery. Twenty-five women subjected to laparotomy for benign gynecological conditions were used as control. The modifications of the immune system were evaluated by monitoring the different circulating T cell subsets by cytofluorimetry and serum cytokines (IL-10, TGF- $\beta$ 1 and IL-6) levels by ELISA. Tumor infiltrating Treg cells were analyzed through confocal microscopy. IFN  $\gamma$  production was revealed by ELISpot.

**Results:** All ovarian cancer patients show an increase in CD4+CD25+FOXP3+ circulating cells (CD4 Treg). CD4/CD8 ratio is increased in primary tumors but not in recurrent neoplasms. Primary cytoreduction is able to increase circulating CD4 and CD8 effector cells and decrease CD4 naïve T cells. CD4+ Treg cells rapidly decreased after primary tumor debulking, while CD8+CD25+FOXP3+ (CD8 Treg) cells are not detectable in peripheral blood. CD4 and CD8 Treg cells are both present in neoplastic tissue. IL-10 serum levels decrease after surgery, while no changes are observed in TGF- $\beta$ 1 and IL-6 levels. Surgically induced reduction of the immunosuppressive environment results in an increased in the capacity of CD8+ T cells to respond to the recall antigens. None of these changes can be observed in patients previously subjected to chemotherapy or affected by recurrent disease.

**Conclusions:** We demonstrate that the reduction of immune suppression is a mechanism through which surgery plays its beneficial role. These results will aid the scientific community to develop new diagnostic and therapeutic strategies and more efficient immunotherapy vaccination schedules.

#### PB06/115 PATIENTS POST HEMATOPOIETIC STEM CELL TRANSPLANTATION HAVE A HIGH PROPORTION OF CD4+CD25++ AND FOXP3 LYMPHOCYTES IN BLOOD AT THE BEGINNING OF AGVHD

D. Dlubek<sup>1,2</sup>, E. Jaskula<sup>1</sup>, M. Sedzimirski<sup>2</sup>, A. Lange<sup>1,2</sup>

<sup>1</sup>Institute of Immunology and Experimental Therapy, Polish Academy of Science, Clinical Immunology, Wrocław, Poland, <sup>2</sup>Lower Silesian Center for Cellular Transplantation with National Bone Marrow Donor Registry, Wrocław, Poland

**Background:** CD4+CD25++ cells having FoxP3 are described as regulatory cells (Treg) exerting immunosuppression.

**Methods:** 74 patients (10 children and 64 adults) were analyzed for the proportions of CD4+CD25++, CD134 and FoxP3 lymphocytes in relation to aGvHD, CMV, EBV and HHV6 reactivation and clinical outcome of transplantation. In 18 patients (62 measurements) lymphocyte profiling included detection of CMV (CD8high, HLA-A\*0201/NLVPMTATV) and EBV (CD8high, HLA-A\*0201/GLCTIVAML) specific cytotoxic cells.

**Results:** aGvHD patients had higher proportions and numbers of CD4+CD25++ cells (0.62%±0.10 vs 0.33%±0.08, p=0.001, 1.8 cells/uL±0.33 vs 1.0 cells/uL±0.2, p=0.05) on the first day of aGvHD manifestation than those lacking aGvHD when examined at the similar time post transplant. CD134+ lymphocytes and CD4+CD25+CD134+ cells followed the pattern of an increase of CD4+CD25++ cells. 76.3%±2.0 of CD4+CD25++ lymphocytes were FoxP3 positive. In investigation of the functional impact of an increase of CD4+CD25++ cells in patients post transplant it was found that (i) higher proportions of Tregs was associated EBV reactivation (0.573%±0.105 vs 0.433%±0.079, p=0.046) and similar trend was observed when all three investigated viruses were considered (p<0.067) (ii) proportions of Tregs and EBV pentamers were inversely correlated (R=-0.18, p=0.09) (iii) patients with the marrow failure or relapse had higher peak values of CD4+CD25++ cells than those without these complications as measured during 82 days lasting observation (0.8%±0.2 vs 0.4%±0.06%, p=0.057) (iv) patients two weeks before the fatal outcome of aGvHD had higher proportions of CD4+CD25++ cells than survivors both groups pts were at the similar time post transplant (0.61%±0.17 vs 0.21%±0.05, p=0.02).

**Conclusions:** CD4+CD25++ cells increase in aGvHD pts associates with EBV reactivation and fatal outcome of transplantation.

Supported by: FP6 Allotest Project (LSHB-CT-2004-503319); Grant 2P05E 037 30 from the Polish Ministry of Science & Higher Education.

#### PB06/116 REGULATORY T CELLS DIRECT THE ADAPTIVE IMMUNE RESPONSE DURING FUNGAL INFECTION

N. Whibley<sup>1</sup>, D. M. MacCallum<sup>2</sup>, N. A. R. Gow<sup>2</sup>, R. N. Barker<sup>1</sup>, A. M. Hall<sup>1</sup>

<sup>1</sup>University of Aberdeen, Section of Immunology and Infection, Aberdeen, United Kingdom, <sup>2</sup>University of Aberdeen, Aberdeen Fungal Group, Aberdeen, United Kingdom

There are a number of outcomes from exposure to potentially pathogenic microorganisms such as fungi. These range from effective immune clearance to stimulation of immune-mediated pathology, and from commensalism to infection. *Candida albicans* is found harmlessly on approximately 80% of humans; although fatal infections can develop if an individual becomes immunocompromised. Effector helper T cells such as Th1, and more recently Th17, have been identified during *C. albicans* infection. Whilst Th1 cells have been associated with protection against fungal pathogens, there is evidence for both protective and pathogenic roles for Th17 cells. It is unclear what controls the effector response; however regulatory T cells (Tregs) have also been identified during *C. albicans* infection and have been found to play a role in limiting these responses. To investigate the relationship between helper T cell subsets during systemic infection, mice were infected with clinical isolates of *C. albicans* known to have differing virulence in murine models. In response to stimulation with fungal antigens a virulent strain (SC5314) induced both Th1 and Th17 cells; comparatively a less virulent strain (HUN96) induced only a Th1 response. Interestingly, the Treg populations induced by each strain were different: the virulent strain promoted the induction of natural Tregs (CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup>) and the less virulent strain induced putative Tr1 cells (CD4<sup>+</sup>IL-10<sup>+</sup>). This data, which is in agreement with other studies, indicates that a Th1 response confers protection against progressive *C. albicans* infection. Our findings suggest that a Th17 response is associated with susceptibility. We hypothesise that the balance between helper T cell subsets has a central role in determining the outcome of *C. albicans* infection, and that the predominant regulatory T cell response may ultimately decide the fate of the host. Modulation of the helper T cell response to re-establish a commensal state could provide a novel target for immunotherapies for treatment of fungal infections.

#### PB06/117 IMMUNOSUPPRESSIVE DRUG CYCLOSPORINE A INHIBITS HUMAN REGULATORY T CELLS ACTIVITY INDEPENDENTLY OF CALCINEURIN PATHWAY

C. Miroux<sup>1</sup>, O. Morales<sup>1</sup>, A. Carpentier<sup>2</sup>, F. Stenard<sup>2</sup>, F. Conti<sup>2</sup>, S. Dharancy<sup>3</sup>, E. Boleslawski<sup>3</sup>, V. Pancre<sup>1</sup>, N. Delhem<sup>1</sup>

<sup>1</sup>CNRS, UMR 8161, Lille, France, <sup>2</sup>UPRES 1833, Hôpital Cochin, Paris, France, <sup>3</sup>Inserm U795, Hôpital Huriez, Lille, France

**Background:** Inevitable Hepatitis C virus (HCV) recurrence after liver transplantation is a major barrier to survival of the transplanted liver and may be promoted by immunosuppression and CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells (Treg). Treg cells are essential for the induction and maintenance of immunological self-tolerance as well as transplant tolerance. The effects of cyclosporine A (CsA), a widely used immunosuppressive agent, on human CD4<sup>+</sup>CD25<sup>+</sup> Treg cells were investigated.

**Methods:** Human CD4<sup>+</sup>CD25<sup>+</sup> cells were isolated from healthy donors and cultured in the presence of 40 or 400 ng/mL CsA or in the presence of non-immunosuppressive analogs of CsA: NIM811 and PSC833. Suppressive activity of regulatory T cells was assessed in Mixed Leukocyte Reaction (MLR) of CD25<sup>+</sup> and autologous activated Peripheral Blood Mononuclear Cells (PBMC). Phenotype analysis (Flow cytometric, Q-PCR) and cytokine production (ELISA) of Treg cells were then performed in culture assays.

**Results:** CsA and NIM, used at 40 and 400 ng/mL, inhibits proliferative capacity of PBMC and CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells, in a dose-dependent manner. Surprisingly, addition of 40 ng/mL CsA in MLR impaired the suppressive activity of CD4<sup>+</sup>CD25<sup>+</sup> cells whereas a high dose of CsA have no effect on Treg cells function. It appears that therapeutical dose of CsA (40 ng/mL) did not change the phenotype of CD4<sup>+</sup>CD25<sup>+</sup> T cells but alters regulatory T cells activity by switching their regulatory cytokine profile in favour of T helper1 cytokines such as IL-2 and IFN- $\gamma$ . Interestingly, NIM 811, which has a calcineurin independent pathway activity, has the same effect as CsA on regulatory T cells activity.

**Conclusions:** CsA significantly impaired the function of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells by inducing IL-2 and IFN- $\gamma$  secretion. The present studies suggest that CsA may block the induction of immune tolerance and decrease the risk of hepatitis C recurrence. Moreover, as NIM also inhibits Treg activity, it suggests that CsA did probably not impair the Treg cells function by interfering with the calcineurin pathway.

#### PB06/118 CTLA-4 (CD152) COMPETENT CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> T CELLS PROLONG THE LIFE-SPAN OF CTLA-4 DEFICIENT MICE

P. Kolar<sup>1,2,3</sup>, K. Knieke<sup>1,2,3</sup>, J. K. E. Hegel<sup>3</sup>, D. Quandt<sup>2</sup>, G.-R. Burmester<sup>1</sup>, H. Hoff<sup>1,2</sup>, M. C. Brunner-Weinzierl<sup>1,2,3</sup>

<sup>1</sup>Charité, Klinik für Innere Medizin mit Schwerpunkt Rheumatologie und Klinische Immunologie, Berlin, Germany, <sup>2</sup>Deutsches Rheuma-Forschungszentrum, Berlin, Germany, <sup>3</sup>Otto-von-Guericke Universität Magdeburg, Germany, Experimentelle Pädiatrie, Universitätskinderklinik, Magdeburg, Germany

Unwanted T cell responses in the periphery can be prevented and suppressed by regulatory CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T cells (T<sub>reg</sub>). These cells express intracellular CTLA-4 (CD152) constitutively suggesting a central role of CTLA-4-mediated functions for regulatory T cells.

Here, we demonstrate that CTLA-4 deficient mice which suffer from severe lymphoproliferative disorder show high numbers of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells, nevertheless these mice die within 3–5 weeks of age.

In terms of costimulation, serological blockade of CTLA-4 on regulatory T cells in vitro leads to enhanced proliferation of these cells showing that the anergic status is controlled by CTLA-4. Additionally, serological blockade of CTLA-4 during activation of regulatory T cells leads to enhanced activation-induced cell death (AICD) dependent on CD95-mediated activation of caspases. Thus, CTLA-4 controls the homeostasis of regulatory T cells.

In vitro, blockade of surface CTLA-4 leads to significantly reduced regulatory T cell mediated suppression of responder T cells. Interestingly, we were able to prolong the life-span of CTLA-4 deficient mice by i. v. injection of CTLA-4 competent CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells stressing the profound impact of CTLA-4 on the suppressive function of regulatory T cells in vivo. Therefore we expect CTLA-4 to be a suitable target for specific strategies to control autoimmune processes.

#### PB06/119 BACTERIAL TRANSLOCATION AND REGULATORY T LYMPHOCYTES IN PATIENTS WITH LIVER CIRRHOSIS

M. Marquez<sup>1</sup>, C. Rodríguez Ramos<sup>2</sup>, M. Montes de Oca Arjona<sup>1,3</sup>, M.J. Blanco<sup>2</sup>, C. Fernandez -Gutierrez<sup>4</sup>, J.A. Giron-Gonzalez<sup>3</sup>

<sup>1</sup>Fundacion para la Investigacion Puerta del Mar, Cadiz, Spain, <sup>2</sup>Puerta del Mar University Hospital, Gastroenterology, Cadiz, Spain, <sup>3</sup>Puerta del Mar University Hospital, Internal Medicine, Cadiz, Spain, <sup>4</sup>Puerta del Mar University Hospital, Microbiology, Cadiz, Spain

**Objective:** Intestinal permeability and bacterial translocation and their influence on T lymphocytes activation and differentiation in T reg were analyzed in patients with compensated and decompensated liver cirrhosis.

**Patients and methods:** Forty patients with liver cirrhosis, 20 of them without previous decompensation (CC) and 20 with ascetic decompensation (DC), and 20 healthy controls (HC) were studied. Bacterial translocation was analyzed by serum concentrations of lipopolysaccharide-binding protein (LBP). Membrane expression of co-stimulatory molecules (CD28), activation markers (CD25 and CD122) and proportion of T regulatory cells (defined as those CD4<sup>+</sup>CD25<sup>high</sup>intracellular Foxp3<sup>+</sup>) were studied by flow cytometry with specific antibodies. Values of the variables were expressed as median [interquartile range]. Comparisons between variables were made by the Mann-Whitney U test. Associations between variables were analyzed by the Pearson's correlation coefficient.

**Results:** Serum concentrations of LBP were significantly elevated in patients with compensated (7.7 [5.7–9.1] microg/ml) and decompensated (28.2 [10.7–40.6]) cirrhosis when compared with healthy controls (3.4 [2.7–4.2]) (p < 0.001 in each case). Significantly higher concentrations of LBP were detected in those patients with higher portal hypertension. Those patients with decompensated cirrhosis shows an activation state characterized by increased percentages of CD25<sup>+</sup> and CD122<sup>+</sup> expression on CD4<sup>+</sup> T cells. A decrease of CD28 expression was detected in T CD4<sup>+</sup> lymphocytes from patients with decompensated cirrhosis (DC, 94 [89–98] %; CC, 97 [92–98] %; HC, 98 [96–99], DC vs HC: p = 0.010). Moreover, T reg lymphocytes, expressed as a proportion of global T CD4<sup>+</sup> cells, were significantly increased in patients with compensated and decompensated cirrhosis (DC, 14.7 [13.3–16.1] %; CC, 10.3 [10.1–11.2]; HC, 8.4 [7.2–8.7], p < 0.001 in each case). A significant and positive correlation was detected between serum LBP concentration and percentage of CD4<sup>+</sup> T reg (r = 0.787, p < 0.001).

**Conclusion:** Patients with liver cirrhosis, fundamentally those with previous decompensation, shows increased intestinal permeability. As a response to those, T lymphocyte activation is detected. Probably as a mean to decrease the continuous antigenic stimuli, a diminution of costimulation and an expansion of suppressor populations, such as T reg, are observed in them.

#### PB06/120 EVALUATION OF REGULATORY T CELLS IN THE IMMUNE RESPONSE AGAINST RV

M.C. Mesa<sup>1</sup>, L. Gutiérrez<sup>2</sup>, C. Duarte<sup>3</sup>, J. Angel<sup>4</sup>, M.A. Franco<sup>4</sup>

<sup>1</sup>Pontificia Universidad Javeriana, Microbiología, Bogotá, Colombia, <sup>2</sup>Merck Colombia, Bogotá, Colombia, <sup>3</sup>Pontificia Universidad Javeriana, Medicina, Bogotá, Colombia, <sup>4</sup>Pontificia Universidad Javeriana, Instituto de Genética Humana, Bogotá, Colombia

**Objectives:** We have previously shown low frequencies of Rotavirus (RV)-specific CD4<sup>+</sup> IFN-γ<sup>+</sup>, IL-13<sup>+</sup> or IL-4<sup>+</sup> and CD8<sup>+</sup> IFN-γ<sup>+</sup> T cells in healthy and RV acute-gastroenteritis (GE) affected adults and even lower or absent in children with RV GE. The objectives of this study were:

- 1) to explore the presence of RV-specific T cells secreting different cytokines;
- 2) to evaluate quantitative changes in peripheral blood leukocytes from children with RV GE; and,
- 3) to evaluate regulatory mechanisms in the immune response against RV.

##### Methods:

- 1) Peripheral blood mononuclear cells were stimulated with the simian rotavirus strain, RRV, for 10h and Brefeldin A to evaluate IFN-γ<sup>+</sup>, IL-13<sup>+</sup>, IL-2<sup>+</sup>, IL-10<sup>+</sup> and IL-17<sup>+</sup> in CD4<sup>+</sup> and CD8<sup>+</sup> T cells by intracellular staining and flow cytometry.
- 2) Absolute counts for peripheral leukocytes subsets were determined in children using electronic analyzers;
- 3) Frequencies of circulating IFN-γ<sup>+</sup> RV-specific T cells were determined before and after removing CD25<sup>+</sup> cells or after blocking TGF-β with its natural inhibitor, LAP, or ALK5i, a TGF-βRI signalling inhibitor; and,
- 4) Numbers of peripheral TGF-β<sup>+</sup> Treg cells were determined in children with acute GE by flow cytometry.

##### Results:

- 1) In a few healthy adults low levels of CD4<sup>+</sup> T cells produced IFN-γ/IL-2 simultaneously but most of the RV<sup>+</sup> specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells produced only IFN-γ. Low frequencies of IL-10<sup>+</sup> and IL-2<sup>+</sup> CD4<sup>+</sup> T cells were found in adults with acute and convalescent RV GE, respectively.
- 2) Lymphopenia was found in 5/12 children with RV acute GE.
- 3) Higher frequencies of IFN-γ<sup>+</sup> RV-specific CD4<sup>+</sup> T cells were observed in healthy adults after CD25<sup>+</sup> cells depletion, treatment with LAP or ALK5i. IFN-γ<sup>+</sup> RV-specific CD8<sup>+</sup> T cells also increased after LAP or ALK5i treatment. CD25-depleted PBMC treated with ALK5i showed even higher frequencies of RV IFN-γ<sup>+</sup> CD4<sup>+</sup> T cells. The ALK5i effect was also observed in adults but not in children with RV GE in spite of the presence of normal numbers of peripheral TGF-β<sup>+</sup> Treg cells.

**Conclusion:** CD25<sup>+</sup> cells and TGF-β mediated regulatory mechanisms can explain partially the low frequencies of peripheral anti-RV T cells observed in adults but not in children.

#### PB06/121 MODULATION OF HUMAN CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> REGULATORY T CELLS BY TLR2 STIMULI

M.H. Nyirenda<sup>1,2</sup>, L. Sanvito<sup>1,2</sup>, C. Constantinescu<sup>1</sup>, B. Gran<sup>1,2</sup>

<sup>1</sup>University of Nottingham, Division of Clinical Neurology, Nottingham, United Kingdom, <sup>2</sup>University of Nottingham, Institute of Infection Immunity and Inflammation, Nottingham, United Kingdom

Naturally occurring CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells (Tregs) are thought to suppress the activity of pathogenic T cells in autoimmune diseases such as Multiple Sclerosis (MS), an inflammatory demyelinating disease of the central nervous system. Toll-like receptors (TLRs) are key components of the innate immune system which have also been detected on Tregs. We hypothesised that in human immune cells, TLR2 stimulation by bacterial lipoprotein (BLP, Pam3cys-SK4, agonist for TLR2/1 heterodimer), may reduce the suppressive activity of Tregs on CD4<sup>+</sup>CD25<sup>+</sup> responder T cells (Tresp), as previously reported in the mouse. We co-cultured purified Tregs and Tresp in the presence of plate-bound anti-CD3/CD28 antibodies with or without BLP. We observed potent suppression of Tresp proliferation, which was dependent on Treg:Tresp ratios. BLP significantly reduced the suppressive activity of Tregs at low Treg:Tresp ratios. Pre-incubation of Tregs or Tresp with BLP before co-culture demonstrated a predominant effect of TLR2 stimulation on Tregs, which were rendered less suppressive. Cell viability staining with Annexin V indicated that loss of suppression was not due to BLP toxicity to Tregs or Tresp. Consistent with these findings, anti-TLR2 surface staining showed that Tregs expressed higher density of TLR2 compared to Tresp. To clarify any dose effect of BLP, Tregs were co-cultured with Tresp at 0:1, 1:4, 1:8 and 1:16 ratios in the presence or absence of increasing concentrations of BLP. Significantly reduced Treg suppressive activity was observed at 5 μg/ml BLP, but not at lower BLP concentrations (1 and 0.1 μg/ml). In separate experiments, we also examined the suppressive effects of FSL-1, agonist for the TLR2/6 heterodimer. Preliminary data indicate weaker inhibition of Treg activity compared with BLP. Data reported here were obtained in co-cultures of purified Tregs and Tresp only, indicating that TLR2 can directly modulate Treg activity in the absence of antigen-presenting cells. We speculate that infections may lead to clinical exacerbations in MS and possibly other immune-mediated diseases by causing transient loss of Treg suppression.

Study supported by Grants from the EU (Marie Curie International Reintegration Grant) and MS Society of the UK to BG. MHN is supported by a PhD studentship from MS Society.

PB06/122 Abstract withdrawn by author

#### PB06/123 CHARACTERIZATION OF CD25<sup>+</sup>FOXP3<sup>+</sup> REGULATORY T CELLS DURING *PLASMODIUM YOELII* AND CYTOMEGALOVIRUS INFECTION

S. Abel<sup>1</sup>, J. Buer<sup>1</sup>, W. Hansen<sup>1</sup>

<sup>1</sup>University Hospital Essen, Medical Microbiology, Essen, Germany

CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells (Tregs) play a critical role in maintaining the immune homeostasis between immune response and self tolerance. It has been well established that Tregs expressing the transcription factor Foxp3, are involved in the suppression of immune responses against self antigens avoiding autoimmune diseases. In addition, over the last few years, Foxp3<sup>+</sup> Tregs were also discussed in the context of different chronic infection diseases. However, the molecular phenotype, the site of induction and/ or expansion as well as the suppressive mechanism of Tregs during infections are rarely understood. To gain further insights into Treg biology during infectious diseases we analyzed mouse models infected with *Plasmodium yoelii* (*P. yoelii*) and murine Cytomegalovirus (mCMV). We isolated Treg cell populations from different organs at various points post infection and detected an increase of Foxp3<sup>+</sup> Tregs in infected mice. According to these results we will isolate Foxp3<sup>+</sup> Tregs from infected Foxp3<sup>+</sup>GFP<sup>+</sup> reporter mice and analyze their functional phenotype as well as their gene expression profile by comparative microarray analysis. Based on these results T cells will be genetically modified and monitored for their influence on the emergence and course of infectious diseases in vivo.



**PB06/124 THE ROLE OF CD4 NEGATIVE CELLS IN THE FORMATION OF TGF-BETA AND RETINOIC ACID-INDUCED RAT ALLOANTIGEN-SPECIFIC REGULATORY T CELLS**

K. Kis<sup>1</sup>, M. Maenz<sup>1</sup>, T. Ritter<sup>1,2</sup>

<sup>1</sup>National University of Ireland, Galway, Regenerative Medicine Institute, Galway, Ireland, <sup>2</sup>National University of Ireland, Galway, Medicine, Galway, Ireland

**Objectives:** Regulatory T (Treg) cells are widely regarded as primary mediators of peripheral tolerance and are characterized by the expression of the transcription factor Foxp3. Factors influencing the generation of CD4<sup>+</sup> Treg cells have been extensively studied in mice models, however the role of CD8<sup>+</sup> cells in this process of immune regulation is less known. The aim of this study was to examine the effect of the presence of CD4<sup>+</sup> cells on the formation of alloantigen-specific Treg cells in vitro in a rat model.

**Methods:** A non-separated rat lymph node cell population or MACS bead-separated CD4<sup>+</sup> or CD4<sup>-</sup> cells were treated with transforming growth factor-beta (TGF) and retinoic acid (RA) or with solvent during alloantigen-specific stimulation. Treg cells were characterized by the expression of different cell markers (CD4, CD8, CD25, Foxp3, CD44H, MHCII and CTLA4), the production of cytokines (IL-2, IL-6, IL-9, IL-10, IFN-gamma and IL-17) and by using an in vitro suppression assay.

**Results:** When non-separated or MACS bead-separated rat lymphocytes were treated with TGF and RA, the increase in Foxp3 expression in CD4<sup>+</sup> cells was less pronounced in the presence of CD4<sup>-</sup> cells, but both CD4<sup>+</sup> cells and non-separated cells suppressed the proliferation of responder T cells. The presence of CD4<sup>-</sup> cells decreased IL-10 production in the non-separated cell population and decreased the cell surface expression of CD25 and CD44H in the CD4<sup>+</sup> fraction of TGF+RA-treated cells. When control or TGF and RA-treated non-separated, CD4<sup>+</sup> or CD4<sup>-</sup> cells were added to the suppression assay, low concentrations of IL-6 and IL-9 were detected, and there was no difference in IL-2 production, but cultures containing TGF and RA-treated cells (non-separated, CD4<sup>+</sup> and CD4<sup>-</sup> as well) produced significantly more IL-10 than control cultures. Interestingly, suppression assay samples containing TGF and RA treated CD4<sup>+</sup> cells produced more IL-17 than the matched control samples, but did there was no increase in IL-17 production when non-separated or CD4<sup>-</sup> cells were used.

**Conclusion:** These data suggest that CD8<sup>+</sup> cells may contribute to the immune regulatory function of CD4<sup>+</sup> Treg cells.

**PB06/125 ROLE OF LOW MOLECULAR WEIGHT RNA IN SUPPRESSION OF CONTACT SENSITIVITY RESPONSE**

K. Bryniarski<sup>1,2,3</sup>, M. Ptak<sup>1</sup>, M. Szczepanik<sup>4</sup>, C. Guerrier-Takada<sup>5</sup>, S. Altman<sup>3</sup>, P.W. Askenase<sup>2</sup>, W. Ptak<sup>1</sup>

<sup>1</sup>Jagiellonian University Medical College, Department of Immunology, Kraków, Poland, <sup>2</sup>Yale University School of Medicine, Department of Internal Medicine, New Haven, United States, <sup>3</sup>Yale University, Department of Molecular Cellular and Developmental Biology, New Haven, United States, <sup>4</sup>Jagiellonian University Medical College, Department of Human Developmental Biology, Kraków, Poland, <sup>5</sup>Yale University, Molecular Cellular and Developmental Biology, New Haven, Poland

**Objectives:** Murine contact sensitivity (CS) a form of T cell-mediated immunity induced by epicutaneous hapten application in mediated by Th1 effector (Te) cells. Intravenous hapten injection induces in the spleen and lymph nodes CD8<sup>+</sup> Ag-specific suppressor T cells (Ts) which produce in vitro suppressor factor (TsF) that on co-incubation with Te cells inhibits their adoptive transfer of CS responses. The molecular nature of TsF is not clear at present but was assumed to be a protein and our experiments suggest that its activity may depend on the presence of a small double-stranded RNA.

**Methods and results:** The suppressive activities of RNA/DNA extracts from supernatants of cultured Ts cells were tested by inhibition of adoptive transfer of CS reaction by effector T cells. The mean inhibition of adoptive transfer varied in different experiments between 40 to 80 percent as compared with control transfer. DNA/RNA phenol-chloroform extracts (PCE) obtained by Chomczynski method were inhibitory and insensitive to DNAase and proteinase (trypsin, proteinase K) treatments while RNase A and RNase III (specific for dsRNA, like pre-miRNA) abolished their suppressor activity. PCE extract was then separated on Qiagen column to obtain pure RNA (TsFQRNA) and pure DNA (TsFQDNA) and only the former was shown to be active as suppressor factor. Further purification of TsFQRNA on acrylamide sequencing gels showed its heterogeneity expressed as 4 separate RNA bands from which only two expressed inhibitory activity in CS assay. The molecular size of active TsFQRNA bands was estimated as 74-80 bases using RNA molecular standards. Their sequence, structure and nature are now under study via corresponding cDNA. In mass spectroscopy a small contamination of TsFQRNA by several different proteins, including proteinases was found. We hypothesize that the antigen-specificity of RNA extracts is associated with bound protein. Its character and structure are now under study and our preliminary results may indicate that it belongs to immunoglobulin family.

**Conclusion:** The contact sensitivity suppressor factor produced by T CD8<sup>+</sup> has structure of low molecular double stranded RNA and express antigen specificity that is associated with bound protein.

**PB06/126 THE EFFECT OF CITRUS PEEL EXTRACT ON THE IMMUNE-MEDIATED LIVER INJURY: INVOLVEMENT OF REGULATORY T CELLS**

I. Pantsulaia<sup>1,2</sup>, A. Sepashvili<sup>1</sup>, M. Iobadze<sup>1</sup>, T. Chikovani<sup>2</sup>, N. Kikodze<sup>2</sup>, I. Chkhikvishvili<sup>2</sup>

<sup>1</sup>Institute of Medical Biotechnology, Biomedicine, Tbilisi, Georgia, <sup>2</sup>Tbilisi State Medical University, Tbilisi, Georgia

**Background:** T cell-mediated immune responses play an essential role in hepatocellular injury induced by autoimmune hepatitis, viral infection, and hepatotoxins. Pharmacological compounds capable of regulating the immune response seem to be suitable candidates for prevention/treatment of this pathology. The peel of citrus fruits is a rich source of polymethoxylated flavones, which exhibit abilities to modulate the inflammatory response and carcinogenesis. Therefore, citrus fruits represent a potentially important source of anti-inflammatory flavonoids in the human diet. **Methods.**

**Purpose:** The aim of this study is to define the effect and mechanism of the action of anti-oxidant, anti-inflammatory and immunosuppressive mixture of Citrus Peel Extract (CPE) on the immune-mediated liver injury.

**Objectives and methods:** The effects of CPE on T regulatory cells (Tregs) in ConA induced hepatitis were evaluated by study the frequency and suppressive capacity of Tregs using FACS. The influence of CPE on liver injury was determined by measurement of transaminase activity in plasma and histological changes in liver. Also, the effect of CPE on pro – and anti-inflammatory cytokines such as TNF-alpha, IL-10 and INF-gamma were studied using Enzyme-Linked Immunosorbent Assay (ELISA).

**Results:** The following outcomes were revealed: a) CPE application notably prevents development of liver injury through decreasing levels of both serum pro-inflammatory cytokines (TNF-alpha, INF) and regulatory T cells, and increasing levels of IL-10; b) The low dose CPE, which proved to be more effective than high dose, may be used to treat T-cell-mediated liver injury such as autoimmune hepatitis, alcoholic hepatitis and chronic viral hepatitis. Thus, the findings lead to the development of fundamentally new ways for prevention and/or arrest of chronic hepatitis and other autoimmune diseases as well as allow developing a new supplemental medicine based on the citrus peel extracts.

**PB06/127 ARF6 – A POTENTIAL MEDIATOR IN T CELL ANERGY**

A. Rakha Arora<sup>1</sup>, M. Gadina<sup>2</sup>, V. Kanamarlapudi<sup>3</sup>, A. Kissenpfennig<sup>1</sup>

<sup>1</sup>Queens University, Center of Infection and Immunity, Belfast, United Kingdom, <sup>2</sup>National Institute of Health, Bethesda, United States, <sup>3</sup>Swansea University, Swansea, United Kingdom

The ADP ribosylation factor (Arf) family of GTPases comprises of small monomeric proteins of 20-25kDa molecular mass, and is classified into 3 subclasses. Among all these family members, the highly expressed Arf6 (Class III) is functionally distinct, and is implicated in various cell types with membrane trafficking, endocytosis, exocytosis, and invasive activities of cancerous cells. Arf6 null embryos are lethal at midgestation which limits to study its regulatory mechanisms. Arf6<sup>-/-</sup> embryos are smaller in size and exhibit hypocellularity due to onset of liver cell apoptosis which leads to lethality due to anemia probably. Recently, work has highlighted that active form of GTPase ADP ribosylation factor 6 (Arf6) is selectively expressed in anergic T-cells, and constitutive expression of active Arf6 interferes with proliferative responses and IL-2 signaling (Tzachanis *et al.* 2003). This led us to hypothesize that Arf6-GTP might be involved in inhibition of T cells activation. Flow cytometric experiments, revealed a significant shift in Arf6 expression levels from CD4<sup>+</sup>CD25<sup>+</sup> cells to CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> cells. To elucidate the precise mechanism of this regulatory cascade, we are following a two pronged strategy: firstly to silence Arf6 in T cells and then to overexpress GTP hydrolysis-defective mutant Arf6 (Q67L) and the GTP-binding defective mutant Arf6 (T27N). We are also investigating NFAT activation following CD3 crosslinking in Jurkats, and to determine the effects of Arf6 knockdown and mutants on the T cell activation. Secondly, we have developed a targeting vector to conditionally delete Arf6 in T cells, regulatory T cells and other immune effector cells to elucidate the exact role of Arf6 in an *in vivo* setting.

**PB06/128 ROLE OF GITR IN NUCLEAR FACTOR OF ACTIVATED T CELLS-2 (NFATC2) IN A MURINE MODEL OF ASTHMA**

R. Karwot<sup>1</sup>, J.H. Maxeiner<sup>1</sup>, T. Bopp<sup>2</sup>, I. Boross<sup>1</sup>, E. Schmitt<sup>2</sup>, H.A. Lehr<sup>3</sup>, S. Finotto<sup>1</sup>

<sup>1</sup>Laboratory of Cellular and Molecular Immunology of the Lung, I. Medical Clinic, University of Mainz, Mainz, Germany, <sup>2</sup>Institute of Immunology, University of Mainz, Mainz, Germany, <sup>3</sup>Institute of Pathology, Centre Hospitalier Universitaire Vaudois, University of Lausanne, Lausanne, Switzerland

Mice with a targeted disruption of NFATc2 show increased Th2 cytokines and maintain the expression of IL-4 transcripts longer than wild-type mice. We have recently found that NFATc2 (-/-) mice develop increased airway hyperresponsiveness (AHR), a hallmark feature of allergic asthma, in the absence of allergen challenge but they surprisingly fail to show increased AHR after allergen challenge as compared to wild-type littermates. We hypothesized that a reason for the missing increase in AHR after allergen challenge might be the fact that NFATc2 mice develop an increased number of functionally intact CD4<sup>+</sup> CD25<sup>+</sup> GITR<sup>+</sup> T regulatory (Tregs) cells along with augmented CD4<sup>+</sup> Th2 and Th17 cells in the lung after allergen challenge. In this study we first demonstrated that in NFATc2 (-/-) mice, spleen CD4<sup>+</sup> CD25<sup>+</sup> GITR<sup>+</sup> T regulatory cells inhibited the hyperproliferation of the target CD4<sup>+</sup> CD25<sup>+</sup> T cell population lacking NFATc2 in vivo.

We then demonstrated that the inhibition of CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> T regulatory cells number and function via the agonist anti GITR mAb resulted in the expansion of the Th17 along with the Th2 and Th1 clone and increased AHR in anti GITR antibodies treated-NFATc2 (-/-) mice as compared to the anti GITR treated wild-type littermates. These data indicate that NFATc2 deficiency resulted in increased CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> T regulatory cell activity in the lung that inhibited the AHR and inflammation.

This work is supported by a DFG Project (Transregio 52-PC1).

**PB06/129 CHARACTERIZATION OF CD25<sup>+</sup>CD4<sup>+</sup>, CD25<sup>+</sup>CD8<sup>+</sup> TREG CELLS DURING MURINE PREGNANCY**C. Kyvelidou<sup>1</sup>, I. Athanassakis<sup>1</sup><sup>1</sup>University of Crete, Biology Department, Heraklion Crete, Greece

**Aim:** Maternal anti-paternal suppressive activity involves regulatory-CD25<sup>+</sup>/foxp3/CTLA-4 T-cells. This study was designed to define the Treg-mediated suppression during pregnancy and correlate marker and cytokine expression to function.

**Methods:** The CD25<sup>+</sup>CD4<sup>+</sup>, CD25<sup>+</sup>CD8<sup>+</sup> cells and their products were isolated from spleens of pregnant or non pregnant mice and tested for their ability to modify cell proliferation and MLR responses in an MHC-restricted manner. These properties were correlated to CTLA-4/CD28, foxp3 expression as well as cytokines and MHC-restricted soluble activity.

**Results:** All types of cells and culture supernatants isolated from pregnant mice provided stimulation, whereas those from control mice suppression. Further characterization of these cells as to the expression of CTLA-4/CD28, foxp3 and MHC-restricted soluble activity, showed that suppression is a much more complicated procedure than initially thought involving a balance of stimulatory and suppressive signals.

**Conclusions:** Suppression is a multi-parametric effect and cannot be attributed to limited cell markers or factors. The presented data showed that the suppressive or the stimulatory activity was mediated not only by soluble factors but also by cell contacts, was MHC-restricted and possibly antigen-specific.

**PB06/130 CD52<sup>hi</sup> EXPRESSION MARKS ANTIGEN-ACTIVATED SUPPRESSOR CD4<sup>+</sup> AND CD8<sup>+</sup> T CELLS IN THE MOUSE**S. Reinwald<sup>1</sup>, J. A. Dromey<sup>1</sup>, L. C. Harrison<sup>1</sup><sup>1</sup>Walter and Eliza Hall Institute of Medical Research, Autoimmunity and Transplantation Division, Parkville, Australia

Regulatory T cells (Tregs) play an essential role in maintaining peripheral tolerance and thereby preventing autoimmune diseases. In both mice and humans there is a relative lack of information about antigen-activated Tregs, yet in the mouse autoantigen-induced Tregs protect against autoimmune disease. We recently identified a marker of human antigen-activated Tregs, CD52, by analysing Treg clones generated to pancreatic islet autoantigens. CD52 is a small glycosylphosphatidylinositol (GPI)-anchored glycoprotein expressed on lymphocytes. It is the antigen for CAMPATH-1H, a humanized monoclonal antibody used clinically to treat lymphoproliferative disorders. Despite the absence of an intracellular domain, CD52 provides a co-stimulatory signal to T cells, but the function of CD52 is still unclear. In the present study, we show that CD52 is a marker of suppressor function of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the mouse. Polyclonal and ovalbumin-specific CD4<sup>+</sup>CD52<sup>hi</sup> T cells displayed suppressor activity on responder T cells, which was contact-independent. Interestingly, CD8<sup>+</sup>CD52<sup>hi</sup> T cells displayed an even stronger suppressor effect. The non-obese diabetic (NOD) mouse model of spontaneous autoimmune diabetes was used to test the suppressor function and migratory capacity of CD52<sup>hi</sup> T cells. Our data indicate that CD52 is a marker of suppressor CD4<sup>+</sup> and CD8<sup>+</sup> T cells involved in regulating autoimmune disease.

PB06/131 Abstract withdrawn by author

**PB06/132 ISOLATION, EXPANSION AND FUNCTIONAL CHARACTERIZATION OF THE CD45RA<sup>+</sup> SUBPOPULATION OF CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> REGULATORY T CELLS**C. Conrads<sup>1</sup>, P. Hoffmann<sup>2</sup>, M. Edinger<sup>2</sup>, A. Scheffold<sup>1</sup>, M. Assenmacher<sup>1</sup>, C. Niemand<sup>1</sup><sup>1</sup>Miltenyi Biotec GmbH, R&D, Bergisch Gladbach, Germany, <sup>2</sup>University Hospital Regensburg, Hematology & Oncology, Regensburg, Germany

**Objectives:** CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> regulatory T (Treg) cells can mediate transplantation tolerance and are therefore a major therapeutic target for cell-based therapies in the context of allogeneic bone-marrow or solid organ transplantation.

For the safe clinical application of Treg cells, tools have to be provided for their rapid isolation and expansion as well as for their standardized functional characterization. Treg cells sorted solely on the basis of their CD25 and CD127 expression levels lose FOXP3 expression during *in vitro* expansion. In contrast, we have shown that flow-sorted CD4<sup>+</sup>CD25<sup>+</sup>CD45RA<sup>+</sup> Treg cells retain their Treg-specific signature including FOXP3 expression during *in vitro* culture. However, flow-sorting is a time consuming task and difficult to adjust to GMP conditions.

**Methods:** We have developed a two-step protocol for the rapid magnetic isolation of CD4<sup>+</sup>CD25<sup>+</sup>CD45RA<sup>+</sup> Treg cells from PBMCs by depletion of non-CD4<sup>+</sup> and CD45RO<sup>+</sup> cells, followed by enrichment of CD25<sup>+</sup> cells using the MACS<sup>®</sup> Technology. Isolated Treg cells were expanded in the presence of high dose recombinant human IL-2 and MACS<sup>®</sup> Bead<sup>™</sup> Particles as artificial antigen-presenting cells. The suppressive activity of isolated Treg cells was evaluated with *in vitro* suppression assays using MACS<sup>®</sup> Bead<sup>™</sup> Particles loaded with CD2, CD3 and CD28 antibodies.

**Results:** Using the newly developed reagents, CD4<sup>+</sup>CD25<sup>+</sup>CD45RA<sup>+</sup> Treg cells could be isolated with a purity greater than 85% among viable WBC. CD4<sup>+</sup>CD25<sup>+</sup>CD45RA<sup>+</sup> Treg cells remained highly positive for FOXP3 after 2-3 weeks of *in vitro* expansion. Applying the bead-based suppression assay a robust dose-dependent suppression of effector T cell proliferation could be demonstrated.

**Conclusion:** The CD45RA<sup>+</sup> subpopulation of CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> regulatory T cells can be obtained using the MACS<sup>®</sup> Technology and can be expanded without significant loss of FOXP3 expression. CD4<sup>+</sup>CD25<sup>+</sup>CD45RA<sup>+</sup> Treg cells are anergic and show suppressive activity against effector T cells.

**PB06/133 ISOLATION, CHARACTERIZATION AND INHIBITION OF WOODCHUCK REGULATORY T CELLS**I. Otano<sup>1</sup>, J. Dotor<sup>1</sup>, S. Menne<sup>2</sup>, J. Prieto<sup>1</sup>, G. Gonzalez-Aseguinolaza<sup>1</sup><sup>1</sup>University of Navarra, Center for Applied Medical Research CIMA, Pamplona, Spain, <sup>2</sup>University of Cornell, Ithaca, United States

**Objectives:** The aim of this study was to characterise woodchuck Tregs in general and to analyze the effect of Treg inhibition on peripheral blood mononuclear cells (PBMCs) activation.

**Methods:** Woodchuck Foxp3 gene was cloned by RT-PCR and sequenced. A quantitative RT-PCR (qRT-PCR) was designed to determine woodchuck Foxp3 expression levels in PBMCs and in the liver of healthy and WHV infected woodchucks. CD25 expressing T cells were purified from woodchuck PBMCs by magnetic isolation system. Foxp3 expression was analysed by FACS on CD25<sup>+</sup> and CD25<sup>-</sup> cells. CD25<sup>+</sup> and CD25<sup>-</sup> cells were activated using plate-bound anti-CD3 antibody, and cytokine expression was analysed by qRT-PCR. The inhibitory activity of CD25<sup>+</sup> cells on PBMC proliferation was determined using CFSE labeled-PBMCs after activation with plate-bound anti-CD3.

**Results:** Significantly higher Foxp3 expression levels were detected in the liver of WHV-chronically infected woodchucks in comparison to non-infected animals. However, no differences were observed in PBMCs. Woodchuck Tregs were isolated from PBMCs and further characterized. We found that about 93% of the woodchuck CD25<sup>+</sup> cells were also Foxp3<sup>+</sup>. Woodchuck Tregs produce TGF-beta but no IFN-gamma upon activation with plate-bound anti-CD3. These Tregs also inhibit the proliferation and IFN-gamma production of anti-CD3 activated woodchuck PBMCs and the inhibition could be blocked *in vitro* with TGF-beta inhibitors.

**Conclusion:** Woodchucks infected with woodchuck hepatitis virus (WHV) develop chronic hepatitis. Chronic WHV infection is considered to be the best model to study the pathogenesis, prevention and treatment of HBV infection. Several studies have shown that regulatory T cells (Treg) contribute to the impairment of virus-specific T cell responses in chronic HBV infection. Tregs are characterised by CD4, CD25 and Foxp3 expression and produce high levels of immunosuppressive cytokines, including active TGF-beta and IL-10. We have isolated and characterized woodchuck regulatory T cells and developed a functional assay to analyze their activity. This will allow us to develop molecules to inhibit woodchuck Treg function.

**PB06/134 IDENTIFICATION OF A NOVEL MODULATOR OF T CELL RESPONSES**M. Papatriantafyllou<sup>1</sup>, G. Moldenhauer<sup>1</sup>, H.-J. Gröne<sup>2</sup>, C. Niehrs<sup>3</sup>, G. Hämmerling<sup>1</sup>, T. Oelert<sup>1</sup>, B. Arnold<sup>1</sup><sup>1</sup>German Cancer Research Center (DKFZ), Department of Molecular Immunology, Heidelberg, Germany, <sup>2</sup>German Cancer Research Center (DKFZ), Department of Cellular and Molecular Pathology, Heidelberg, Germany, <sup>3</sup>German Cancer Research Center (DKFZ), Department of Molecular Embryology, Heidelberg, Germany

Protection of tissues from the devastating effects of immune responses is essential for the integrity of an organism. Tolerance mechanisms are controlling potentially autoreactive lymphocytes. In addition, organs like the brain that are particularly sensitive to damage by inflammation, so called immune privileged sites, are protected by tissue barriers and contain an immunosuppressive microenvironment. Aim of this study was the identification of molecules involved in both local processes establishing immune privilege and systemic tolerance induction. In our model of systemic tolerance, regulatory CD8 T cells specific to a peripheral self antigen are induced by parenchymal cells during the neonatal phase. Starting with this transgenic mouse model, molecules that are important for the maintenance of systemic T cell regulation were determined by gene expression analysis and functional tolerance assays. Candidate molecules were then tested for their contribution to the regulation of polyclonal T cell responses in the tissues. Immune privileged organs such as the brain, the eye, the placenta and the uterus, as well as the embryo were screened for the expression of selected molecules in mRNA and protein level. As a result, a potential ERK-MAPK inhibitor was found to be both crucial for the maintenance of CD8 T cell tolerance in the transgenic mouse model and highly expressed in the immune privileged tissues. Its regulatory effect in functional *in vitro* T cell assays and in the *in vivo* model of experimental autoimmune encephalitis (EAE) will be presented and its role as a modulator of T cell responses will be discussed.

**PB06/135 INTERFERON ALPHA AND C-PHYCOCYANIN COMBINATION INDUCES REGULATORY T CELLS IN MULTIPLE SCLEROSIS PATIENTS**

G. Penton-Rol<sup>1</sup>, M. Cervantes-Llanos<sup>1</sup>, R. Alonso-Ramirez<sup>2</sup>, R. Lara-Rodriguez<sup>3</sup>, J.A. Cabrera-Gómez<sup>4</sup>, C. Valenzuela-Silva<sup>1</sup>, E. Rodriguez-Jimenez<sup>1</sup>, K. Romero-García<sup>4</sup>, E. de Armas<sup>5</sup>, G. Guillen-Nieto<sup>1</sup>, P.A. Lopez-Saura<sup>1</sup>, E. Penton-Arias<sup>1</sup>

<sup>1</sup>Center for Genetic Engineering and Biotechnology, Havana, Cuba, <sup>2</sup>Center of Molecular Immunology, Havana, Cuba, <sup>3</sup>Calixto García Hospital, Havana, Cuba, <sup>4</sup>International Center for Neurological Restoration, Havana, Cuba, <sup>5</sup>Cuban Spirulina production enterprise, Havana, Cuba

**Introduction:** Multiple Sclerosis (MS) relapses or “attacks” are likely caused by activated auto-reactive myelin-specific T lymphocytes trafficking into the CNS and recruitment of peripheral mononuclear phagocytes, causing inflammation associated to brain and spinal cord oedema and demyelization, ultimately leading to impaired neuronal transmission. In the healthy host, regulatory mechanisms of the immune system prevent and control such immuno-pathologies. MS relapses and remissions indicate that even during relapses control mechanisms are triggered. EAE studies in rodents suggest that immunological acute attacks are self-limited by regulatory T cells (rTc).

**Materials & Methods:** Peripheral blood mononuclear cells (PBMC) were separated from MS patient peripheral blood by Ficoll gradient centrifugation and mononuclear cells were collected from the ring. PBMC were divided into four experimental groups: IFN-alpha treated cells, C-Phycocyanin treated cells, IFN-alpha/C-Phycocyanin treated cells and untreated cells. The cells were incubated for 4 hours and the RNA extracted. The genes of rTc markers (CD25, Foxp3, IL-10 and TGF-β) were amplified and the CD4+CD25<sup>high</sup>Foxp3+ subset of the different experimental groups was measured by flow cytometry.

**Results:** We found that the IFN-alpha/C-Phycocyanin combination provokes a CD4+CD25<sup>high</sup>+Foxp3+ rTc response in PBMC from MS patients and healthy controls, demonstrated by RT-PCR and flow cytometry studies.

**Conclusions:** These results show that the IFN-alpha/C-Phycocyanin combination could be expected beneficial for MS treatment, in agreement with reports suggesting that rTc would limit acute MS attacks. Other autoimmune and neurological diseases could also benefit from these findings.

**PB06/136 CD4+CD25<sup>HI</sup>FOXP3+ T REGULATORY CELLS IN PATIENTS WITH DIABETES MELLITUS TYPE 1**

F. Petropoulos<sup>1</sup>, K. Tsalimalma<sup>1</sup>, N. Kafassi<sup>1</sup>, N. Tentolouris<sup>2</sup>, E. Choremi-Papadopoulou<sup>1</sup>

<sup>1</sup>Laikon General Hospital of Athens, University of Athens, Dpt of Immunology and Histocompatibility, Athens, Greece, <sup>2</sup>University of Athens, 1st Department Pro-paedeutic Medicine, Athens, Greece

The understanding of the defects in immune regulation that allow through the uncontrolled self-reactivity the destruction of insulin producing pancreatic β-cells in type 1 diabetes is of great importance. T regulatory cells (Tregs) seem to be involved in maintaining peripheral tolerance and preventing organ specific autoimmune diseases. Tregs have high CD25 expression, the more lineage specific intracellular marker FOXP3 and CD127<sup>lo/-</sup> expression.

The aim of this study is the detection of CD4+CD25<sup>HI</sup>FOXP3+Treg cells as well as of CD4+CD25<sup>HI</sup>CD127<sup>lo/-</sup>. The suppressive function of CD4+CD25+ T cells was also determined in patients with DM1 and in healthy subjects.

The detection of CD4+CD25<sup>HI</sup>FOXP3+Tregs was performed in whole blood with flow cytometry (FACSCANTO) and monoclonal antibodies (MoAbs, BD). The suppressive activity of CD4+CD25+ Tcells was estimated by co-cultures with CD4+CD25- T cells after their stimulation with MoAbs against CD3 and CD28 antigens. The CD4+CD25+ and CD4+CD25- separation was performed with appropriate magnetic beads (Miltenyi Biotec). Mann-Whitney U test was used for statistical analysis.

Five DM1 patients, mean age 26, were compared to five healthy subjects, mean age 32. Their corresponding cell populations percentages were: % CD4+CD25<sup>HI</sup> on CD4+ 0.9 vs. 1.6 (p=NS), % CD4+CD25<sup>HI</sup>FOXP3+ on CD4+ 0.76 vs. 1.21 (p=0.076) and % CD4+CD25<sup>HI</sup>CD127<sup>lo/-</sup> on CD4 0.86 vs. 1.52 (p=NS). The suppressive activity in co-cultures CD4+CD25+ with CD4+CD25- cells for DM1 patients and healthy subjects was determined to be 79.2% and 95.3% respectively (p=0.083).

In this group of patients no significant statistically difference was found in the number and suppressive activity of CD4+CD25<sup>HI</sup>FOXP3+ Tregs compared to healthy subjects. FOXP3 antigen is probably a more sensitive marker for the detection of Tregs.

**PB06/137 THE INFLUENCE OF CYCLOSPORINE A AND RAPAMYCIN ON THE LEVEL OF HUMAN CD8+CD28- CELLS IN MLR IS ASSOCIATED WITH HLA CLASS I MISMATCHES**

A. Korecka-Polak<sup>1</sup>, A. Jalbrzykowska<sup>2</sup>, J. Wyzgal<sup>2</sup>, G. Korczak-Kowalska<sup>1,2</sup>

<sup>1</sup>Faculty of Biology, University of Warsaw, Department of Immunology, Warsaw, Poland, <sup>2</sup>The Medical University of Warsaw, Transplantation Institute, Warsaw, Poland

The percentage of CD8+CD28- cells in peripheral blood of renal allograft recipients seems to be associated with the function of graft. It seems to be essential to evaluate the effect of immunosuppressive drugs on the level of this population. Mixed Leukocyte Reaction (MLR) is a model used in vitro to mimic the situation after organ transplantation.

Peripheral blood was obtained from healthy volunteers. HLA genotypes were determined by PCR using commercially available kits. Peripheral Blood Mononuclear Cells (PBMCs) were cultured in two-way MLR (n=29), in the presence or without cyclosporine A (CsA) or rapamycin (RAPA). After six days cells were labeled with mouse anti-human mAbs conjugated with fluorochromes and analyzed by flow cytometry (FACSCalibur).

Investigated drugs decreased the level of activated CD8+CD28- cells average from 26% in control to 5% in cultures with RAPA and 6% in cultures with CsA (Wilcoxon Matched Pairs Test: CsA p=0.000007, RAPA p=0.000003) and had no significant effect on resting CD8+CD28- cells. There was a negative correlation between the change of the percentage of CD8+CD28- cells caused by drugs and the level of activated cells within this population (Spearman Rank Order Correlations: CsA p=0.005, r=-0.500; RAPA p=0.010, r=-0.470). In cultures with high activated cells level (above 40% of CD8+CD28-) we observed a significant decrease in the percentage of CD8+CD28- whereas in cultures with low activated cells level (below 40% of CD8+CD28-) there were possible three situations: decrease, no change or increase in the percentage of CD8+CD28- cells. The level of activated cells within CD8+CD28- population was connected with the presence of HLA I mismatches between donors of PBMCs. In cultures without HLA I mismatches the percentage of activated cells was higher than in cultures with one common HLA I.

CD8+CD28- cells recognize antigens in the context of MHC class I so the presence/lack of HLA class I mismatches in MLR can be essential for the level of activated cells and therefore the influence of CsA and RAPA on the percentage of CD8+CD28- lymphocytes. It should be taken into consideration in studies evaluating the effect of immunosuppressive drugs on the level of CD8+CD28- cells in vitro.

**PB06/138** Abstract withdrawn by author

**PB06/139 STUDY ON THE EFFECTS OF PENTOXIFYLLINE AND TRIPTOLIDE ON IMMUNE EFFECTOR CELLS**

Y.-J. Cheng<sup>1</sup>, B.-L. Chiang<sup>2</sup>

<sup>1</sup>Graduate Institute of Oral Biology School of Dentistry National Taiwan University, Taipei, Taiwan, Republic of China, <sup>2</sup>Graduate Institute of Clinical Medicine, College of Medicine, National Taiwan University, Taipei, Taiwan, Republic of China

**Objectives:** Regulatory T cells are a small subset of T cells in vivo; however, they exert some important suppressive functions in the immune system such as self-antigens tolerance and immune homeostasis. Also, some diseases have been demonstrated to be caused by the defects in regulatory T cells. Pentoxifylline and Triptolide, widely used in clinical treatment, have been reported that they show some immunosuppressive effects. We aim to study the suppressive functions of these two drugs and subsequently the relationship with regulatory T cells induction.

**Methods:** We isolated CD4<sup>+</sup> T cells from spleen of 4-week-old OVA-T-cell receptor transgenic mice (DO11.10) and also treated these cells with Pentoxifylline and Triptolide *in vitro*. We further analyzes several functional feature such as cytokines secretion, cell surface marker expression, and proliferation of these T cells.

**Results:** In our study, the IL-2 secretion and cell proliferation of the CD4<sup>+</sup> T cells, which were activated by anti-CD3/CD28 antibody, would be suppressed by Pentoxifylline or Triptolide in a dose dependent manner. The expression of Foxp3 and CD25, which are the specific markers of regulatory T cells, had no significant difference under drug treatment.

**Conclusion:** Pentoxifylline and Triptolide have some immunomodulatory function, however, our data showed that these effects were not caused by the induction of regulatory T cells induction directly. It is possible that other immunomodulatory mechanisms might be involved, such as the suppression of dendritic cells or T cells functions.

**PB06/140 ISOLATION AND EXPANSION OF HUMAN CD4<sup>+</sup>CD25<sup>HI</sup> REGULATORY T CELLS AND THEIR EFFECTS ON ALLOREACTIVITY**

D. Keller<sup>1</sup>, S. Thomas<sup>1</sup>, H.-D. Volk<sup>1</sup>

<sup>1</sup>Charité – Universitätsmedizin Berlin, Institute of Medical Immunology, Berlin, Germany

During the last decade, a broad set of immunosuppressive drugs has been developed which improve short-term transplant results (1yr survival, acute graft rejection), but not long-term results (graft half life, e.g. kidney rejection after approx. 10-12 years). In this regard, the therapeutic aim is to minimise costs and long-term side effects caused by immunosuppressive drugs on the one hand, and on the other to considerably improve the long term survival of transplanted patients. To avoid dose-dependent damage of the transplant by immunosuppressive drugs, a specific development of additional tolerance protocols is mandatory. One top candidate for the tolerance induction in transplantation is the population of CD4+CD25<sup>high</sup> regulatory T cells (Treg's).

Because Tregs constitute 1-2% of peripheral CD4<sup>+</sup> T cells in human, it may not be possible to purify sufficient cells *ex vivo*. Therefore it is necessary to establish appropriate protocols for isolation and expansion of CD4<sup>+</sup>CD25<sup>high</sup> Treg's. In our system we are able to isolate CD4<sup>+</sup>CD25<sup>high</sup> cells with a very high purity (approx. 80% CD4<sup>+</sup>CD25<sup>high</sup>Foxp3<sup>+</sup>). Afterwards we expand the isolated Treg's by different expansion protocols such as expansion with CD3-, CD2- and CD28-expansionbeads as well as allogeneic feeder cells.



The suppressive potential of the expanded CD4<sup>+</sup>CD25<sup>+</sup> Treg's was determined by three different functional assays, a CFSE-based proliferation assay, a Cytokine-Bead-Array, and an IFN $\gamma$ -Elispot. Based on mentioned methods, the proliferative potential of allogenic stimulated responder T cells, IFN $\gamma$  production and Th1/Th2 cytokine profile, as well as the IFN $\gamma$  producing T-cells in a mixed lymphocyte reaction with different amounts of the expanded regulatory T cells were tested. Another aspect of our project is the expansion of the isolated regulatory T cells with additional rapamycin. The immunosuppressive drug rapamycin is a powerful pharmacological agent which blocks IL2-dependent T cell proliferation by accumulation with mTOR and induces expansion of regulatory T cells.

**PB06/141 LACTIC ACID BACTERIA DIFFER IN THEIR ABILITY TO INDUCE FUNCTIONAL REGULATORY T CELLS IN HUMANS**

S. de Rooij<sup>1,2</sup>, M. van Elk<sup>1</sup>, M. van Dijk<sup>1</sup>, H. Timmerman<sup>3,4</sup>, G. Rijkers<sup>4,5,6</sup>, B. Prakken<sup>1</sup>, M. Hoekstra<sup>2</sup>, I. de Klee<sup>1,7</sup>

<sup>1</sup>University Medical Centre Utrecht; Wilhelmina Children's Hospital, Pediatric Immunology, Utrecht, Netherlands, <sup>2</sup>University Medical Centre Utrecht; Wilhelmina Children's Hospital, Pediatric Dermatology/Allergology, Utrecht, Netherlands, <sup>3</sup>Winclive Bio Industries, Amsterdam, Netherlands, <sup>4</sup>University Medical Center Utrecht, Surgery, Utrecht, Netherlands, <sup>5</sup>University Medical Centre Utrecht; Wilhelmina Children's Hospital, Pediatric Immunology, Utrecht, Netherlands, <sup>6</sup>St. Antonius Hospital, Nijmegen, Netherlands, <sup>7</sup>University Medical Centre Utrecht; Wilhelmina Children's Hospital, Pediatric Dermatology/Allergology, Utrecht, Netherlands

Trials with probiotic lactic acid bacteria have rendered different results which may be due to the strains used. Lactobacilli and bifidobacteria are known to be potent modulators of the immune system. The capacity of these bacteria used as probiotics to influence both T<sub>H</sub>1 and T<sub>H</sub>2 mediated diseases has been shown before. However, the ability of strains to induce forkhead box P3 expressing (FOXP3<sup>+</sup>) regulatory T cells has not been investigated yet. We tested the inherent differences between strains in their capacity to induce functional regulatory T cells in human peripheral blood mononuclear cells PBMC. Therefore, human PBMC were co-cultured *in vitro* with either *Bifidobacterium lactis* W51, *Lactobacillus acidophilus* W55 or *L. plantarum* W62 or an *Escherichia coli* control strain. The percentage of FOXP3<sup>+</sup> cells, the origin of the induced cells and the functionality of these cells were assessed. Probiotic strains differed in their capacity to induce regulatory T cells. This was reflected by differences in the concentration of IL-10 found in the supernatant. No differences between lactic acid bacteria were found in IL-17, IFN- $\gamma$  or IL-13. FOXP3<sup>+</sup> cells were induced from CD25<sup>+</sup> cells and were able to suppress effector T cells. Naturally occurring regulatory T cells were not affected by co-culture with lactobacilli. We conclude that some probiotic strains are potent inducers of regulatory cells, while others are not. The clear differences between strains implicate that an *in vitro* characterization of probiotic strains prior to application is necessary.

**PB06/142 CD4+CD25+CD127LOW REGULATORY T CELLS IN PATIENTS WITH BEHÇET'S DISEASE**

F. İlhan<sup>1</sup>, N. Demir<sup>1</sup>, T. Demir<sup>2</sup>, A. Godekmerdan<sup>1</sup>

<sup>1</sup>Firat University, Immunology, Elazığ, Turkey, <sup>2</sup>Firat University, Ophthalmology, Elazığ, Turkey

Recently the low expression of CD127, a chain of interleukin 7 receptor, was described as another specific marker of regulatory T cells. CD4<sup>+</sup> CD25<sup>+</sup>CD127 low T cells in Behçet's disease were some higher than healthy controls. The current study investigates the role of regulatory T (Treg) cells in the pathogenesis of patients with Behçet's disease (BD) in ocular attack, inflammatory arthritis or mucocutaneous activation. Twenty-two BD patients and 15 healthy control were included in this study. Patient group was statistically compared with control group. BD patients had significantly higher CD4<sup>+</sup>CD25<sup>+</sup>CD127 low T cells, as compared with healthy controls ( $p < 0.05$ ). These findings were found similar to our research CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> cells on this group. These findings suggest that Treg cells play an important role in BD patients presented with different attacks.

**PB06/143** Abstract withdrawn by author

**PB06/144 CD4+CD25+FOXP3+ REGULATORY T CELLS SUPPRESS THE CD4+CD25- TH RESPONSE TO PLASMODIUM BERGHEI ANKA IN CEREBRAL MALARIA**

A.-L. Blanc<sup>1</sup>, O. Gorgette<sup>2</sup>, S. Pied<sup>3</sup>, P.-A. Cazenave<sup>1,3</sup>

<sup>1</sup>Institut Pasteur de Paris, Département d'Immunologie, Paris, France, <sup>2</sup>Institut Pasteur de Paris, Département de Parasitologie, Paris, France, <sup>3</sup>Institut Pasteur de Lille, Equipe PIME CNRS, Inserm U547, Lille, France

The role of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> naturally occurring regulatory T cells (nTreg) in the course of malaria, which involves both protective and pathogenic T cell responses, is still under scrutiny.

Using the model of *Plasmodium berghei* ANKA (PbA) infection, which leads to death by cerebral malaria (CM) in 80% of B6 mice within a week, we previously showed that CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T cells expand and acquire an activated phenotype during infection. Yet although these cells were able to suppress proliferative Th responses *in vitro*, early depletion using anti-CD25 antibody *in vivo* did not interfere with the neuropathogenesis.

However, the experimental protocol of CD25 depletion has since proven not to be Treg-specific, leaving us with incomplete answers.

To further decipher the role of nTreg in CM pathogenesis, we chose a reverse approach that consists of enriching in nTreg cells of either B6 [CM susceptible (CMS)] or B6-CD4KO [CM resistant (CMR)] mice by transferring splenic CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells on the day of infection. Foxp3-GFP<sup>+</sup> mice were used as donors for *in vivo* cell tracking in wild type B6 mice and for cell sorting.

Following transfer, naïve nTreg migrated to the spleen, where they expanded, but not to the brain. Surprisingly, transfer of nTreg accelerated the development of CM in a dose-dependent manner and also decreased survival of CMR mice. This was confirmed by *in vivo* suppression assays, where enrichment of nTreg inhibited the protective effect to CM mediated by transferred CD4<sup>+</sup>CD25<sup>+</sup> T cells. Finally, injection of nTreg previously activated *in vivo* by PbA also showed a pathogenic effect at lower doses. Currently, we are establishing the cytokine polarization of nTreg following activation by PbA *in vivo*.

Our results confirm that nTreg are activated and functional during PbA infection. To our knowledge, this is the first direct demonstration of a pathogenic role for nTreg in the development of cerebral malaria in the PbA infection model. One possible mechanism could be the suppression of the Th protective response.

**PB06/145 MICROGLIA INDUCED REGULATORY T CELLS (TREGS) SUPPRESS EAE**

F. Ebner<sup>1</sup>, P. Thiele<sup>1</sup>, B. Sawitzki<sup>2</sup>, R. Nitsch<sup>1</sup>, C. Brandt<sup>1</sup>

<sup>1</sup>Institute of Cell- and Neurobiology, Center for Anatomy, Charité, Berlin, Germany, <sup>2</sup>Institute of Immunology, Charité, Berlin, Germany

Microglial cells are the major immune competent cells of the brain. They are activated in various CNS affecting diseases and injury models including brain ischemia, entorhinal cortex lesion (ECL), or experimental allergic encephalomyelitis (EAE). Moreover, they play a crucial role in initiating innate and adaptive immune responses. The present study aims to elucidate the complex cross-talk between microglia and brain infiltrating immune cells, specifically the direct microglia – T cell interaction.

Here, we demonstrate for the first time that cultured microglia have the potential to induce CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup>, IL-10 and IL-13 secreting, regulatory T cells (Tregs). In detail, microglia cultured from C57BL/6-J mice were stimulated with 100 U/ml IFN $\gamma$  for 24h to express intermediate levels of MHCII. Subsequently, cells were pulsed with low levels (1  $\mu$ g/ml) of an EAE eliciting peptide such as myelin oligodendrocyte glycoprotein (MOG<sub>35-55</sub>) and co-cultured with CD4<sup>+</sup> T cells derived from T cell receptor (TCR) transgenic mice specific for MOG. Co-cultures of microglia and T cells were performed for further 7 days to induce Tregs. Interestingly, the induced relative frequency of Tregs versus activated effector cells depended directly on the amount of myelin antigen presented by microglia cells revealing only a tolerogenic phenotype of these cells when cultured with low doses of peptide. Stimulated and antigen primed microglia revealed intermediate expression of co-stimulatory molecule CD40 and iNOS. Moreover, after 7 days of co-culture CD40 expression on microglia was strongly downregulated whenever microglia induced a relatively high frequency of Tregs. In contrast, high levels of MHCII expression and high concentration of MOG peptide led to the induction of effector cells characterised by prolonged CD40 expression on microglia. We could also demonstrate neogenesis of microglia-induced Tregs by differentiating naïve and Treg depleted T cells into antigen-specific Tregs.

Finally, to test the regulatory potential of microglia-induced MOG-specific Tregs *in vivo*, adoptive transfer of Tregs into EAE models was performed. Therefore C57BL/6-J mice were immunized with MOG<sub>35-55</sub> peptide. Time point of disease onset and severity of EAE were diminished in recipient mice. In sum, our data demonstrate that activated microglia contribute to an antigen-dependent regulation of the adaptive immune response.

**PB06/146 CONTACT-DEPENDENT REGULATION OF THE MUCOSAL IMMUNE RESPONSE TO NEISSERIA MENINGITIDIS**

L.S. Brackenbury<sup>1</sup>, S.J. Glennie<sup>2</sup>, R.S. Heyderman<sup>2</sup>, N.A. Williams<sup>1</sup>

<sup>1</sup>Bristol University, Cellular and Molecular Medicine, Bristol, United Kingdom, <sup>2</sup>Malawi-Liverpool-Wellcome Trust Clinical Research Programme, Chichiri, Blantyre, Malawi

*Neisseria meningitidis* (Nm) is a gram-negative diplococci, carried by approximately 10% of the population (Caugant *et al.*, 1994) as a common commensal of the upper respiratory tract. Carriage is predominantly asymptomatic but can result in septicaemia and meningitis. Naturally acquired mucosal immunity to Nm peaks during the latter teenage years and is maintained in adults (Davenport *et al.*, 2003). T cell responses can also be detected in younger children following the depletion of CD25<sup>+</sup> cells from tonsillar but not peripheral blood mononuclear cells (Davenport *et al.*, 2007). We have characterised the mechanism of regulation to Nm by regulatory T cells (Treg) in order to define their antigen-specificity and to determine whether vaccination with Nm-derived outer membrane vesicles is likely to alter this regulation. Using tonsillar CD4<sup>+</sup> T cells from adults, we detect proliferative responses to Nm and influenza antigens, and now show that only the former is significantly regulated by antigen-specific CD4<sup>+</sup>CD25<sup>hi</sup> Treg in a contact-dependent manner. The profile of cytokines produced in these regulated cultures and the involvement of a range of individual Nm antigens has also been assessed. These data suggest that contact-dependent CD4<sup>+</sup>CD25<sup>hi</sup> Treg are induced peripherally by Nm mucosal colonisation.

Caugant *et al.*, 1994 J. Clin. Microbiol. 55: 887-896

Davenport *et al.*, 2003 J. Immunol. 171: 4263-70

Davenport *et al.*, 2007 Cell Microbiol. 9: 1050-61

**PB06/147 INFILTRATION OF REGULATORY T CELLS (TREGS) IN THE BRAIN AFTER TRAUMATIC INJURY**T. Stubbe<sup>1</sup>, D. Richter<sup>1</sup>, C. Meisel<sup>2</sup>, R. Nitsch<sup>1</sup>, C. Brandt<sup>1</sup><sup>1</sup>Charité, Center for Anatomy, Institute for Cell Biology and Neurobiology, Berlin, Germany, <sup>2</sup>Charité, Institute of Immunology, Berlin, Germany

Regulatory T cells (Tregs) play a major role in controlling immune responses under physiological conditions as well as in various systemic and central nervous system inflammatory diseases. However, little is known about the mechanisms of the induction of Tregs after traumatic brain injury. So far, investigations from several animal models such as entorhinal cortex lesion (ECL) revealed that although autoreactive T cells are present in traumatic CNS injury, white matter damage such as found in disseminated autoimmune encephalomyelitis, does not occur, even when highly susceptible mouse strains were employed. Thus, the initial expansion of myelin-specific T cells and subsequent inflammation in regions of (traumatic) axonal degeneration seems to be transient and eventually is kept under tight control, implying active maintenance of immune tolerance. Aim of this study was to investigate the temporary infiltration and localization of Tregs after ECL.

ECL was performed in Foxp3EGFP tg mice, and T cell and Treg infiltration was analyzed by flow cytometry on day 7, 14, and 30 after ECL. Tregs were identified by the expression of CD4 and Foxp3. We could find at each day a significant increase of T cells as well as Tregs in the ipsilateral hemisphere with a maximum on day 14 compared with infiltrates of sham operated mice. Additionally, an increase of Tregs in the CD4+/Tregs ratio in the ipsilateral hemisphere at day 14 compared to day 7 occurred. Interestingly, CD25 was downregulated in the Tregs subset on day 7 and 14, which raises the question whether proliferation or neogenesis of Tregs takes place in the brain. In order to localize Foxp3+ T cells in the brain, cryosections were stained with antibodies directed against CD3 and EGFP on day 14 after ECL. CD3+Foxp3+ cells were detected directly within the lesion and in the parenchyma adjacent to the lesion.

Further investigations are necessary to differentiate between infiltrations versus neogenesis and to determine the regulatory potential of the Tregs after ECL. Elucidating the trafficking and function of Tregs in the context of ECL may lead to a better understanding of general mechanisms involved in adaptive immune responses after CNS trauma.

**PB06/148 INFLUENZA-INDUCED TREG CELLS PREVENT PARTIAL CLONAL EXHAUSTION BUT CONTRIBUTE TO ENHANCED IMMUNOPATHOLOGY DURING SUBSEQUENT HETEROLOGOUS LCMV CLONE 13 INFECTION**A.R. Kraft<sup>1</sup>, M. Włodarczyk<sup>1</sup>, L. Kenney<sup>1</sup>, P. Durost<sup>1</sup>, L.K. Selin<sup>1</sup><sup>1</sup>University of Massachusetts Medical School, Pathology, Worcester, United States

Prior immunity to an unrelated pathogen in the form of heterologous immunity can dramatically alter the outcome to a subsequent viral infection influencing viral clearance and severity of immunopathology. Previous work has demonstrated that cross-reactive memory T cells activated during the second infection play a major role in mediating these effects. However, Treg cells have been shown to be important in modulating immune responses to viruses so we questioned whether Tregs induced during heterologous infections were also contributing to influencing disease outcome. Using a respiratory infection model, influenza-immune mice challenged with LCMV clone 13 have enhanced viral titers and severe pathology in the form of consolidating mononuclear pneumonia with bronchiolization while control mice only have mild pneumonitis. CD8 T cell responses have been found to play a major role in mediating this greatly increased immunopathology. Influenza-immune mice were noted to have an increased frequency of Tregs with a skewed Tcr repertoire in the lung as compared to naïve or LCMV-immune mice. When these influenza induced Tregs were depleted prior to LCMV infection the LCMV-specific CD8 T cell response appeared to be partially exhausted as demonstrated by decreased frequency, loss of TNF production and increased PD-1 expression. The immunopathology in the lung was greatly decreased consistent with the loss of highly activated CD8 T cells which would normally mediate the pathology. Treg depletion of naïve mice infected with LCMV clone 13 did not show any alteration in their CD8 T cell responses or immunopathology. These results suggest that Tregs induced during the influenza infection could temper the activation of LCMV clone 13 specific CD8 T cells preventing hyperactivation which could lead to partial exhaustion and potentially a persistent infection. It also suggests that Treg cells generated during a past infection can influence the balance between the qualitative characteristics of effector T cell responses and their ability to contribute to immunopathology and possibly viral clearance during a subsequent heterologous virus infection.

**PB06/149 A PERIPHERAL DEFECT OF REGULATORY T CELL HOMEOSTASIS IN PATIENTS WITH AUTOIMMUNE POLYENDOCRINOLOGY- CANDIDIASIS -ECTODERMAL DYSTROPHY**S.M. Salonen<sup>1</sup>, L.H. Rossi<sup>2</sup>, T.T. Laurinola<sup>1</sup>, J. Perheentupa<sup>3</sup>, T.P. Arstila<sup>2</sup><sup>1</sup>Helsinki Biomedical Graduate School and University of Helsinki, Immunology, Helsinki, Finland, <sup>2</sup>University of Helsinki, Immunology, Helsinki, Finland, <sup>3</sup>Helsinki University Hospital, Hospital for Children and Adolescents, Helsinki, Finland

**Objectives:** Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy, APECED, is a rare, monogenic disorder of recessive inheritance, caused by mutations in the AIRE gene. In Aire – knockout mice, ectopic transcription of tissue-specific antigens in the thymus is disturbed, leading to impaired negative selection. The mice however remain clinically healthy, suggesting further defects of tolerance in APECED patients. Notably, regulatory T (Treg) cells have been shown to be defective in patients but not in Aire-knockout mice, and this defect may thus contribute to the pathogenesis of APECED. Regulatory T cells develop in the thymus, but their maintenance in humans is at least partly dependent on peripheral conversion of conventional T cells into Treg cells. It is not known, whether the Treg cell defect in APECED patients reflects impaired thymic development, peripheral conversion, or activation. We have addressed this question by multicolor flow cytometric analysis of Finnish APECED patients.

**Methods:** We collected blood samples from 12 APECED patients and 12 age- and sex-matched controls. Peripheral blood mononuclear cells were isolated and stained with 9 different, directly labeled monoclonal antibodies. The samples were analyzed using the FACSAria instrument and the data analyzed using FACS Diva software. Statistical significance of the differences was calculated with Student's two-tailed t-test, using the SPSS software.

**Results:** The main analytical measure was FOXP3 expression level, previously shown to correlate with suppressive function. The mean fluorescence intensity of FOXP3 was significantly lower in APECED patients, confirming earlier findings. Interestingly, the difference was more pronounced in CD45RO+ Treg cells than in Treg cells expressing markers characteristic of recent thymic emigrants. Moreover, the data suggested that the peripheral turnover of Treg cells was increased in the patients.

**Conclusion:** These data indicate that in APECED the peripheral maintenance and conversion of Treg cells is impaired. This does not exclude the possibility that a thymic defect contributes to the observed differences, but suggests that peripheral factors may be more important. Our results elucidate novel aspects of the pathogenesis of APECED and hold lessons for more common autoimmune diseases, as well.

**PB06/150 POTENTIAL MECHANISM OF ADENOSINE REGULATING T CELL ACTIVATION**A. Yang<sup>1</sup>, M. Desrosiers<sup>1</sup>, A. Muchi<sup>1</sup>, Y. Shi<sup>1</sup><sup>1</sup>University of Calgary, Microbiology and Infectious Diseases, Calgary, Canada

Adenosine is a nucleoside present in all cells and body fluids of all living organisms. Its production, both intracellularly and extracellularly, is coupled to G-protein receptor resulting in increasing extracellular adenosine levels with changing energy consumption. Adenosine has been regarded as a crucial anti-inflammatory agent that protects the host from excessive damage. It has been shown that adenosine suppresses CD4<sup>+</sup> T cells activation by inhibiting IL-2 secretion. However, it is a general observation that induction of T cell activation is an efficient event despite the high adenosine levels that are often present in the affected host due to injury or stress. This study disclosed that prior to antigenic stimulation via TCR/CD3, exposure to T cells to adenosine desensitizes adenosine receptors, so as to create a window of several hours where T cells are "blind" to this ubiquitous suppressor. T cells from mice that were pre-exposed to this manipulation showed stronger responses to antigenic stimulation; therefore, the P1R desensitization demonstrated an adjuvant like effect. The results suggest that adenosine receptor desensitization may be an important mechanism for T cells to escape the general suppression during early points of T cell activation, and may present as a potential alternative of vaccine adjuvant.

**PB07 – B CELL SUBSETS****PB07/1 REGULATORY B CELLS SHAPE THE DEVELOPMENT OF TH2 IMMUNE RESPONSES IN BALB/C MICE INFECTED WITH LEISHMANIA MAJOR THROUGH IL-10 PRODUCTION**C. Ronet<sup>1</sup>, Y. Hauyon La torre<sup>2</sup>, M. Revaz-Breton<sup>2</sup>, B. Mastelic<sup>2</sup>, F. Tacchini-Cottier<sup>2</sup>, J. Louis<sup>2,3</sup>, P. Launois<sup>2</sup><sup>1</sup>WHO-IRTC, University of Lausanne, Département de Biochimie, Epalinges, Switzerland, <sup>2</sup>WHO-IRTC, University of Lausanne, Epalinges, Switzerland, <sup>3</sup>Institut Pasteur, Département de Parasitologie and Mycologie, Paris, France

Recent evidence indicates that B cells are required for susceptibility to infection with *Leishmania major* (*L. major*) in BALB/c mice. In this study, we analyzed the role of the IL-10 produced by B cells in this process. We first showed that B cells purified from the spleen of BALB/c mice produced IL-10 in response to stimulation with *L. major* in vitro. In vivo, early IL-10 mRNA expression is detected in B cells from draining lymph nodes of susceptible BALB/c but not of resistant C57BL/6 mice after *L. major* infection. Although adoptive transfer of naïve wild type B cells prior to infection in B cell deficient BALB/c mice restored Th2 cell development and susceptibility to infection with *L. major* of these otherwise resistant mice, adoptive transfer of IL-10<sup>-/-</sup> B cells mice did not. Both in vitro and in vivo, the B cells producing IL-10 in response to *L. major* stimulation expressed the CD1d and CD5 molecules suggesting that these B cells are regulatory B cells. Finally the IL-10 produced by B cells in response to *L. major* downregulated IL-12 production by *L. major* stimulated dendritic cells. Altogether these results indicate that the IL-10 produced by regulatory CD1d<sup>+</sup> CD5<sup>+</sup> B cells in response to *L. major* stimulation is critical for Th2 cell development in BALB/c mice by regulating the IL-12 production.

**PB07/2 IL-10 PRODUCED BY NATURAL T2B REGS IS CRUCIAL FOR THE INDUCTION OF TR1 AND AND FOR THE SUPPRESSION OF TH17 BUT NOT FOR THE DE NOVO GENERATION OF T2BREGS**N.A. Carter<sup>1</sup>, R. Vasconcellos<sup>1</sup>, C. Tulone<sup>2</sup>, E.C. Rosser<sup>1</sup>, M.R. Ehrenstein<sup>1</sup>, D. Gray<sup>3</sup>, C. Mauri<sup>1</sup><sup>1</sup>University College London, Division of Medicine, Department of Inflammation, London, United Kingdom, <sup>2</sup>University College London, Department of Virology, London, United Kingdom, <sup>3</sup>University of Edinburgh, Institute of Immunology and Infection Research, Edinburgh, United Kingdom**Objectives:** The immune system contains natural regulatory cells important in the maintenance of tolerance. Although this suppressive function is usually attributed to CD4+ Tregs, we and others have shown that IL-10-producing B cells are equally important in the maintenance of tolerance. To discern the effect that IL-10 produced specifically by Bregs, has on T cell differentiation and pro-inflammatory cells such as Th1 and Th17, important modulators of autoimmunity, we generated chimeric mice that lack IL-10 specifically on B cells.**Methods:** We generated mice with IL-10 deficient B cells by adoptive transfer of a bone marrow mixture 80% from B cell null and 20% from IL-10-/- mice into an irradiated DBA/1 recipient. Therefore all B cells lacked the capacity to secrete IL-10 whilst the majority of other cells in the immune system had a normal phenotype. Mice were then immunized with collagen (CII) emulsified in Complete Freund's adjuvant (CFA). Cytokine secretion and cell phenotype were analyzed by FACS analysis, ELISA, in vivo cytokine capture assay and thymidine incorporation assays. Clinical score, antibody production, histology and paw swelling assessed the disease severity of the mice.**Results:** Here we demonstrate that IL-10 produced by B cells is essential in dampening Th17 and Th1 responses and in promoting the generation of CD4+FoxP3-IL-10+ T cells. In addition, upon immunization with CII in CFA IL-10 B cell KO mice develop an exacerbated arthritis compared to IL-10+ B cell mice. No difference in the absolute number of Bregs was detected between the IL-10 B cell KO and WT B cell mice.**Conclusions:** These data suggest that IL-10 produced by Bregs is integral in the induction of Tr1 regulatory cells and that IL-10 produced by B cells inhibit Th17 and Th1 development.**PB07/3 POLYCHROMATIC IMMUNOPHENOTYPING AND PROBABILITY BINNING ALGORITHM REVEAL DISTINCT DISEASE CLUSTERS IN PATIENTS WITH COMMON VARIABLE IMMUNODEFICIENCY**T. Kalina<sup>1,2</sup>, J. Stuchly<sup>1,2</sup>, A. Janda<sup>1,2</sup>, S. Ruzickova<sup>3</sup>, J. Litzman<sup>4</sup>, A. Sediva<sup>5</sup>, M. Vlkova<sup>4</sup><sup>1</sup>Charles University Prague 2nd Faculty of Medicine, Pediatric Hematology Oncology, Prague, Czech Republic, <sup>2</sup>CLIP, Childhood Leukemia Investigation Prague, Czech Republic, <sup>3</sup>Institute of Biotechnology of Academy of Sciences of the Czech Republic, p. r. i., Laboratory of Diagnostics of Autoimmune Diseases, Prague, Czech Republic, <sup>4</sup>St. Anne University Hospital in Brno, Dept. of Immunology, Brno, Czech Republic, <sup>5</sup>Motol University Hospital, Dept. of Immunology, Prague, Czech Republic**Objectives:** Common Variable Immunodeficiency (CVID) is a heterogeneous disorder with defined molecular defect in only a few cases. Currently used CVID classification EUROClass [1] is based on quantification of selected B-cell subsets.

Using 6-colour polychromatic flow cytometry, we analyzed B-cell phenotype in a cohort of 47 patients and 50 controls. The aim of the study was to find an expert free approach for phenotype analysis covering all differences in the 6-colour space and form groups of patients with similar phenotype.

**Methods:** We used "probability binning" [2] algorithm to create 1024 bins (i.e. six-colour gates) that optimally covered the cells' distribution within the entire B-cell compartment. We created a matrix file recording cellular content in all bins.

The hierarchical clustering of the individual samples was analyzed using Pearson correlation of the bins' values.

**Results:** The Cut Tree algorithm has found 11 clusters, six of them dominated by healthy individuals, one by EUROClass IA patients, two by EUROClass IB and IIB, remaining two were mixed.

The overall reproducibility of this approach was evaluated by testing replicates of two samples from the same donor 98 to 336 days apart. When the B-cell profile of replicates was used to match the original cohort using the similarity matrix of Pearson correlation, 15 replicates matched the same individual, 3 replicates matched a different individual within the same cluster and 3 replicates matched to a different cluster. We were able to define B cell subsets over- or under-represented in a particular cluster and display them back in the flow-cytometry software thus resembling manually created gating.

**Conclusion:** We describe a new analytical approach enabling us to search in an expert-free environment for patients' cohorts defined by similar B-cell profiles and thus contribute to description of differences between CVID patients' groups.

(1) Roederer – Cytometry 2001

(2) Wehr – Blood 2008

This work was supported by grants IGA NR/9198-3 and MZCR 000064203.

**PB07/4 B-1 CELLS ALTERED GENE EXPRESSION PROFILE IN MURINE METASTATIC MELANOMA CELLS**P. Xander<sup>1</sup>, R.R. Novaes e Brito<sup>1</sup>, F. Aliperti<sup>1</sup>, R. Pellegrino<sup>2</sup>, M.G. Jasiulonis<sup>3</sup>, V. Bernardo<sup>4</sup>, M. Mariano<sup>1</sup>, J.D. Lopes<sup>1</sup><sup>1</sup>Universidade Federal de São Paulo, Departamento de Microbiologia, Imunologia e Parasitologia, São Paulo, Brazil, <sup>2</sup>Universidade Federal de São Paulo, Departamento de Psicobiologia, São Paulo, Brazil, <sup>3</sup>Universidade Federal de São Paulo, Departamento de Farmacologia, São Paulo, Brazil, <sup>4</sup>Universidade Federal de São Paulo, Departamento de Informática em Saúde, São Paulo, Brazil**Introduction:** Recent technological advances in the analysis of the gene expression pattern in cancer have improved the understanding the mechanisms underlying the process of metastatic progression. Despite this, the acquisition of invasive behaviour in melanoma is poorly understood. In the tumor microenvironment, inflammatory cells can influence almost every aspects of cancer progression, but the knowledge on the cellular and molecular mechanism are not completely clarified. Recent studies have attributed an important role to B-1 cells, a subset group of B lymphocytes, in melanoma progression. In vitro heterotypic cell-cell interaction between B16 melanoma cells and B-1 lymphocytes induced increase in metastatic potential of B16 lineage. In addition, B-1 cells led to up-regulation in melanoma cells of several molecules involved with metastasis as well as increased phosphorylation levels of ERK/MAPK.**Objective:** Using microarray approach we assessed gene expression profiling in melanoma cells before and after contacting B-1 lymphocytes to identify genes involved with metastatic progression.**Methods:** We used gene microarray analysis to compare melanoma cells before and after B-1 cells contact. RNA samples were hybridized to the mouse genome Affymetrix GeneChip 430-2Array. Probe signal summarization, normalization, and background subtraction were performed using robust multichip analysis. We performed the statistical tests for differentially expressed genes using the Significance Analysis of Microarrays (SAM). Finally, we identified genes with significant different expression in biologic comparison.**Results:** First, we performed in vitro experiments of co-culture between B-1 lymphocytes and B16 melanoma cells. We observed that both cells physically interact, and that led to increased metastatic potential in B16 lineage in an experimental metastasis model. RNA of B16 cells, before and after co-culture, were extracted and used for microarray analysis. Screening of the 30,000 genes identified 100 genes differentially expressed in melanoma cells after B-1 lymphocytes contact. Some genes selected were involved with metastatic progression, such as MMP12, CTSS, CTSH, CD74, STAT3, FN1 and LGALS3.**Conclusion:** We found up-regulation of many genes related with metastatic progression after B16 cells contacting B-1 lymphocytes. These findings can contribute to elucidate the biological effect induced by B-1 lymphocytes in melanoma cells and eventually to the molecules responsible for the process.**PB07/5 SPECIALLY B-LYMPHOCYTES FROM PATIENTS WITH SJÖGREN'S SYNDROME SHOW A DYSREGULATION IN THE GENE EXPRESSION OF COMPONENTS OF THE PROTEASOME SYSTEM**L. Martinez Gamboa<sup>1</sup>, K. Lesemann<sup>1</sup>, S. Scheffler<sup>1</sup>, U. Kuckelkorn<sup>2</sup>, K. Egerer<sup>3</sup>, T. Dörner<sup>4</sup>, G.R. Burmester<sup>5</sup>, D.L. Faustman<sup>6</sup>, E. Feist<sup>6,7</sup><sup>1</sup>Charité Universitätsmedizin Berlin, Forschungslabor Rheumatologie, AG Feist, Berlin, Germany, <sup>2</sup>Charité Universitätsmedizin Berlin, Dept. of Biochemistry, Berlin, Germany, <sup>3</sup>Charité Universitätsmedizin Berlin, Rheumatologisches/Immunologisches Labor, Berlin, Germany, <sup>4</sup>Charité Universitätsmedizin Berlin, Inst. f. Transfusionsmedizin, Berlin, Germany, <sup>5</sup>Charité Universitätsmedizin Berlin, Rheumatologie und Klinische Immunologie, Berlin, Germany, <sup>6</sup>Massachusetts General Hospital, Harvard Medical School, Immunobiology Laboratory, Charlestown, United States, <sup>7</sup>Charité Universitätsmedizin Berlin, Rheumatologie und Klinische Immunologie, Berlin, Germany**Background:** A dysregulation in expression of some components of the proteasome system in patients with primary Sjögren's syndrome (pSS) has been already shown in total peripheral blood mononuclear cells (PBMCs), but it is not yet known which specific blood cell subsets are involved. Due to the central role of the proteasome for the immune response through antigen processing/presentation and in apoptosis, elucidation of the involved cell subset(s) in relation to the proteasome alteration could be important for understanding of disease pathogenesis.**Objectives:** To compare gene expression of constitutive and inducible catalytic subunits of the 20S proteasome in isolated blood immune competent cellular subsets. Additional investigations in vitro included analysis of proteasome activity and of apoptosis after exposure to the proteasome inhibitor Bortezomib.**Methods:** CD4+ and CD8+ T-lymphocytes, CD19+ B-lymphocytes, CD14+ monocytes and total dendritic cells were sorted from peripheral blood samples of patients and controls. Transcript levels of proteasome system components, including the constitutive catalytic subunits Delta, Z and MB1, and their corresponding inducible ones LMP2, MECL and LMP7, were relative quantified by real time PCR. For statistic analysis, Mann-Whitney test and P-values < 0.005 were applied.**Results:** Specially B-lymphocytes from pSS showed a marked activation of the proteasome system, with significant up-regulation of all constitutive and inducible subunits. Apart from B-cells, transcript levels of LMP7 were also highly increased in CD8+ T-cells and in monocytes from patients, reflecting the systemic autoimmune process. Preliminary data from further in vitro studies confirm the dysregulation of the proteasome in B-cells: after exposition to Bortezomib, proteasome activity is reduced in a similar degree in PBMCs and T-lymphocytes, but to a lesser extent in B-lymphocytes, and apoptosis is stronger induced in total PBMCs and T-cells than in B-cells.



**Conclusion:** Our results show that the proteasome system is strongly activated specially in B-lymphocytes from patients with Sjögren's syndrome. In general, B-cells seem to be more resistant to the effects of proteasome inhibition by Bortezomib. This results should encourage further investigations in the field of proteasome inhibition in patients with Sjögren's syndrome.

**PB07/6 INTERLEUKIN-10 KNOCK-OUT MICE B-1 CELLS DO NOT INCREASE THE METASTATIC POTENTIAL OF B16 MURINE MELANOMA CELLS AN IN VITRO CO-CULTURE MODEL**

E. C. Pérez<sup>1</sup>, J. J. Machado<sup>1</sup>, M. Mariano<sup>1</sup>, J. D. Lopes<sup>1</sup>

<sup>1</sup>Universidade Federal de São Paulo – UNIFESP, Microbiology, Immunology and Parasitology, São Paulo, Brazil

B-1 cells are the prevalent lineage of B cells in the peritoneal and pleural cavities of adult mice. These cells have a strong self-renewal capacity and are one of the main sources of interleukin-10 (IL-10). Moreover, IL-10 is involved in B-1 cells survival, suggesting a positive autocrine regulatory loop. Previous studies in our group demonstrated that co-cultivation of B-1 cells with B16 murine melanoma cells increases the metastatic potential of the latter and physical contact between these two cells induces augment the levels of phospho-extracellular signal regulated kinase (p-ERK) in B16 cells. Since B-1 cells constitutively secrete IL-10 and IL-10 can promotes ERK activation, we asked whether this interleukin also plays a role in the increased metastatic effect of B16 melanoma cells after contact with B-1 lymphocytes. To test this, B16 melanoma cells were co-cultured for 48 h with B-1 cells either from wild-type or IL-10 knock-out C57BL6 mice. After this period, B16 melanoma cells were isolated from B-1 lymphocytes and injected in the tail vein of both wild-type and IL-10 knock-out C57BL6 mice for evaluation of pulmonary nodules as indicative of metastases. We showed that, irrespective of mice genetic background, a significant increase in the number of pulmonary nodules was observed only when B16 melanoma cells were co-cultured with B-1 cells from wild-type but not from IL-10 knock-out mice. In addition, B-1 cells from IL-10 knock-out mice did not induce increase in the levels of p-ERK in melanoma cells after co-culture both cells. These results suggest that IL-10 favors the effect of B-1 cells to increase the metastatic potential of murine melanoma cells induced by previous contact with B-1 lymphocytes.

**PB07/7 DISTURBED PERIPHERAL B LYMPHOCYTE HOMEOSTASIS IN CHRONIC SARCOIDOSIS**

N. Lee<sup>1</sup>, Y. P. Kataria<sup>1</sup>, M. J. Thomassen<sup>1</sup>, M. S. Kavuru<sup>1</sup>, S. Arce<sup>1</sup>

<sup>1</sup>Brody School of Medicine at East Carolina University, Internal Medicine/Pulmonary, Greenville, United States

Sarcoidosis is a prototypical cell-mediated immunological disorder characterized by granuloma development and production of inflammatory cytokines by activated macrophages and CD4<sup>+</sup> T-cells. In spite of the predominant involvement of cellular immunity in the pathogenesis of this disease, sarcoidosis frequently associates with hypergammaglobulinemia, autoantibody production, and circulating immune complexes. These humoral disturbances are thought to be a "by product" of the presence of activated T-cells at the sites of disease activity. It is therefore assumed that B-cells play little role in the pathogenesis of sarcoidosis. B-cells, however, can regulate inflammatory conditions independently of their ability to produce antibodies. We thus hypothesized that B-cell could regulate granuloma formation in sarcoidosis via mechanisms that does not depend on antibody production (i.e. antigen presentation and cytokine production). As a first step to elucidate this, we studied the distribution of peripheral B-cell populations in sarcoid patients using flow cytometry (FC). The FC analysis relied on the expression of IgD and CD27 on B-cells. Western blotting was also utilized to study signaling pathways in purified preparations of sarcoid B-cells. Compared to healthy controls (n=15), sarcoid patients (n=20) exhibited significant B-cell lymphopenia ( $5.5 \pm 2.1$  vs  $3.7 \pm 2.4$ ,  $p=0.009$ ), and significantly increased frequencies of double negative (CD19<sup>+</sup>IgD<sup>+</sup>CD27<sup>+</sup>) B-cells ( $2.6 \pm 1.5$  vs  $4.3 \pm 3.1$ ,  $p=0.028$ ), and plasmablasts (CD19<sup>+</sup>CD20<sup>+</sup>CD27<sup>+</sup>) ( $0.6 \pm 0.5$  vs  $1.7 \pm 2.2$ ,  $p=0.038$ ). Frequencies of unswitched memory (CD19<sup>+</sup>IgD<sup>+</sup>CD27<sup>+</sup>) B-cells ( $14.4 \pm 8.8$  vs  $7.4 \pm 7.8$ ,  $p=0.01$ ), were significantly reduced in sarcoid patients. Sarcoid B-cells also showed drastic reductions in the intracellular content of phosphoproteins, and in the levels of the p65 subunit of NF- $\kappa$ B. These B-cell anomalies concurred with significantly increased frequencies of central (CD4<sup>+</sup>CD45RA<sup>+</sup>CD62L<sup>+</sup>) ( $33.9 \pm 9.4$  vs  $46.5 \pm 11.5$ ,  $p<0.0001$ ), and effector (CD4<sup>+</sup>CD45RA<sup>+</sup>CD62L<sup>+</sup>) ( $8.0 \pm 2.5$  vs  $15.3 \pm 10.1$ ,  $p=0.009$ ) memory T-cells that, after CD3/CD28 cross-linking, expressed enhanced levels of HLA-DR ( $15.3 \pm 2.1$  vs  $29 \pm 4.5$ ,  $p=0.094$ ), and of the B-cell co-stimulatory molecules CD134 ( $113.4 \pm 11.6$  vs  $153.9 \pm 14.83$ ,  $p=0.0439$ ) and CD278 ( $39.2 \pm 5.4$  vs  $56 \pm 10.2$ ,  $p=0.1362$ ). Our results strongly suggest ongoing (auto) immune responses with imprints of B-cell-T-cell collaboration in sarcoidosis.

**PB07/8 THE HUMAN IMMUNOMODULATORY CD25<sup>+</sup> B CELL POPULATION BELONGS TO THE MEMORY B CELL POOL**

S. Amu<sup>1</sup>, A. Tarkowski<sup>1</sup>, T. Dörner<sup>2</sup>, M. I. Bokarewa<sup>1</sup>, M. Brissler<sup>1</sup>

<sup>1</sup>Sahlgrenska Academy at University of Gothenburg, Rheumatology and Inflammation Research, Gothenburg, Sweden, <sup>2</sup>Deutsches Rheumaforschungszentrum Berlin, Berlin, Germany

**Objective:** We have shown that human CD20<sup>+</sup>25<sup>+</sup> B cells display immunomodulatory properties. The aim of this study was to investigate if CD25<sup>+</sup> B cells are found within the CD27 memory B cell population, and to analyse pattern of their cytokine production.

B cells isolated from healthy subjects.

**Material and methods:** RA- and SLE patients were analysed regarding the frequency of CD25<sup>+</sup> B cells within certain B cell subsets. Purified CD25<sup>+</sup> B cells from healthy subject were used in vitro to evaluate their production of immunomodulatory cytokines.

**Results:** In healthy subjects the majority (60%) of memory B cells (CD20<sup>+</sup>27<sup>+</sup>) also co-expressed CD25 while only 10-20% of the naïve B cells (CD20<sup>+</sup>27<sup>-</sup>) and plasmablasts (CD20<sup>+</sup>27<sup>-</sup>) expressed CD25. In RA and SLE patients, we found that 51% and 48%, respectively, co-expressed CD25 in the memory population, whereas only 11% and 9% co-expressed CD25 in the naïve B cell population.

Phenotypic analysis of the CD20<sup>+</sup>25<sup>+</sup>27<sup>+</sup> and CD20<sup>+</sup>25<sup>+</sup>27<sup>-</sup> cells using CD10, CD24, CD38, CD45, CD71, CD80, CD86, CD95, CD138, BAFF-R, TACI, IgA, IgD, IgG and IgM showed that CD20<sup>+</sup>25<sup>+</sup>27<sup>+</sup> B cells preferentially represent highly activated, Ig class switched memory B cells.

Cytokine profile analysis showed that CD25<sup>+</sup> B cells secreted significantly higher levels of IL-10 vs. CD25<sup>-</sup> B cells. In contrast, TGF- $\beta$ 1 secretion was similar between the CD25<sup>+</sup> and CD25<sup>-</sup> sub-populations.

**Conclusion:** CD20<sup>+</sup>25<sup>+</sup> B cells constitute a unique subpopulation preferentially occurring among CD20<sup>+</sup>27<sup>+</sup> memory B cells. We suggest that CD25 can be used as a marker for a memory B cell subset.

**PB07/9 GENE EXPRESSION STUDIES IN B-1 CELL FROM LUPUS PRONE-MICE**

R. R. Novaes e Brito<sup>1</sup>, P. Xander<sup>1</sup>, R. Aliperti<sup>1</sup>, R. Pelegrino<sup>2</sup>, M. G. Jasiulionis<sup>3</sup>, V. Bernardo<sup>4</sup>, J. D. Lopes<sup>1</sup>, M. Mariano<sup>1</sup>

<sup>1</sup>Universidade Federal de São Paulo, São Paulo, Brazil, <sup>2</sup>Universidade Federal de São Paulo, Psicobiologia, São Paulo, Brazil, <sup>3</sup>Universidade Federal de São Paulo, Farmacologia, São Paulo, Brazil, <sup>4</sup>Universidade Federal de São Paulo, Informática em Saúde, São Paulo, Brazil

**Introduction:** B-1 cells participate in various autoimmune diseases. These cells, typically IgM<sup>high</sup>, IgD<sup>low</sup>, CD23<sup>+</sup>, CD43<sup>+</sup>, CD19<sup>+</sup>, CD11b<sup>+</sup>, are found mainly in peritoneal and pleural cavities in mice and are known to be radiosensitive. (NZB/NZW)F1 mice develop an autoimmune condition resembling human Systemic Lupus Erythematosus (SLE). We have investigated the role of B-1 cells in the genesis of SLE in these animals using an irradiation prophylactic protocol. Unexpectedly, B-1 cells were not depleted in (NZB/NZW)F1, despite several rounds of irradiation. We found that radioresistant B-1 cells from these mice overexpress the anti-apoptotic protein Bcl-2 (e Brito *et al.* 2007, Lupus 16, 947-954). However, even though these cells seemed to be physiologically unaltered by radiation, the data on the participation of B-1 cells in SLE in this model are not conclusive.

**Objectives:** Using microarray technology, we analyzed expression of genes putatively involved in SLE and radioresistance in B-1 cells from irradiated and non-irradiated (NZB/NZW)F1 mice.

**Methods:** B-1 cells RNA samples were hybridized with the mouse genome AffymetrixGeneChip 430Array. Probe signal summarization, normalization, and background subtraction were performed using robust multichip analysis. Statistical test for differentially expressed genes using the Significance Analysis of Microarrays was performed. Genes with significantly different expression were selected.

**Results:** Within ~30,000 genes tested by microarray, the expression of 34 genes in the irradiated mice differed significantly from non-irradiated. Eight of these genes are involved in apoptosis. Five pro-apoptotic and two anti-apoptotic genes were up-regulated, and 1 gene anti-apoptotic was down-regulated in irradiated animals.

**Conclusion:** This study is the first to examine the global gene expression profile of the B-1 cells in the (NZB/NZW)F1 background. Our results point to two possibilities:

- (1) There is a peculiar adjustment between pro- and anti-apoptotic factors in the B-1 cells of irradiated animals, leading to greater survival, or
- (2) There are two sub-populations of B-1 cells in (NZB/NZW)F1 mice, one being radioresistant and the other sub-population responding to radiation by undergoing apoptosis.

These data increases the understanding of radioresistance mechanisms in these B-1 cells and will be decisive for the elucidation of SLE genesis in this model.

**PB07/10 AUTOREACTIVE ANTI-DNA TRANSGENIC B CELLS IN LUPUS-PRONE NZB/NZW MICE SHOW NEAR PERFECT L-CHAIN ALLELIC EXCLUSION<sup>1</sup>**

E. Makdasi<sup>1</sup>, R. Fischel<sup>1</sup>, I. Kat<sup>1</sup>, D. Eilat<sup>1</sup>

<sup>1</sup>Hebrew University-Hadassah Medical School, Medicine, Jerusalem, Israel

Recent work on B cell tolerance and autoimmunity has suggested the L-chain allelic inclusion is a property of autoreactive B cells and is closely linked to receptor editing. Allelic inclusion could rescue autoreactive B cells from clonal deletion by reducing their effective BCR surface density. We have investigated this phenomenon in anti-DNA producing hybridomas, derived from different strains of immunoglobulin (Ig) gene-targeted, lupus-prone NZB/NZW mice. Our results indicate that isotype and allelic exclusion was strictly maintained in most high and low affinity, edited and non-edited anti-DNA transgenic B cells. However, a substantial fraction of the anti-DNA hybridomas expressed a very restricted set of non-productively rearranged L-chain mRNA, in addition to the productive anti-DNA L chain. The aberrant L chains could have a role in the selection and survival of autoreactive B cells in these autoimmune mice.

## PB07/11 ONTOGENY OF CD19CD5 EARLY IN THE LIFE OF HIV-1 EXPOSED INFANTS

B. M. Abramczuk<sup>1</sup>, I. Lorand-Metze<sup>1</sup>, E. Borges-Almeida<sup>1</sup>, F. G. P. Cunha<sup>1</sup>, H. Milanez<sup>1</sup>, K. Metzke<sup>1</sup>, M. T. N. da Silva<sup>1</sup>, M. M. S. Vilela<sup>1</sup><sup>1</sup>State University of Campinas, Campinas, Brazil

**Introduction and Objectives:** Exposure in utero to HIV proteins, maternal cytokine transplacental transfer, as well as the use of HAART by the mother, affects the ontogeny of the immune system. Thus we evaluated CD19+, CD19+CD5+, CD3+CD4+ and CD3+CD8+ cells in vertically HIV exposed children in their cord blood and at the 7-27 months of age.

**Methods and Results:** We investigated the cord blood from newborns of 36 HIV+ mothers (9 were drug user) and 15 newborns of healthy mothers. Among the HIV exposed group, we studied 21 infants (7-23 months) when their diagnosis was defined as not infected. We performed immunophenotyping using monoclonal antibodies combinations (CD5/CD19/CD45 and CD8/CD4/CD3) and data were acquired in a FACS Calibur (Beckton Dickinson) flow cytometer. B lymphocytes were increased in the newborns of HIV+ mothers, especially of drug users, due to an increase in CD19+/CD5+ cells. The medians of CD19+CD5+ lymphocyte in cord blood were 59.5 (42.8-91.1) for the controls and 75.8 (47.7-97.0) for newborns from HIV+ mothers ( $p = 0.006$ ). The median percentage of CD19+CD5+ in the peripheral blood of the 21 infants (62.46 (17.99-96.98)) was different of that one present in their cord blood (75.8 (48.58-96.98)) ( $p = 0.01$ ). There was no correlation between the CD19CD5+ percentage and the age of the children ( $p = 0.524$ ). In the cord blood, the percentage of CD3+CD8+ cells was different between the control group and the newborns of HIV+ mothers (Kruskall Wallis' test,  $p = 0.04$ ). The percentage of CD3+CD4+ cells from the HIV exposed uninfected group showed difference between the peripheral blood of the infants and their cord blood ( $p = 0.026$ ).

**Conclusion:** Newborns from HIV+ mothers present alteration in T and B subsets. The high percentage of CD19/CD5+ cells present in the cord blood from HIV exposed newborns persists in some infants. These changes can interfere with the ability to respond to T-cell dependent antigens and may affect effectiveness of neonatal vaccination.

**Financial Support:** FAPESP and CNPq

PB07/12 CHARACTERIZATION OF *BORDETELLA PERTUSSIS* PROTEIN-SPECIFIC MEMORY B-CELL RESPONSES IN VACCINATED CHILDRENL. H. Hendriks<sup>1,2</sup>, L. de Rond<sup>1</sup>, G. A. M. Berbers<sup>1</sup>, E. A. M. Sanders<sup>3</sup>, A. M. Buisman<sup>1</sup><sup>1</sup>National Institute for Public Health and the Environment (RIVM), LIS, Bilthoven, Netherlands, <sup>2</sup>Spaarne Hospital Hoofddorp, Pediatrics, Hoofddorp, Netherlands,<sup>3</sup>University Medical Centre Utrecht; Wilhelmina Children's Hospital, Pediatric Immunology, Utrecht, Netherlands

**Objective:** Antibody levels against *Bordetella pertussis* vaccine antigens wane rapidly after immunization. In addition, whooping cough is re-emerging worldwide, with peak incidences every 3 years in the Netherlands. Long-term cellular memory immunity might play a major role in protection against pertussis. In this study we identified numbers of pertussis protein-specific memory B-cells and their relation to IgG responses produced by plasma cells in children.

**Methods:** Children 3 to 9 years of age who were vaccinated according to the Dutch National Immunization program participated in a cross-sectional study performed in 2007 to 2008. B-cells were purified from PBMCs and polyclonally stimulated by CpG via TLR9. After 5 days antibody-secreting cells were detected by protein-specific ELISpot assays. B-cells were characterized by FACS analysis of CD19, CD20, CD27 and CD38 expression. Plasma IgG levels and avidities to pertussis-specific proteins were measured by fluorescent bead-based multiplex immunoassay.

**Results:** Two to three years after pertussis vaccination in the first year of life, IgG responses to the pertussis proteins were low. At day 10 after booster vaccination at 4 years of age the IgG levels and avidity indexes to the pertussis antigens had increased and remained high at day 28 after vaccination. PBMCs of children contained  $13 \pm 3\%$  B-cells (CD19+/CD20+). After polyclonal stimulation the population of memory B-cells (CD19+/CD27+) increased from  $27 \pm 19\%$  to  $46 \pm 6\%$ . In the stimulated memory B-cell population we found  $15 \pm 3\%$  IgG producing cells. From this total IgG-producing memory B-cells, we identified low amounts of pertussis protein-specific cells compared to tetanus toxoid specific cells.

**Conclusion:** In conclusion, although numbers were low, *Bordetella pertussis* specific memory B-cells could be determined in children in which plasma pertussis-specific IgG levels had waned. These children showed antibody booster responses which confirm the existence of memory B-cells. No correlation between pertussis specific memory B-cell numbers and pertussis IgG levels in plasma was found, indicating that memory B-cells and plasma cells are two distinct B-cell populations.

## PB07/13 LACK OF IL-12 AND IL-35 SECRETION DETERMINES RESISTANCE TO ANTIGEN INDUCED ARTHRITIS THROUGH INHIBITION OF TH17 RESPONSE AND INCREASE IN THE REGULATORY T AND B CELL POPULATIONS

R. Vasconcellos<sup>1,2</sup>, N. A. Carter<sup>1</sup>, C. A. Notley<sup>1</sup>, E. C. Rosser<sup>1</sup>, C. Mauri<sup>1</sup><sup>1</sup>University College London, Division of Medicine, Department of Inflammation, London, United Kingdom, <sup>2</sup>Universidade Federal Fluminense, Departamento de Imunobiologia, Rio de Janeiro, Brazil

**Objectives:** Despite sharing the common p35 chain, IL-12 and IL-35 have contrasting biological effects in the regulation of immune responses. Whereas IL-12 is pivotal in the polarization of Th1 driven immune responses, IL-35 is essential in the maximal suppressive activity exerted by regulatory T cells. Mice deficient in IL-12p35 have been shown to be more susceptible to intracellular parasite infections and are highly susceptible to the induction of autoimmune diseases including collagen induced arthritis and experimental autoimmune encephalomyelitis. In the present study we investigate the deficiency in the IL-12p35 chain to define the requirement for p35 chain signaling in the development and function of Th17 and Th1 response cells in a model of acute inflammation (antigen-induced arthritis).

**Methods:** C57BL/6 mice deficient in IL-12p35 (p35KO) and wild-type were intradermally immunized with methylated BSA (mBSA) emulsified with CFA and arthritis was induced by intraarticular inoculation of mBSA in one of the knees. Clinical score, antibody production, histology and knee swelling assessed the disease severity. Expansion of antigen-specific lymphocytes, cytokine secretion and cell phenotype were analyzed by flow cytometry, ELISA and thymidine incorporation assays.

**Results:** We report here that in the absence of IL-35 and IL-12, mice displayed reduced arthritis and inflammation compared to wild type. Th17, but not Th1, development was significantly impaired during the development of arthritis. In addition, both T2 regulatory B cell subset and the FoxP3+ CD25+ regulatory T cells were numerically increased in the p35KO mice compared to the wild type.

**Conclusion:** Contrary to the previously reported effects of IL-35, our results suggest that the deficiency in both IL-12 and IL-35 secretion determines resistance to antigen induced arthritis through a remarkable decrease in Th17 differentiation and an increase in the regulatory lymphocytes population likely involved in the inhibition of inflammatory mediators.

## PC01 – GENETICS OF AUTOIMMUNITY

## PC01/1 THE DISEASE SUSCEPTIBILITY IN A NEW 'HUMANIZED' MULTIPLE SCLEROSIS MODEL IN HLA-DR15 (DRB1\*1501;DQB1\*0602) TRANSGENIC MICE IS DETERMINED BY HLA-DQB1\*0602

N. Kaushansky<sup>1</sup>, D. M. Altmann<sup>2</sup>, C. S. David<sup>3</sup>, H. Lassman<sup>4</sup>, A. Ben-Nun<sup>1</sup><sup>1</sup>The Weizmann Institute of Science, Rehovot, Israel, <sup>2</sup>Imperial College, Hammersmith Hospital, London, United Kingdom, <sup>3</sup>Mayo Clinic, Rochester, MN, United States, <sup>4</sup>University of Vienna, Vienna, Austria

Multiple sclerosis (MS) is an autoimmune disease of the central nervous system (CNS) characterized by perivascular inflammation accompanied by primary demyelination, axonal damage, and neuronal loss. Numerous studies show that both genetic and environmental factors play a role in the etiology of MS. Although the genetic and environmental risk factors have been extensively studied in the context of MS, the major histocompatibility complex (MHC) is the only genomic region consistently associated with MS and several MHC alleles predispose to the disease. In patients of North European, Caucasian origin, the MHC class II alleles that are most prevalent among MS patients come from the HLA-DR15 haplotype. The 'DR15 haplotype' encodes three functional HLA-class II heterodimers, DR15 (DRA1\*0101/DRB1\*1501 pair), DRB5 (DRA1\*0101/DRB5\*0101 pair), and the DQ6 (DQA1\*0102/DQB1\*0602 pair). Although several other HLA and non-HLA disease predisposing alleles have been identified, alleles of the HLA-DR15 haplotype remain the strongest susceptibility factor. Many studies have suggested that the HLA-DRB1\*1501 allele determines MS-associated susceptibility. However, due to strong linkage disequilibrium within the HLA class-II region, there has been a lack of agreement on whether the HLA- DRB1\*1501 or the HLA- DQB1\*0602 class II alleles, or both, attribute to the genetic risk of susceptibility to MS. Here we used HLA-class II-transgenic (Tg) mice to illuminate the relative potential contribution of DRB1\*1501 or DQB1\*0602 heterodimers, or their combination, to myelin-associated oligodendrocytic basic protein (MOBP)-associated pathogenic autoimmunity relevant to MS. We show that while the HLA-DRB1\*1501 transgenics are refractory to disease induction, the HLA-DQB1\*0602 Tg mice are susceptible to EAE induction by hMOBP, through pathogenic T-cells reactive against MOBP15-36 and MOBP55-77 encephalitogenic epitopes. Although both transgenics react against these epitopes, the MOBP15-36 and MOBP55-77-reactive T-cells are of Th2-type in HLA-DRB1\*1501 transgenics, and pathogenic Th1/Th17 cells in the HLA-DQB1\*0602 transgenic mice. These findings, which are the first to show DQ6-associated pathogenic anti-myelin autoimmunity, also offer a rationale for HLA-DQB1\*0602 association with MS, and indicate that DQ6 rather than DR15 may account for genetic susceptibility to MOBP-related pathogenesis in MS.

PC01/2

**NOVEL ASSOCIATION OF THE *INTERLEUKIN 2-INTERLEUKIN 21* REGION WITH INFLAMMATORY BOWEL DISEASE**A. Marqu<sup>1</sup>, G. Orozco<sup>2</sup>, A. Martínez<sup>1</sup>, R. Palomino-Morales<sup>2</sup>, M. Fernández-Arquero<sup>1</sup>, J.L. Mendoza<sup>3</sup>, C. Taxonera<sup>3</sup>, M. Díaz-Rubio<sup>3</sup>, M. Gómez-García<sup>4</sup>, A. Nieto<sup>5</sup>, M.A. López-Nevo<sup>6</sup>, E.G. de la Concha<sup>1</sup>, J. Martín<sup>2</sup>, E. Urcelay<sup>1</sup><sup>1</sup>Hospital Clínico San Carlos, Immunology Department, Madrid, Spain, <sup>2</sup>Instituto de Parasitología y Biomedicina López-Neyra, CSIC, Granada, Spain, <sup>3</sup>Hospital Clínico San Carlos, Digestive Department, Madrid, Spain, <sup>4</sup>Hospital Virgen de las Nieves, Digestive Department, Granada, Spain, <sup>5</sup>Hospital Puerta del Mar, Immunology Department, Cádiz, Spain, <sup>6</sup>Hospital Virgen de las Nieves, Immunology Department, Granada, Spain**Objectives:** Genome-wide association studies reported the role of the *IL2-IL21* chromosomal region at 4q27 in several autoimmune conditions. Mice deficient for interleukin-2 develop a disease with clinical and histological similarity to ulcerative colitis in humans. Modest evidence of linkage with ulcerative colitis was tentatively proposed for the *IL2* gene more than a decade ago. Therefore, we decide to investigate the association of polymorphisms in the *IL2* axis (*IL2*, *IL2RA* and *IL2RB* genes) with inflammatory bowel diseases (IBD).**Methods:** Seven hundred and twenty eight white Spanish unrelated IBD patients (356 Crohn's disease and 372 ulcerative colitis) and 549 ethnically matched controls were included in a case-control study. Additionally, a Spanish replication cohort with 562 Crohn's disease and 430 ulcerative colitis patients and 1310 controls was analyzed. Eight single nucleotide polymorphisms previously associated with different autoimmune diseases were analyzed by TaqMan chemistry (rs11938795, rs17388568 and rs6822844 located in the *IL2-IL21* block; rs2104286, rs11594656 and rs41295061 located in the *IL2RA* region; and rs743777 and rs9622555 located in the *IL2RB* gene) in a 7900HT Fast Real-Time PCR system. The statistical analysis to compare allelic and genotypic distributions was performed using chi-square test or Fisher's exact test included in a standard statistical package (Epi Info v. 6.02; World Health Organization, Geneva, Switzerland).**Results:** The *IL2*-rs6822844 polymorphism modified Crohn's disease predisposition [ $p=0.002$ ; OR(95% CI)=0.61 (0.44-0.84)]; this signal was replicated in other Spanish cohort, resulting in a strong protective effect of the minor allele in the merged samples [ $p=0.0002$ ; OR(95% CI)=0.70 (0.58-0.85)]. A similar effect of rs6822844 was detected for ulcerative colitis. Another marker, rs11938795, also evidenced association with Crohn's disease [ $p=0.006$ ; OR(95% CI)=0.73 (0.58-0.92)]. However, no evidence of association with IBD was found for any of the analyzed SNPs in the *IL2RA* or *IL2RB* loci.**Conclusions:** Polymorphisms within the *IL2-IL21* linkage disequilibrium block demonstrate a novel association with IBD, concordantly with suggestive previous results of whole genome analysis in celiac disease and type 1 diabetes. Our data agree with the effect formerly observed for other conditions and delineate a shared underlying mechanism.

PC01/3

**MINIMAL SUB-CONGENIC MICE HARBORING FCGR (CIA9 LOCUS) DEVELOPS SEVERE ARTHRITIS BUT DIFFERS IN PATHOGENIC IGG SUBCLASS SUSCEPTIBILITY**D. Klaczowska<sup>1</sup>, R. Holmdahl<sup>1</sup>, K. S. Nandakumar<sup>1</sup><sup>1</sup>Karolinska Institutet, Medical Inflammation Research, Department of Medical Biochemistry and Biophysics, Stockholm, Sweden

Earlier, we have identified an arthritis promoting locus (Cia9) on chromosome 1 (LOD 5.6) using genome wide analysis of F2 intercross between the arthritis susceptible C57BL/10.Q and resistant NOD.Q mice. We confirmed the arthritis promoting effect of Cia9 locus by backcross and partially advanced intercross strategies. One of the possible arthritis promoting candidate genes proposed in this locus is Fcgr cluster. To positionally identify the arthritis promoting gene, we have generated three minimal sub-congenic mice having Cia9 locus containing 2-10 Mb fragments, and characterized these mice for arthritis phenotypes using collagen induced arthritis (CIA) and collagen antibody induced arthritis (CAIA), models for rheumatoid arthritis (RA). Sub-congenic mice containing Fcgr region are highly susceptible to severe arthritis and developed a very high level of anti-collagen IgG antibody response. These mice also showed a significantly higher incidence and severity of antibody (IgG2a/2b) induced arthritis compared to B10.Q control mice. Interestingly, IgG2a monoclonal antibodies induced a disease with a significantly reduced incidence and severity compared to IgG2b monoclonal antibodies. On the other hand, the sub-congenic mice devoid of Fcgr cluster genes are susceptible only to CIA but not CAIA. Moreover, homozygous but not the heterozygous Fcgr congenic mice had severe arthritis in both these models suggested recessive gene(s) operating in the arthritis development. However, present results demonstrate the possibility of at least two different genes located within the original Cia9 locus operating at the priming and effector phases of arthritis. Furthermore, differential susceptibility to CAIA induced with IgG2a and IgG2b isotypes indicate the presence of FcgrIV gene polymorphism in our sub-congenic mice. Heterogeneous stock mice are being used to select the mice containing recombination(s) within the Fcgr gene cluster.

PC01/4

**FINE MAPPING OF THE HLA REGION SHOWS THAT OTHER GENES BESIDES HLA-B\*27 COULD CONTRIBUTE TO THE SUSCEPTIBILITY TO ANKYLOSING SPONDYLITIS: A POSSIBLE ROLE OF THE NATURAL KILLER FUNCTION**F. Paladini<sup>1</sup>, C. Elisa<sup>1</sup>, C. Isabella<sup>2</sup>, F. Belfiore<sup>3</sup>, C. Alberto<sup>3</sup>, F. Maria Teresa<sup>1</sup>, M. Alessandro<sup>3</sup>, S. Rosa<sup>1,4</sup><sup>1</sup>University of Rome 'Sapienza', Department of Cell Biology and Development, Rome, Italy, <sup>2</sup>Cell Biology Institute, National Research Council, Monterotondo Scalo, Italy, <sup>3</sup>University of Cagliari, Department of Medical Sciences, Cagliari, Italy, <sup>4</sup>Istituto Pasteur-Fondazione Cenci Bolognietti, 'Sapienza' University of Rome, Rome, Italy**Objectives:** Ankylosing Spondylitis (AS) is a rheumatic disorder strongly associated with HLA-B\*27. However, HLA-B\*2709, an allele that occurs at high frequency in Sardinia, is not associated with the disease and is in the context of a haplotype different from that harboring the B\*2705 allele. This leaves open the possibility that other genes mapping in the same haplotype could contribute to the association. A previous study in the sardinian population allowed the identification of an extended B\*2709 haplotype, showing no major differences between AS patients and B\*2705-matched controls.**Methods:** A total of 121 patients with AS, 174 HLA-B\*27-positive controls (of whom 40 were positive for HLA-B\*2709), and 255 randomly selected controls were genotyped for microsatellites and single-nucleotide polymorphisms (SNPs) spanning the HLA region. Fisher's 2-tailed exact test and the FDR correction were applied in the statistical analysis.**Results:** Haplotypes carrying either the B\*2705 or the B\*2709 allele were found to share a conserved region downstream the HLA B gene and a functional polymorphism in the HLA-E gene (R128G), while differing in all other markers. Notably, the presence of an A at SNP rs1264457, encoding for Arg-128, was significantly ( $P < 0.0001$ ) increased in the cohort of patients but not in B\*2705- or B\*2709-positive controls. Comparing the alleles co-occurring at each HLA marker, we identified a region differentiating patients with AS and B\*2705-matched controls. In particular, there was a markedly increased prevalence of heterozygosity at rs1264457 among B\*27-positive controls suggesting a protective role of G128 in AS.**Conclusions:** These results demonstrate a significant difference in the frequency of some HLA markers between AS patients and B\*2705-positive controls, which could be attributed to the opposite chromosome. In particular, the differential distribution of a functional polymorphism in the HLA-E gene suggests a possible role of natural killer function in AS pathogenesis. Experiments are under way to confirm this hypothesis.

PC01/5

**ASSESSMENT OF SNP SCREENING METHODS : RELEVANCE TO AUTOIMMUNE DISEASES**A. Pera<sup>1</sup>, I. Gayoso<sup>1</sup>, R. Nevado<sup>1</sup>, C. Hernández-Chico<sup>2</sup>, R. Solana<sup>1</sup><sup>1</sup>Reina Sofia University Hospital, Immunology, Córdoba, Spain, <sup>2</sup>Ramon y Cajal University Hospital, Molecular Genetics, Madrid, Spain**Aim:** The SNPs (single nucleotide polymorphisms) comprise a pool of genetics markers that can be used for association studies with autoimmune diseases. It has been estimated that in human genome exist more than 10.000.000 SNPs, many of whom are intragenic. Although SNPs are mostly neutral, a number of them might influence the phenotype of autoimmune diseases (pathogenic mutations), underlining the importance of SNPs screenings.**Methods:** A total of 644 probands were included in this study. For SLC26A4 mutation screening, we carried out PCR amplification of the 21 SLC26A4 exons and their flanking intronic sequences. PCR products were screened for mutations by Heteroduplex analysis, DHPLC (denaturing high performance liquid chromatography) and/or direct sequencing. Detection of known SLC26A4 variants was performed by primer extension analysis (SNaPshot) and/or restriction enzyme digestion.**Results and conclusions:** The identification of new SNPs can be accomplished in a variety of ways, however all techniques are not equally accurate, timely or cost effective. We have performed a mutational analysis of SLC26A4 gene using various screening methods. The results of our study showed that the Heteroduplex analysis was 83% effective in detecting SLC26A4 SNPs when compared to other techniques like DHPLC and direct sequencing. On the other hand, direct sequencing can be time consuming and expensive, especially for large genes, thus we studied DHPLC as an alternative screening method. When comparing both methods, we found DHPLC as accurate and reliable as direct sequencing but to be more rapid and cost effective. Besides, we optimized different methods in order to identify known SNPs and the most worthwhile technique was the primer extension analysis that allowed us the detection of up to four SNPs, occurring in different amplicons, in a single reaction.

PC01/6

Abstract withdrawn by author

PC01/8

**RAC2 GENE SINGLE NUCLEOTIDE POLYMORPHISMS MODULATE MYELIN BASIC PROTEIN-SPECIFIC IMMUNE RESPONSES AND CORRELATE WITH DISEASE STATUS IN MULTIPLE SCLEROSIS**C. Agliardi<sup>1</sup>, F.R. Guerini<sup>1</sup>, M. Saresella<sup>1</sup>, R. Mancuso<sup>1</sup>, D. Caputo<sup>2</sup>, M. Clerici<sup>3,4</sup><sup>1</sup>Fondazione don C.Gnocchi IRCCS S.Maria Nascente, Laboratory of Molecular Medicine and Biotechnology, Milano, Italy, <sup>2</sup>Fondazione don C.Gnocchi IRCCS S.Maria Nascente, Multiple Sclerosis Unit, Milano, Italy, <sup>3</sup>Fondazione don C.Gnocchi IRCCS S.Maria Nascente, Milano, Italy, <sup>4</sup>University of Milan, Department of Biomedical Sciences and Technology, Milano, Italy

RAC-2 (RAS-related C3 botulinum toxin substrate 2), a member of the superfamily of Rho small GTPase proteins, is involved in signal transduction in T lymphocytes resulting in the stimulation of chemotaxis, proliferation, and the preferential production of TH1 cytokines. The involvement of this protein in immune activa-



tion stimulated us to study 3 SNPs (single nucleotide polymorphism) located in the 3' region (rs5756570(C/G), rs2899284(A/G)) and in an intron (rs739041(A/G)) mapping into the regulatory region of the RAC-2 gene in Relapsing Remitting Multiple Sclerosis (RRMS). Thus, 335 RRMS patients and 222 healthy controls (HC) were analyzed by allelic discrimination Real time PCR. Results showed that, whereas frequency distributions of alleles and genotypes was comparable in RRMS and HC, the G/G genotype in rs5756570 -resulting in an increased expression of the Rac2 gene- was associated with higher mean MSSS scores (OR:1.9 IC(95%): 1.05-2.77  $p=0.03$ ) and a poorer response to immunomodulators. Myelin basic protein (MBP)-specific proliferation was increased ( $p<0.05$ ) and the T frequency of 4+/25+/+/FOXp3/PD1- T regulatory lymphocytes (Treg) was reduced ( $p<0.01$ ) in patients expressing the rs5756570 G/G genotype. In these same individuals, programmed cell death-1 (PD-1)-expressing MBP-specific CD4+ and CD8+ T lymphocytes were reduced ( $p<0.05$ ) and apoptosis of MBP-specific lymphocytes was diminished ( $p<0.05$ ).

These data suggest that particular SNPs modulating the activity of RAC-2 influence the clinical outcome of RRMS, possibly secondarily to an augmented immune reactivity associated with an impairment of Treg-mediated immune suppression. These results, although needing validation in bigger cohorts, indicate that RAC-2 might play a role in the immunopathogenesis of MS.

#### PC01/9 ASSOCIATION OF *IL18RAP* AND *CCR3* WITH CELIAC DISEASE IN THE SPANISH POPULATION

B. Dema<sup>1</sup>, C. Maluenda<sup>2</sup>, I. Polanco<sup>3</sup>, C. Núñez<sup>1</sup>

<sup>1</sup>Hospital Clínico San Carlos, Clinical Immunology, Madrid, Spain, <sup>2</sup>Hospital Clínico San Carlos, Paediatric, Madrid, Spain, <sup>3</sup>Hospital Universitario La Paz, Gastroenterology Paediatric, Madrid, Spain

**Objective:** Celiac disease (CD) is a chronic inflammatory gut disorder that affects to genetically predisposed individuals. The strongest genetic association described is with the *HLA* locus, but recently, genome wide association studies showed eight new genetic regions associated with CD susceptibility. However, a replication study performed in the Italian population could not confirm the association with two of those new regions: 2q12, containing the *IL18RAP* gene, and 3p21, holding a cluster of chemokine receptors genes (being *CCR3* the nearest one). They postulated that a genetic population difference may exist across Europe. We aimed at studying the role of those two genetic regions in CD risk in a different Mediterranean population, the Spanish one.

**Methods:** A case-control study with 722 white Spanish CD patients and 794 ethnically matched healthy controls was performed. CD patients were diagnosed following ESPGHAN criteria, 62% are female and 97% are positive for HLA-DQ2 and/or HLA-DQ8. Two single nucleotide polymorphisms, rs917997 (2q12) and rs6441961 (3p21), were genotyped by TaqMan technology. Genetic frequencies were compared between cases and controls with the chi-square test using EpiInfo v5.00. For *IL18RAP*, a meta-analysis using published data was carried out with EPIDAT 3.1, which uses the Mantel-Haenszel test for calculating combined ORs.

**Results:** A significant association with rs6441961 (3p21) was found: OR=1.32 95% CI 1.13-1.54,  $p=0.0004$ . When we studied rs917997 (2q12), a non-significant result was obtained (OR=1.10 95% CI 0.94-1.30,  $p=0.23$ ), concordantly with the observed Italian result. In addition, the meta-analysis performed by adding our *IL18RAP* data to the negative previously published data showed a borderline significance: OR=1.12 95% CI 1.00-1.24,  $p=0.044$ .

**Conclusion:** We confirmed the association of the 3p21 genetic region with CD susceptibility in the Spanish population. In 2q12, the initially described OR is most probably overestimated, and therefore the real situation may be the existence of a genuine but weak risk factor, which generates statistical power limitations.

#### PC01/10 GLYPICAN 5 IS AN INTERFERON-BETA RESPONSE GENE IN MULTIPLE SCLEROSIS PATIENTS

M.C. Ceni<sup>1</sup>, F. Blanco<sup>1</sup>, V. de las Heras<sup>2</sup>, M. Bartolomé<sup>2</sup>, R. Arroyo<sup>2</sup>, A. Martínez<sup>1</sup>

<sup>1</sup>Hospital Clínico San Carlos, Clinical Immunology, Madrid, Spain, <sup>2</sup>Hospital Clínico San Carlos, Multiple Sclerosis Unit, Neurology Department, Madrid, Spain

**Objective:** Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system. Interferon-beta is the most usual therapy in relapsing-remitting MS. Nevertheless, approximately 50% of the treated patients do not respond adequately. Very recently, a genome-wide association study on interferon-beta pharmacogenetics has described polymorphisms at several genes that are associated with response to this treatment. Our aim is to replicate the results obtained at the two loci most strongly associated with the response to interferon-beta treatment, *HAPLN1* and *GPC5*.

**Methods:** We performed a case-control study, analysing 199 MS patients treated with interferon-beta for at least two-years and at least two documented relapses over the two years previous to treatment onset. Responders had no relapses and no increase in EDSS over the 2-year follow-up period; non-responders had at least 2 relapses or an increase in EDSS of at least 1 point. We studied three SNPs in the *GPC5* locus and three SNPs in the *HAPLN1* locus by TaqMan technology. Allelic frequencies between responders and non-responders were compared by a  $\chi^2$  test.

**Results:** An association was found between *GPC5* polymorphisms and the response to interferon-beta therapy in MS patients, in agreement with earlier data (responder vs. non-responder patients: rs10492503,  $p=0.0005$ ). The other locus studied (*HAPLN1*) did not show association with response to interferon-beta (all SNPs  $p>0.05$ ).

**Conclusions:** We confirm the association of polymorphisms within *GPC5* with response to interferon-beta therapy in MS patients. The *GPC5* polymorphisms studied are located in intronic regions. Some of these polymorphisms associated with response to treatment may be the direct cause of the degree of response to treatment or, alternatively (and perhaps more likely), they can merely act as genetic markers of another nearby polymorphism in tight linkage disequilibrium with it. *Glypican5* encodes a proteoglycan whose synthesis seems to increase due to interferon-beta. This proteoglycan may intervene in the binding of interferon-beta to its receptor, increasing or decreasing the affinity for interferon-beta. There are few pharmacogenetic studies and they are very interesting to delineate in future patients a personalized therapy tailored to their genetic profile.

#### PC01/11 ASSOCIATION OF POLYMORPHISMS IN THE *IL2*, *IL2RA* AND *IL2RB* GENES WITH MULTIPLE SCLEROSIS

M.L. Cavanillas<sup>1</sup>, V. de las Heras<sup>2</sup>, M. Bartolomé<sup>2</sup>, R. Arroyo<sup>2</sup>, F. Matesanz<sup>3</sup>, E. Urcelay<sup>1</sup>

<sup>1</sup>Hospital Clínico San Carlos, Immunology Department, Madrid, Spain, <sup>2</sup>Hospital Clínico San Carlos, Neurology Department, Madrid, Spain, <sup>3</sup>Instituto López-Neyra, CSIC, Granada, Spain

**Objective:** IL-2/IL-2R signalling has an essential non-redundant role in the development and function of regulatory T cells (Treg), maintaining, therefore, the peripheral T-cell tolerance. Reduced Treg function has been detected in Multiple Sclerosis (MS) patients. Genome-wide association studies (GWAS) have recently found single nucleotide polymorphisms (SNPs) in the linkage disequilibrium block which includes the *IL2/IL21* genes (4q27), *IL-2RA* (10p15) and *IL-2RB* (22q13) loci, associated with an increased susceptibility towards several autoimmune diseases. Considering a possible common mechanism in the pathogenesis of such diseases, and given the essential role of the IL-2 pathway in the control of autoimmunity, we studied previously associated SNPs within these regions to test its genetic implication in Spanish MS patients.

**Methods:** We recruited 430 Spanish MS patients diagnosed according to Poser criteria and 550 ethnically matched controls. Genotyping was performed with TaqMan assays. We studied SNPs from the *IL-2/IL-21* block (rs11938795, rs17388568 and rs6822844); *IL-2RA* gene (rs2104286, rs41295061 and rs11594656) and *IL-2RB* locus (rs743777 and rs9622555). The statistical analysis was performed using a chi-squared test implemented on Epi Info v.6.02. Haplotypic frequencies were estimated with the Expectation-Maximization algorithm included in the Haploview v.6.0 software.

**Results:** We found a significant reduction of the minor allele frequency of rs2104286 located in the *IL-2RA* region in MS patients compared with controls [ $p=0.0002$ ; OR (95% CI)= 0.64 (0.50-0.82)], as previously described. Besides, the genotypic frequencies of rs6822844 within the *IL-2/IL-21* block showed that carriers of the minor allele were significantly reduced in MS patients [ $p=0.03$ ; OR (95% CI)=0.72 (0.52-0.98)]; and a marginally significant difference was found in our population for the allele frequency of rs9622555, positioned in the *IL-2RB* gene [ $p=0.06$ ; OR (95% CI)=0.83 (0.68-1.02)]. The novel effect of *IL2RB* rs9622555 in MS risk was intensified after stratification by *IL2RA* rs2104286 [ $p=0.0006$ ; OR (95% CI)=0.13 (0.02-0.5)].

**Conclusion:** Our results confirm rs2104286 in the *IL-2RA* locus as a genetic susceptibility factor for MS in the Spanish population, and we describe here for the first time a possible association of *IL-2RB* gene with MS. These findings are consistent with the relevant role of the IL2 pathway on autoimmune risk.

#### PC01/12 CELL SPECIFIC DEFICIENCY OF MOUSE A20/TNFAIP3, AN INHIBITOR OF NF-KB AND APOPTOSIS, IS ASSOCIATED WITH INCREASED SUSCEPTIBILITY TO INFLAMMATION AND AUTOIMMUNITY

R. Bevaert<sup>1</sup>, L. Verecke<sup>1</sup>, M. Sze<sup>1</sup>, C. McGuire<sup>1</sup>, M. Matmati<sup>1</sup>, M. Pasparakis<sup>2</sup>, G. van Loo<sup>1</sup>

<sup>1</sup>Ghent University – VIB, Molecular Biomedical Research, Gent, Belgium, <sup>2</sup>Institute for Genetics/University of Cologne, Cologne, Germany

The transcription factor NF- $\kappa$ B plays an important role in a variety of biological functions, most notably in the initiation and amplification of immune and inflammatory responses and the regulation of apoptosis. Excessive NF- $\kappa$ B activation or its inappropriate termination have been implicated in the pathogenesis of many autoimmune and inflammatory diseases. Multiple cellular and molecular mechanisms normally ensure the proper termination of NF- $\kappa$ B activation and resolution of inflammation. In this context, the intracellular ubiquitin-editing protein A20 (also known as TNFAIP3) is a key player in the negative feedback regulation of NF- $\kappa$ B signalling in response to TNF and pattern recognition receptors. Moreover, A20 also regulates TNF-induced apoptosis. Recent genome wide association studies suggest human A20/TNFAIP3 as a susceptibility locus for common inflammatory diseases such as Crohn's disease, rheumatoid arthritis, systemic lupus erythematosus, psoriasis, and type 1 diabetes. Understanding the physiological role of A20 in different tissues and cell types is therefore of crucial importance. Using the Cre/LoxP recombination system in which the A20 gene is flanked by LoxP consensus sites we have generated multiple tissue and cell specific A20 knockout mice. Conditional A20 deficient mice develop normally and in contrast to full A20 knockout mice do not die prematurely. Cells lacking A20 are hypersensitive to TNF-induced NF- $\kappa$ B activation and apoptosis. A20 deletion in immune cells but also in non-immune cells results in severe autoimmunity. A20 deficient mice are currently also analyzed in different mouse models of autoimmune disease, of which the results will be presented. Our data further support the suggestion that A20 deficiency in humans might contribute to the development and progression of autoimmune diseases, making A20 an interesting therapeutic target.

**PC01/13 GENETIC CONTRIBUTION OF CELIAC1 (HLA), CELIAC3, CELIAC4, TNF- $\alpha$  AND LINFOTOXIN *LOC1* TO CELIAC DISEASE SUSCEPTIBILITY**

A. Capilla<sup>1</sup>, D. Planelles<sup>2</sup>, E. Donat<sup>3</sup>, C. Espinós<sup>4</sup>, C. Ribes-Koninckx<sup>3</sup>, F. Palau<sup>1</sup>, R. Roig<sup>2</sup>, J.A. Montoro<sup>2</sup>

<sup>1</sup>Instituto de Biomedicina, Consejo Superior de Investigaciones Científicas (CSIC), Genetics and Molecular Medicine, Valencia, Spain, <sup>2</sup>Centro de Transfusión de la Comunidad Valenciana, Histocompatibilidad, Valencia, Spain, <sup>3</sup>Hospital Universitario La Fe, Paediatric Gastroenterology Unit, Valencia, Spain, <sup>4</sup>Centre for Biomedical Research on Rare Diseases (CIBERER), Valencia, Spain

Celiac disease is a complex immunologic disease with multiple contributing genes. The association between celiac disease and HLA class II genes on chromosome 6p21 (CELIAC1 region) is well known, with DQ haplotype DQA1\*0501-DQB1\*0201 as a primary risk factor. However, contribution of HLA-DQ8 molecule is unclear. On the other hand, several lines of evidence suggest that other non-HLA genetic risk factors must be involved in the pathogenesis of this disease. The aim of this study has been to investigate how relevant regions contribute to celiac disease susceptibility: CELIAC1 (HLA class II genes), CELIAC3 (CD28/CTLA4/ICOS region on 2q33) and CELIAC4 (19p13) as well as the tumour necrosis factor  $\alpha$  (TNF- $\alpha$ ) and the linfoxin *loci*.

Our study was carried out in a series of 168 paediatric patients from Eastern Spain: 13 familial cases and 155 isolated cases. As a control population, a total of 378 healthy individuals were included. We performed association studies, case-control approaches and sib transmission disequilibrium tests. In the CELIAC1 region we studied HLA-DRB1, -DQA1 and -DQB1 genes and the synergistic effect between associated alleles was evaluated. Two polymorphisms were analysed in TNF- $\alpha$  and linfoxin *loci*, -308\*A/G and NcoI\*A/G respectively. In the CELIAC3 region we studied four polymorphisms on CTLA4 *locus*, -1147\*C/T, +49\*A/G, CT60\*A/G and CT61\*A/G, and the c.602\*A/C polymorphism within the ICOS gene. In the CELIAC4 region, we investigated the STR marker D19S899 and two SNPs, rs2305764 and rs2305767.

Our results confirm in our population that the main associated HLA haplotype was DRB1\*0301-DRB3\*-DQA1\*0501-DQB1\*0201. However, no association with DQ haplotype DQA1\*0301-DQB1\*0302 (DQ8 molecule) was noted. It is noteworthy that haplotypes DRB1\*07-DRB4-DQA\*0201-DQB1\*0202 and DRB1\*11-DRB3-DQA1\*0505-DQB1\*0301, by themselves, have a negative relative risk; however, this risk increases to 2.1 when combined. We highlight the association with the +49\*A allele of cytotoxic T-lymphocyte-associated antigen 4 *locus*, and the -308\*A of TNF- $\alpha$  *locus* in HLA-DQ2 individuals, although an independent role for TNF- $\alpha$  as risk factor has not been proven. Moreover, we do not confirm the association with the CELIAC4 region polymorphisms described in other populations.

We have concluded that both the CELIAC1 and CELIAC3 regions, besides the TNF- $\alpha$  *locus*, confer susceptibility for celiac disease.

**PC01/14 ASSOCIATION BETWEEN IDIOPATHIC ACHALASIA AND *IL23R* GENE**

J.L. Santiago<sup>1</sup>, C. Sevilla<sup>1</sup>, A. Ruiz de León<sup>1</sup>, A.G. Vigo<sup>1</sup>

<sup>1</sup>Hospital Clínico San Carlos, Madrid, Spain

**Objectives:** Idiopathic achalasia is an esophageal motor disorder of unknown etiology characterized by esophageal aperistalsis and abnormal lower esophageal sphincter relaxation. Autoimmune etiology of achalasia is supported by three lines of evidence: the presence of inflammatory T cells in the myenteric plexus, high titers of circulating autoantibodies against the myenteric plexus and increased prevalence of certain human leukocyte antigen (HLA) class II antigens. However, few reports studying non-HLA loci in achalasia susceptibility can be found in the literature. Recent studies have shown association of the *IL23R* gene with different chronic inflammatory or autoimmune disorders, including Barrett's esophagus. The *IL23R* coding variant Arg381Gln displayed the strongest association in the original study and association with this polymorphism has been extensively replicated. Our aim in the present study was to assess whether the *IL23R* Arg381Gln polymorphism is involved in susceptibility to idiopathic achalasia.

**Methods:** We performed a case-control study including 262 patients with idiopathic achalasia and 802 healthy subjects, all of them white Spaniards. Achalasia patients were diagnosed on the basis of clinical, radiographic, endoscopic and manometric criteria. All samples were genotyped for the *IL23R* Arg381Gln polymorphism (rs11209026) by TaqMan technology. Comparisons of genetic frequency were performed using the chi-squared test and the conferred risk was expressed as odds ratios (OR) with 95% confidence intervals (CI).

**Results:** The minor allele (coding for Gln) of the Arg381Gln polymorphism was significantly increased in achalasia patients compared with healthy controls (OR=1.46, 95% CI 1.01-2.11, p=0.036).

**Conclusion:** Our results suggest a role of *IL23R* in idiopathic achalasia predisposition and, therefore, extend the evidence of the general influence of this gene in autoimmune and inflammatory diseases.

**PC01/15 ASSOCIATION OF A NKP44 ALLELE WITH PRIMARY SJÖGREN'S SYNDROME**

S. Nothdorff<sup>1</sup>, R.M. Thomas<sup>1</sup>, G. Kabalak<sup>1</sup>, P. Schirmer<sup>2</sup>, N. Marquardt<sup>1</sup>, R. Jacobs<sup>1</sup>, R.E. Schmidt<sup>1</sup>, T. Witte<sup>1</sup>

<sup>1</sup>Hannover Medical School, Clinic for Immunology and Rheumatology, Hannover, Germany, <sup>2</sup>Hannover Medical School, Clinic for Gastroenterology, Hepatology and Endocrinology, Hannover, Germany

**Objectives:** Persisting viral infections are suspected to trigger autoimmune diseases. Alleles of receptors involved in virus control might be risk factors for virus persistence and autoimmunity. NKP44 is almost exclusively expressed on activated NK cells, triggering cytotoxicity and cytokine release. Hence we investigated a possible association of a SNP of NKP44 and primary Sjögren's Syndrome (pSS).

**Methods:** We chose rs9471577 (NKP44 (syn. NCR2), A310G, M75V, in the Ig-like domain with supposable impact on ligand binding). Genotyping was performed using ABI TaqMan<sup>®</sup> SNP Genotyping Assays. 335 healthy controls were compared with 292 patients suffering from pSS, matching the US/European consensus criteria. Contingency was analyzed using Fisher's exact test.

**Results:** The 'G'-allele of NKP44 was present (heterozygous or homozygous) in 9.9% of the blood donors, but in 16.8% of the pSS patients (p=0.0124; Odds Ratio: 1.85). The odds ratio increased even further, when two rather than only one objective test of glandular function (Schirmer's and Saxon's test) were pathological, and also, when antibodies against alpha-fodrin were present.

**Conclusion:** The M75V substitution in NKP44 is associated with primary Sjögren's Syndrome. Since the site is localized on the surface of NKP44 and protrudes away from the cell surface, it may well be involved in binding of NKP44 ligands and thus be important in virus defence or NK-cell activation.

**PC01/16 THE FUNCTION AND APOPTOSIS OF MONOCYTES/MACROPHAGES DEPEND ON THE POLYMORPHISM OF PPAR-GAMMA GENE**

I.P. Kaidashev<sup>1</sup>, A. Rasin<sup>1</sup>, M. Rasin<sup>1</sup>, N. Kutzenko<sup>1</sup>, M. Mikitjuk<sup>1</sup>

<sup>1</sup>Ukrainian Medical Stomatological Academy, Research Institute for Genetic and Immunological Grounds of Pathology and Pharmacogenetics, Poltava, Ukraine

**Rationale:** Regulation of immune cells activity is one of the important problems of modern immunology. Human macrophages expressed mainly one from three isomers of peroxisome-proliferator activating receptors – PPAR-gamma. It was shown that agonists of PPAR-gamma – thiozolidinediones can influence some functions of macrophages. In the same time there is evidence for polymorphism of PPAR-gamma-1 gene which led to the change of proline on alanine in 12 position. Today there are no data for prevalence of Pro12Ala polymorphism of PPAR-gamma1 in Ukrainian population and little is known about the functional consequences of this polymorphism on function of macrophages.

**Methods:** The group of observation was comprised by 49 mail patients (age 51 $\pm$ 4) with metabolic syndrome. The control group included 46 health volunteers (age 49 $\pm$ 3.4). Monocytes/macrophages (Mmf) were obtained from peripheral blood stabilized by heparin. Mmf were cultured with PPAR-gamma agonist rosiglitazone (R) in final concentration of 10, 30 and 100 mkmol/l during 72h in parallel with appropriate controls. Before and after the culturing the cells and cultural medium were tested. Mmf morphology was investigated by MGG and Hoechst 33342 staining. Phagocytosis was estimated by latex uptake and NBT test. In supernatants the level of TNF-alpha was measured. The Pro12Ala polymorphism of PPAR-gamma1 gene was studied by PCR with further restriction analysis. All data were estimated by nonparametric statistics.

**Results:** The prevalence of 12Ala allele of PPAR-gamma1 gene is 20.7% in healthy mail subjects in Ukrainian population. In patient with metabolic syndrome 12Ala allele is rare (-11.9%, p=0.092). All patients were divided in three groups according to genotypes: Pro12Pro, Pro12Ala and Ala12Ala. R induced the apoptosis of Mmf in all groups but the persons with 12Ala allele were the most susceptible. The similar results were obtained for the production of TNF-alpha and reactive oxygen species (40.5% and 17% decrease in patients with 12Ala allele, respectively).

**Conclusions:** Patients with metabolic syndrome had decreased frequency of 12Ala allele of PPAR-gamma1 gene and increased – 12Pro allele. Mmf from patients bearing 12Ala allele are the most susceptible to agonists of PPAR-gamma. Agonist PPAR-gamma rosiglitazone induced Mmf apoptosis and decreased TNF-alpha and reactive oxygen species production in polymorphism specific manner.

**PC01/17 TYPE 1 DIABETES SUSCEPTIBILITY GENES IN THE ANCESTRAL HAPLOTYPE 18.2 DIFFERENT FROM THE MHC CLASS II GENES**

J.L. Santiago<sup>1</sup>, W. Li<sup>2</sup>, A. Lee<sup>2</sup>, E.G. de la Concha<sup>1</sup>, E. Urcelay<sup>1</sup>, P.K. Gregersen<sup>2</sup>

<sup>1</sup>Hospital Clínico San Carlos, Immunology, Madrid, Spain, <sup>2</sup>Feinstein Institute for Medical Research, North Shore LIJ Health System, Manhasset, United States

**Objectives:** The genetic susceptibility to Type 1 diabetes mellitus (T1D) is strongly associated with the MHC class II genes: DR3-DQ2 and DR4-DQ8. Several studies have proven that not all of the DR3-DQ2 haplotypes predispose equally to the disease. Two conserved extended DR3-DQ2 haplotypes were associated with diabetes susceptibility: the AH8.1 and the AH18.2. The aforementioned studies have reported that the AH18.2 haplotype confers significantly increased risk, therefore may harbor an additional susceptibility gene to T1D different from MHC class II alleles. The aim of this study was trying to locate the additional susceptibility gene to T1D carried by the AH18.2 haplotype.

**Methods:** We analyzed 10 AH18.2 homozygous subjects using the Illumina's HumanHap550 Bead chip in order to establish to what extent the linkage disequilibrium (LD) is conserved. Then we compared this region with 20 AH8.1 homozygous healthy controls, from the New York Cancer Project (NYCP) to find differences in markers.

**Results:** After a 95% call rate clean up performed with the HelixTree software, 2022 SNPs were studied in the MHC region (2.60–3.36 Mb interval) and we found that the LD was higher between DDR1 and HLA-DQA1 genes. If we excluded the DRB1, DQA1 and DQB1 loci, the region extended 1.65 Mb, which is narrower than the one reported by Johansson et al. suggesting that the additional gene was located in the 2.35 Mb interval between D6S2707 and MHC-DOB.

In the comparison between AH18.2 and CAH8.1 haplotypes, hundreds of SNPs were different in the aforementioned region. However, when we grouped the markers by genes we found 17 genes different in class I; 31 in class III and only 2 in class II. Surprisingly, the class III region showed no differences between both conserved haplotypes in 11 out of 42 genes, which we would be able to discard as candidates.

**Conclusion:** We consider that the MHC region between DDR1 and HLA-DRA genes is likely to carry the second susceptibility gene in the AH18.2 haplotype. This region comprises 50 genes which showed differences in the analyzed markers and hence all of them are good candidates.

#### PC01/18 INVESTIGATION OF *TLR5* AND *TLR7* AS CANDIDATE GENES FOR SUSCEPTIBILITY TO SYSTEMIC LUPUS ERYTHEMATOSUS

E. Sanchez<sup>1,2</sup>, J.L. Callejas-Rubio<sup>3</sup>, J.M. Sabio<sup>4</sup>, M.A. Gonzalez-Gay<sup>2</sup>, J. Jimenez-Alonso<sup>5</sup>, L. Micó<sup>6</sup>, E. de Ramón<sup>6</sup>, M. Camps<sup>6</sup>, A. Suarez<sup>7</sup>, C. Gutierrez<sup>7</sup>, R. Garcia-Portales<sup>8</sup>, C. Tolosa<sup>9</sup>, N. Ortego-Centeno<sup>3</sup>, J. Sanchez-Roman<sup>10</sup>, F.J. Hernandez<sup>10</sup>, M.F. Gonzalez-Escribano<sup>10</sup>, J. Martin<sup>1</sup>, M.A. Lopez-Nevot<sup>4</sup>  
<sup>1</sup>Instituto de Parasitología y Biomedicina Lopez-Neyra, CSIC, Armilla, Spain, <sup>2</sup>Hospital Xeral Calde, Lugo, Spain, <sup>3</sup>Hospital Clínico San Cecilio, Granada, Spain, <sup>4</sup>Hospital Virgen de las Nieves, Granada, Spain, <sup>5</sup>Hospital La Fe, Valencia, Spain, <sup>6</sup>Hospital Carlos Haya, Malaga, Spain, <sup>7</sup>Hospital Central de Asturias, Oviedo, Spain, <sup>8</sup>Hospital Virgen de la Victoria, Malaga, Spain, <sup>9</sup>Hospital Parc Tauli, Sabadell, Spain, <sup>10</sup>Hospital Virgen del Rocío, Seville, Spain

**Background:** Systemic lupus erythematosus (SLE) is a systemic autoimmune disease characterised by the production of a wide variety of autoantibodies. Autoantibodies to DNA, RNA and associated proteins are common targets of the autoimmune response in SLE. This autoantibodies have the capacity to stimulate the innate immune system directly via Toll-like receptors (TLRs). The TLR family constitutes an important group of pattern-recognition receptors that play an essential role in the activation and regulation of innate and adaptive immunity through the recognition of specific molecular patterns of pathogens. Because of their central role in the regulation of inflammation and the immune response to pathogens, TLRs are excellent candidate genes in genetic susceptibility studies for autoimmune diseases, such as SLE. A stop codon polymorphism in the ligand-binding domain of *TLR5* (rs5744168) is able to mediate resistance to SLE. *TLR7* has recently been described as a potential functional relevance gene in SLE. In addition, *TLR7* has the ability to induce the release of interferon- $\alpha$  (IFN $\alpha$ ), a cytokine that has been shown to have a relevant role in SLE.

**Objective:** The aim of this study was to evaluate the relevance of genetic variants of *TLR5* (rs5744168) and *TLR7* (rs179008) gene in systemic lupus erythematosus (SLE) in a Spanish population.

**Material and methods:** Our study population consisted of 752 SLE patients and 1107 healthy controls. All individual were of Spanish Caucasian origin. The *TLR5* and *TLR7* polymorphisms were genotyped using a PCR system with pre-developed TaqMan allelic discrimination assay.

**Results:** No statistically significant differences were observed when the allele and genotype distribution of *TLR5* rs5744168 and *TLR7* rs179008 polymorphisms was compared between SLE patients and healthy controls. A significant increase frequency in the CC genotype of the *TLR5* rs5744168 polymorphism among SLE patients without nephritis was found (93.4% vs 87% in SLE patients with nephritis,  $P = 0.03$ , OR = 2.11 95%CI 0.93–3.51). However, this difference did not reach statistical significance in the allele frequencies ( $P = 0.08$ ).

**Conclusion:** These results suggest that the tested variations of *TLR5* and *TLR7* genes do not confer a relevant role in the susceptibility or severity of SLE in the Spanish population.

#### PC01/19 KIAA0350 AS A SUSCEPTIBILITY GENE IN SPANISH TYPE 1 DIABETES PATIENTS

L. Espino-Paisán<sup>1</sup>, M.A. Figueredo<sup>1</sup>, H. de la Calle<sup>2</sup>, J.L. Santiago<sup>1</sup>

<sup>1</sup>Hospital Clínico San Carlos, Immunology, Madrid, Spain, <sup>2</sup>Hospital Ramon y Cajal, Endocrinology, Madrid, Spain

**Objectives:** Underlying genetics of type 1 diabetes (T1D) has been extensively studied in the last few years through linkage-disequilibrium and genome-wide studies. Though half of the genetic background of the disease can be explained by the HLA class II susceptibility haplotypes, other genes such as *PTPN22* or *CTLA-4* have been involved in the mechanisms of the disease.

Genome-wide studies have the advantage of studying the whole genome, thus pointing at genes and chromosomal regions of unknown function so far. *KIAA0350*, also termed *CLEC16A*, is one of these genes. Located on chromosome 16 (16p13), *KIAA0350* encodes a protein with a predicted C-type lectin domain, a structure found in many proteins involved in adhesion and pathogen recognition in the immune response.

*KIAA0350* polymorphism rs2903692 has been found strongly associated with T1D susceptibility in European populations of Northern ancestry with pediatric debut of the disease. Our aim was to replicate this finding in a set of patients from southern Europe and with early and late onset of the disease. Additionally, we studied polymorphism rs6498169, previously associated with multiple sclerosis (MS), under the hypothesis of a common basis of autoimmune diseases.

**Methods:** We recruited 316 T1D patients with an age at disease onset ranging from 1 to 55 years (median = 17) and 550 healthy unrelated controls from the Madrid area. Genotyping was performed for two SNPs, rs2903692 and rs6498169, using Taqman Genotyping Assays. Genotype and allele frequencies were compared by Chi-square and Fisher tests.

**Results:** We found a protective effect of the minor allele of polymorphism rs2903692 in our general study ( $p = 0.047$ , OR = 0.81 [0.66–1.00]). Polymorphism rs6498169 did not provide any additional information.

**Conclusion:** We replicated the T1D protective effect previously described in polymorphism rs2903692. Statistical difference between pediatric and adult groups was not found, so we propose that *KIAA0350* polymorphism is a protective factor to T1D independent of the age at disease onset.

The difference in T1D association of both polymorphisms might suggest the existence of different etiologic factors in 16p13, each one conferring susceptibility to one disease.

#### PC01/20 THE AUTOIMMUNE-ASSOCIATED ALLELES OF *CTLA-4* GENE ARE ALSO RISK FACTORS FOR DEVELOPMENT OF MULTIPLE MYELOMA

A. Tomkiewicz<sup>1</sup>, L. Karabon<sup>1</sup>, E. Pawlak<sup>1</sup>, A. Jedynak<sup>1</sup>, M. Kielbinski<sup>2</sup>, D. Woszczyk<sup>3</sup>, A. Jonkisz<sup>4</sup>, K. Kuliczowski<sup>2</sup>, I. Frydecka<sup>1,2</sup>

<sup>1</sup>Institute of Immunology and Experimental Therapy, Wrocław, Poland, <sup>2</sup>Medical University, Department of Hematology, Neoplastic Diseases & Bone Marrow Transplantation, Wrocław, Poland, <sup>3</sup>State Hospital, Department of Hematology, Opole, Poland, <sup>4</sup>Medical University, Department of Forensic Medicine, Wrocław, Poland

**Objectives:** Multiple myeloma (MM) is a B-lineage malignancy characterized by an accumulation of isotype-switched, immunoglobulin-producing monoclonal plasma cells. Various phenotype and functional abnormalities of B and T cell are observed in MM patients.

The aim of the study was to investigate the association between polymorphisms of the gene encoding cytotoxic T-lymphocyte antigen 4 (*CTLA-4*) – negative regulator of the T-lymphocyte immune response and susceptibility to MM in a Polish population.

**Methods:** Two hundred and two MM patients and 380 healthy subjects were examined. The following polymorphisms in *CTLA-4* gene: c.49A>G, g.319C>T, and CT60 (g.\*6230G>A) were typed by PCR-RFLP method using *BseXI*, *TruI*, and *TaII* enzymes, respectively. Jo31 (g.\*10223G>T) SNP was genotyped by allelic discrimination method on the Applied Biosystems 7300 -Real -Time PCR using TaqMan SNP Genotyping Assay. The dinucleotide repeat polymorphism g.\*642AT(8–33) was studied by PCR and fluorescence based technique.

**Results:** We observed that the presence of the c.49A>G [G] allele and [GG+GT genotypes] ( $p = 0.04$ , OR: 1.30, and  $p = 0.02$ , OR: 1.56, respectively) and Jo31 [G] allele and [GG] genotype ( $p = 0.03$ , OR: 1.3, and  $p = 0.04$ , OR: 1.45, respectively) were associated with an increased risk of MM. Moreover we noticed higher frequency of CT60 [G] allele, and [GG] genotype in MM patients in comparison with controls ( $p = 0.04$ , OR: 1.30, and  $p = 0.08$ , OR: 1.66, respectively). Other studied polymorphism did not correlate with MM. No association between age of onset and clinical course of the disease and studied gene polymorphisms was found.

**Conclusion:** For the first time our results indicate that the polymorphism in the *CTLA-4* gene might be susceptibility loci for MM.

#### PC01/21 LACK OF ASSOCIATION BETWEEN *STAT4* GENE POLYMORPHISM AND BIOPSY-PROVEN GIANT CELL ARTERITIS

R.J. Palomino-Morales<sup>1</sup>, T. Vazquez-Rodriguez<sup>2</sup>, I.C. Morado<sup>3</sup>, C. Santos<sup>4</sup>, N. Ortego-Centeno<sup>3</sup>, J. Miranda-Filloy<sup>2</sup>, J.R. Lamas<sup>3</sup>, J. Martin<sup>1</sup>, M.A. Gonzalez-Gay<sup>2</sup>  
<sup>1</sup>Institute of Parasitology and Biomedicine Lopez-Neyra, CSIC, Cellular Biology and Immunology, Granada, Spain, <sup>2</sup>Hospital Xeral-Calde, Rheumatology, Lugo, Spain, <sup>3</sup>Hospital Clínico San Carlos, Rheumatology, Madrid, Spain, <sup>4</sup>Hospital de la Princesa (Univ. Autonoma), Rheumatology, Madrid, Spain, <sup>5</sup>Hospital Clínico San Cecilio, Internal Medicine Unit, Granada, Spain

**Background:** Giant cell arteritis (GCA) is the most common systemic vasculitis in individuals over the age of 50 years in Western countries. It is characterized by the granulomatous involvement of large and medium-sized blood vessels of the aorta. An important step forward in our understanding of the pathogenesis of autoimmune diseases may be to establish the presence of common mechanisms that may lead to a variety of very different complex autoimmune diseases. In this regard, the Janus kinase and signal transducer and activator of transcription (Jak-STAT) pathway is the signalling target of a multitude of cytokines, which are thought to have biologically significant roles in autoimmunity. Interestingly, *STAT4* gene polymorphism rs7574865 has been recently found associated with multiple autoimmune diseases. Taking all these considerations together, *STAT4* gene has been proposed as a general susceptibility marker for autoimmunity.

**Objectives:** To investigate the potential implication of the *STAT4* gene polymorphism rs7574865 in the predisposition to or the clinical expression of giant cell arteritis (GCA).



**Methods:** A total of 212 patients diagnosed with biopsy-proven GCA were included in this study. DNA from patients and controls matched by age and sex and ethnicity was obtained from peripheral blood. Samples were genotyped for *STAT4* rs7574865 polymorphism using a TaqMan 5' -allele discrimination assay on the ABI PRISM 7900 Sequence Detection Systems and SDS 2.3 software.

**Results:** No statistically significant differences in the allele frequencies for the *STAT4*

rs7574865 polymorphism were observed between biopsy-proven GCA patients and controls. Although we observed an increased frequency of the T/T genotype in GCA patients (6.0%) compared to healthy controls (3.9%), this difference did not achieve statistical significance (odds ratio = 1.57; 95% confidence interval: 0.72-3.41). Moreover, no statistically significant differences in the allele or genotype frequencies were observed when biopsy-proven GCA patients were stratified according to the presence of typical features of the disease such as polymyalgia rheumatica, severe ischemic manifestations or visual ischemic complications in the setting of this vasculitis.

**Conclusion:** Our results do not support a major role of the *STAT4* rs7574865 gene polymorphism in the susceptibility or clinical manifestations of GCA.

#### PC01/22 GENES SHARING SEQUENCES HOMOLOGOUS TO THE ALPHA SEQUENCE IN CD28 PROMOTER BEHAVE SIMILARLY IN CD4+ CELLS OF RA PATIENTS AND HEALTHY ELDERLY

M. I. Soroczynska-Cybula<sup>1</sup>, Z. Smolenska<sup>2</sup>, E. Bryl<sup>1</sup>, J. M. Witkowski<sup>1</sup>

<sup>1</sup>Medical University of Gdansk, Pathophysiology, Gdansk, Poland, <sup>2</sup>Medical University of Gdansk, Family Medicine, Gdansk, Poland

There are still discrepancies in the available literature regarding the participation of CD4+ lymphocytes in the pathomechanism of rheumatoid arthritis (RA). They concern mostly phenotypical changes and proliferative dynamics of these cells. It is suggested that the CD4+ lymphocytes of RA patients undergo an accelerated aging, manifested for example by partial or total loss of CD28 from their surface.

Based on the knowledge on the TNF-controlled, specific nucleotide sequence called the  $\alpha$ , present in the promoter region of the CD28 gene and known to be necessary for its expression and functionally impaired in the lymphocytes of RA patients, we have searched the GENBANK<sup>TM</sup> for possible other genes possessing  $\alpha$ -homologous sequences. We found them in the KLOTHO (the 'aging hormone' with  $\beta$ -glucuronidase activity), RAR $\beta$ 2 (the retinoic acid receptor affecting cellular proliferation and differentiation), GRAP-2 (an adapter molecule specific for T cells) and finally a zinc finger regulatory protein, ZNF334. The expression of KLOTHO at both mRNA and protein levels as well as its enzymatic activity is strongly reduced in the CD4+ cells of healthy elderly and of all RA patients as compared to healthy young [JI 2007]. The RA disease activity and type of pharmacological treatment had no influence on the phenomenon. Lowered KLOTHO expression was correlated with the decreased expression of CD28 and of RAR $\beta$ 2 and ZNF334. It was recently shown that KLOTHO interferes with the p53/p21 protein system controlling the progression of the cell cycle; thus, its suppression in the RA and elderly lymphocytes might affect their proliferative dynamics. Similarly, decreased activity of RAR $\beta$ 2 gene expression in the CD4+ cells of RA patients may be (partially) responsible for the modulation of the dynamics of the cell cycle observed in the RA cells.

The age- or RA-related change in expression was apparently correlated with the level of homology between the alpha sequence and those found in the abovementioned genes. Thus, it was the strongest for Klotho, significant for ZNF334 and RAR $\beta$ 2, and non-significant for the expression of GRAP-2. This may suggest a common mechanism controlling (to various extent) the expression of all four genes.

#### PC01/23 ASSOCIATION OF 4Q27, 10P15, 22Q13 CHROMOSOMIC REGIONS WITH TYPE 1 DIABETES IN SPANISH POPULATION

L. Espino-Paisán<sup>1</sup>, M. A. Figueredo<sup>1</sup>, H. de la Calle<sup>2</sup>, J. L. Santiago<sup>1</sup>

<sup>1</sup>Hospital Clínico San Carlos, Immunology, Madrid, Spain, <sup>2</sup>Hospital Ramon y Cajal, Endocrinology, Madrid, Spain

**Objectives:** Type 1 diabetes (T1D) is an autoimmune disease characterized by the destruction of the insulin-producing  $\beta$ -cells in the pancreas mediated by autoreactive T lymphocytes. Numerous studies have been conducted to uncover the genetic basis of the disease and three chromosomal regions comprising the gene *IL2* and its receptor subunits *IL2RA* and *IL2RB* (4q27, 10p15, 22q13 respectively) are currently under in-depth study. Both the interleukin-2 and its receptor are interesting candidates given that they have an important role in the onset and regulation of the cell mediated immune response.

The first analyses performed in European populations found signals in 4q27, 10p15 and 22q13. Our aim was to replicate previous observations and determine whether these associations are limited to pediatric patients or are common to all individuals regardless of the age at disease onset.

**Methods:** We studied 389 T1D patients and 547 healthy controls from the Madrid area. All patients were insulin-dependent and age at disease onset ranged from 1 to 59 years (median = 16). Samples were genotyped for nine SNPs localized in 4q27 (rs11938795, rs17388568, rs6822844), 10p15 (rs2104286, rs41295061, rs11594656) and 22q13 (rs3218253, rs743777, rs9622555). Genotyping was performed with Taqman Genotyping Assays. Genotype and allele frequencies were compared by Chi-square and Fisher tests.

**Results:** We confirmed the association observed in three of the nine polymorphisms studied. In 4q27, we found significant differences in rs6822844 ( $p=0.02$ , OR=0.71 [0.53-0.96]) while rs17388568, also in 4q27 ( $p=0.07$ , OR= 1.21 [0.98-1.49]), and rs41295061 in 10p15 ( $p=0.07$ , OR=0.68 [0.44-1.06]) showed a trend towards statistical significance. The SNP rs3218253 (22q13) was weakly associated only in the age-stratified analysis ( $p=0.06$ , OR=0.73 [0.52-1.03]).

**Conclusion:** We replicated the previously known associations in 4q27 and 10p15, both found in pediatric patients. In our study, we described for the first time that polymorphisms rs6822844 (4q27) and rs41295061 (10p15) are associated with both early and late onset of the disease, while the 4q27 signal (polymorphism rs17388568) is exclusive of pediatric cohorts. Results in 22q13 are inconclusive, probably due to lack of statistical power.

#### PC01/24 HAEMATOLOGIC AUTOIMMUNE MANIFESTATIONS IN CVID PATIENTS: POTENTIAL PROTECTIVE ROLE OF A NOVEL CXCL16 VARIANT

G. Tampella<sup>1</sup>, M. Baronio<sup>1</sup>, M. Vitali<sup>1</sup>, V. Zattoni<sup>1</sup>, J. Marchesi<sup>1</sup>, S. Bolognini<sup>1</sup>, A. Soresina<sup>1</sup>, V. Lougaris<sup>1</sup>, A. Plebani<sup>1</sup>

<sup>1</sup>Department of Pediatrics and Laboratory for Molecular Medicine 'A. Nocivelli', Brescia, Italy

**Objectives:** CVID is the most frequent primary humoral immunodeficiency with an incidence ranging from 1:20.000 to 1:100.000. Autoimmune manifestations frequently complicate the natural history of CVID. In particular, haematologic autoimmune manifestations, ie AIHA and/or ITP have been reported in almost 10% of CVID patients.

CXCL16 is a small cytokine belonging to the CXC family of chemokines. Recently, it was shown that CXCL16 influences the specific response of B cells to CpG via the TLR9 pathway. Various studies have shown that CpG stimulation may be involved in the pathogenesis of autoimmune phenomena.

In this study, we evaluated a cohort of CVID patients for haematologic autoimmune manifestations. In addition, direct sequence analysis for the gene encoding CXCL16 was performed in order to evaluate the potential presence of genetic variants associated with autoimmunity in CVID.

**Methods:** Sixty three patients affected with CVID were included in this study. All patients were under regular Ig replacement therapy. Informed consent was obtained from all patients. Direct gene sequencing of the exons and flanking regions of the CXCL16 were sequenced using an ABI PRISM sequencer.

**Results:** In a total of 63 patients, 9 patients (14.2%) presented either AIHA or/and ITP, while 53 patients (85.8%) did not. The distribution of these haematologic abnormalities was 30% for AIHA and 70% for ITP. Interestingly, eight out of nine patients with these abnormalities presented splenomegaly. Another characteristic of this group included the presence of granulomatous lesions (lungs, liver, spleen): such lesions were present in 5 out of 9 patients (55.5%). Direct gene sequencing of CXCL16 allowed us to identify a novel variant, A220V. This variant is present in 31/53 CVID patients without haematological abnormalities (58%), while it's only present in 1 out of 9 patients with such abnormalities (11%).

**Conclusions:** Autoimmune complications are a rather frequent finding in CVID and may complicate the clinical history of these patients. It is possible that the presence of the A220V variant of CXCL16 may play a protective role in the development of autoimmune haematologic manifestations in CVID. A larger cohort of patients with CVID needs to be studied in order to validate this hypothesis.

#### PC01/25 DISTRIBUTION OF KILLER IMMUNOGLOBULIN-LIKE RECEPTOR 3DL1 ALELLES IN BEHÇET'S DISEASE

J. Duyumaz Tozkir<sup>1</sup>, A. Ugurlu<sup>2</sup>, A. Uyar<sup>2</sup>, P. Norman<sup>3</sup>, P. Parham<sup>3</sup>, G. Saruhan Direskeneli<sup>2</sup>, A. Gul<sup>2</sup>

<sup>1</sup>Trakya University, Immunology, Edirne, Turkey, <sup>2</sup>Istanbul University Faculty of Medicine, Istanbul, Turkey, <sup>3</sup>Stanford University School of Medicine, Stanford, United States

**Objectives:** Behçet's disease (BD) is a chronic, systemic inflammatory disorder of unknown etiology. The pathogenic significance of the strong association with HLA-B\*51 and a weak association with B\*2702 has yet to be identified. HLA-B\*51 and B\*2702 share the Bw4 epitope that can bind to killer immunoglobulin-like receptors 3DL1 (KIR). In this study, we aimed to analyse the role of KIR3DL1/DS1 polymorphisms in BD pathogenesis in patients with and without HLA-Bw4 ligands.

**Methods:** A group of 241 patients with BD and 235 healthy controls comprised the study group. All individuals were genotyped for the KIR3DL1/DS1 polymorphism. Thereafter KIR3DL1\*001, \*002, \*01502, \*004, \*005, \*007, \*008 and KIR3DS1\*013 alleles were determined by PCR- SSP method. Allele frequencies and their positivity along with their respective ligands (HLA-Bw4 or -B\*51) have been analysed in BD patients and controls using a chi-square test.

**Results:** In HLA-Bw4 ligand positive individuals, homozygous 3DL1 genotype was found to be associated with 1.7-times increased the risk for BD (95% CI 1.1-2.6,  $P=0.013$ ). In the comparison of HLA-Bw4 positive BD patients and controls according to their HLA-B51 status, a significant increase of 3DL1/3DL1 genotype (68.1% vs. 54.5%,  $P=0.03$ ; OR = 1.8, 95%CI 1.1-3.0) was detected in B51-negative patients compared to B51-negative controls. The overall group comparison of KIR3DL1/DS1 alleles, KIR3DL1\*001 allele was significantly increased (51.2% vs. 39.8%, OR: 1.58, 95% CI: 1.2-32,  $p=0.018$ ), whereas KIR3DS1\*013 allele was significantly less frequent in BD patients compared to controls (34% vs. 46.3% OR: 0.59, 95% CI: 0.4-0.88,  $p=0.009$ ). Among the Bw4 carrying groups, the increase of KIR3DL1\*001 and decrease of KIR3DS1\*013 allele frequencies were more prominent in HLA-B51 negative patients (64.6% vs. 32.1%, OR: 3.8, 95% CI: 1.8-8.1,  $p<0.0001$  for KIR3DL1\*001 and 25% vs. 60.5%, OR: 0.2, 95%CI: 0.09-0.48  $p<0.0001$  for KIR3DS1\*013).

**Conclusions:** The KIR3DL1/S1 alleles exhibit different expression levels and different inhibitory capacities, and association of HLA-Bw4 epitopes with particular KIR alleles may have a functional significance in the development of BD.

The study is supported by grants from the Istanbul University Research Fund: T752/13052005.

#### PC01/26 KIR GENOTYPING OF ITALIAN PAROXYSMAL NOCTURNAL HAEMOGLOBINURIA PATIENTS

E. Cosentini<sup>1</sup>, S. Lastraio<sup>1</sup>, P. Bruno<sup>1</sup>, L. Gargiulo<sup>2</sup>, M. Cerra<sup>3</sup>, F. Alfinito<sup>3</sup>, R. Notaro<sup>4</sup>, G. Ruggiero<sup>5</sup>, G. Terrazzano<sup>1,5</sup>

<sup>1</sup>Università di Napoli 'Federico II', Biologia e Patologia Cellulare e Molecolare, Napoli, Italy, <sup>2</sup>Istituto Tumori di Genova, Laboratorio di Genetica Umana, Genova, Italy, <sup>3</sup>Università di Napoli 'Federico II', Biochimica e Biotecnologie Mediche, Napoli, Italy, <sup>4</sup>Istituto Toscano Tumori, Firenze, Italy, <sup>5</sup>Università della Basilicata, Potenza, Italy

Paroxysmal Nocturnal Haemoglobinuria (PNH) is a very rare haematopoiesis disorder characterised by the expansion of a stem cell bearing a somatic mutation in the phosphatidylinositol glycan-A (PIG-A) gene, which is involved in the biosynthesis of the glycosyl-phosphatidylinositol (GPI) anchor. Haemolytic anaemia, thrombophilia and cytopenia characterise this disease. A number of data suggest the inability of PIG-A mutation to account alone for the clonal dominance of the GPI-defective clone and for the development of PNH. In this context, the occurrence of immune-mediated mechanisms have been hypothesized.

Our previous data (Hum Immunol 2008, 69:202-6) revealed the association of PNH with the HLA Class I haplotype B\*1402, Cw\*0802 as well as with the extended Mediterranean haplotype A\*33, B\*1402, Cw\*0802, DRB1\*0102. In order to analyse the biological mechanisms underlying PNH pathogenesis we are addressing the analysis of KIR molecules, whose role in the regulation of immune response and self-tolerance has been established.

KIR distribution in 48 patients affected by PNH (estimated to represent almost the half of all Italian PNH patients) and in 72 controls of the same ethnical origin was analysed by PCR-SSP typing. Preliminary results showed no significant associations with the KIR haplotype A or B as well as with significant lack of KIR-HLA matched ligands in our patient cohort. Moreover, a significant decrease of KIR-2DS3 in PNH patients, as compared to healthy controls (26.41% vs 45.83%;  $p < 0.05$ ) was observed. Notably, such KIR allele is present only in 1 out of the 12 patients (8.33%) bearing the PNH HLA associated haplotype B\*1402, Cw\*0802. At variance KIR 2DS3 was observed in 3 out of the 8 (37.5%) healthy controls, till now analysed, bearing the same haplotype.

This association may provide new insights into the autoimmune pathogenesis of PNH.

#### PC01/27 LACK OF ASSOCIATION OF CROHN'S DISEASE SUSCEPTIBILITY POLIMORPHISMS IN THE NKX2-3, ATG16L1 AND IRGM GENES WITH CELIAC DISEASE

B. Dema<sup>1</sup>, C. Maluenda<sup>2</sup>, I. Polanco<sup>3</sup>, C. Núñez<sup>1</sup>

<sup>1</sup>Hospital Clínico San Carlos, Clinical Immunology, Madrid, Spain, <sup>2</sup>Hospital Clínico San Carlos, Paediatric, Madrid, Spain, <sup>3</sup>Hospital Universitario La Paz, Gastroenterology Paediatric, Madrid, Spain

**Objective:** Celiac disease (CD) is an inflammatory and chronic gut disorder that affects genetically susceptible individuals after gluten ingestion. Last advances in CD predisposing genes have resulted from investigating the involvement of genetic polymorphisms implicated in other autoimmune diseases as type 1 diabetes, rheumatoid arthritis or inflammatory bowel disease. Based on this idea, new interesting CD candidate genes could be *NKX2-3*, *ATG16L1* and *IRGM*, which are well-established Crohn's disease risk factors. *NKX2-3* gene encodes a transcription factor that, in mice, seems to be involved in gut development. *ATG16L1* and *IRGM* genes encode proteins involved in autophagy, a process that could be related to innate and adaptive immunity. For that reason, we aimed at studying the implication of several polymorphisms located in those genes in CD susceptibility.

**Methods:** A case-control study was performed with 725 Spanish celiac disease patients diagnosed using ESPGHAN criteria, and 956 ethnically matched healthy individuals used as controls. Three hundred and nine cases were also included, with both parents, in a familial study. We studied five polymorphisms by TaqMan technology: rs10883365 and rs888208, located in the *NKX2-3* gene; rs2241880 in *ATG16L1*; and rs10065172 and rs4958847 in the *IRGM* gene. Genetic frequencies were compared with the  $\chi^2$  test offered by the statistical package EpiInfo v.6.02 and haplotypic frequencies were estimated, when necessary, with the EM algorithm implemented in the Haploview v.4.1 software. The familial study was performed with the transmission disequilibrium test. The statistical power for the case-control study was above 80% for detecting the risk observed in Crohn's disease susceptibility.

**Results:** Genotypic and allelic frequencies did not differ significantly between CD patients and healthy control individuals. In *NKX2-3* and *IRGM* genes, haplotypic frequencies did not show statistically significant differences among both groups either. No differential transmission of alleles or haplotypes from heterozygous parents to affected children was observed in the familial study.

**Conclusion:** No evidence of association with CD has been found for the Crohn's disease susceptibility studied polymorphisms in *NKX2-3*, *ATG16L1* and *IRGM* genes.

#### PC01/28 ROLE OF NOS2A POLYMORPHISMS IN RHEUMATOID ARTHRITIS SUSCEPTIBILITY

J. Varadé<sup>1</sup>, J.R. Lamas<sup>2</sup>, B. Fernández-Gutierrez<sup>2</sup>, E. Gómez de la Concha<sup>1</sup>

<sup>1</sup>Hospital Clínico San Carlos, Immunology, Madrid, Spain, <sup>2</sup>Hospital Clínico San Carlos, Rheumatology, Madrid, Spain

**Objective:** Nitric oxide has been described as a trigger for the synthesis of proinflammatory mediators and as a cytotoxic molecule with a pivotal role in apoptosis at the joints of rheumatoid arthritis (RA) patients. Polymorphisms in the *NOS2A* gene, which codes for the inducible nitric oxide synthase (iNOS), have been tested for association with several autoimmune diseases such as Crohn's disease or type 1 diabetes<sup>1,2</sup>. Moreover, the existence of correlated levels of iNOS protein and synovial cell apoptosis in RA patients<sup>3</sup>, pointed to *NOS2A* as a good candidate gene involved in RA predisposition.

**Methods:** The role of *NOS2A* was studied in 405 Spanish RA patients and in 398 ethnically matched healthy controls, through the analysis of five SNPs: two at the *NOS2A* promoter (rs2779251 and 2779248), other two exonic markers [Asp<sup>346</sup>Asp (rs1137933) and Ser<sup>608</sup>Leu (rs22518)] and the last one located at intron 7 (rs3729508). We also included other two widely-used promoter polymorphisms: the insertion/deletion (TAAA/-) and the (CCTTT)n microsatellite.

**Results:** No association of each individual single-marker or haplotype with RA susceptibility was found. Our data show the low linkage disequilibrium between these *NOS2A* SNPs and the alleles of the (CCTTT)n microsatellite corroborating, the observation previously described in British and Gambian population in a Spanish population.

**Conclusion:** A major role of these *NOS2A* polymorphisms in RA predisposition can be discarded based on the present data.

1. Johannesen, J., Pociot, F., Kristiansen, O. P., Karlsen, A. E. & Nerup, J. No evidence for linkage in the promoter region of the inducible nitric oxide synthase gene (*NOS2*) in a Danish type 1 diabetes population. *Genes Immun* 1, 362-6 (2000).

2. Martin, M. C. et al. Influence of the inducible nitric oxide synthase gene (*NOS2A*) on inflammatory bowel disease susceptibility. *Immunogenetics* 59, 833-7 (2007).

3. van't Hof, R. J. & Ralston, S. H. Nitric oxide and bone. *Immunology* 103, 255-61 (2001).

#### PC01/29 GENETIC DETERMINANTS IN MHC REGION AND ENVIRONMENT RISK FACTORS CONTROL CONTRIBUTE TO FOLLOW-UP OF PSORIASIS PATIENTS RELATIVES

R. F. Magalhaes<sup>1</sup>, A. C. Biral<sup>1</sup>, M. H. Kraemer<sup>1</sup>

<sup>1</sup>University of Campinas, Campinas, Brazil

Psoriasis is a chronic inflammatory skin disease of unknown cause but involves the genes of the Major Histocompatibility Complex (MHC) and environmental factors such as alcoholism, infections, stress and drugs. How to select individuals at risk, do genetic triage, considering the costs involved, and design prevention programs? Psoriasis is more prevalent in family members, a risk that may reach 25%. The objective of this study was to demonstrate the results of a prevention program, considering genetic risk factors for psoriasis in the Brazilian population. A total of 572 individuals, first-degree relatives of 324 psoriasis patients, are enrolled in an orientation program about environmental risk factors for the disease since 1997. Of these, 13 families with more than one generation affected, reaching the total of 59 individuals, were submitted to typing of HLA class I alleles and received more personalized follow-up. No new cases were observed in the studied families. In the prevention program, it is important to acquire knowledge about psoriasis, and improve skin care practices. Measures to fight obesity, smoking habit and alcohol ingestion should be strict. Early detection and treatment of infections, mainly in childhood and adolescence (*Streptococcus*, *Staphylococcus*, *Candida*, etc.) might minimize the possibility of the onset of an undesired immune response. The use of certain medications that may trigger the disease, such as lithium and antimalarial, should be avoided. The psychological and personality aspects of the patients should be considered in prevention programmes. Impulsive behaviour, lack of positive effect, alcohol abuse, sadness, helplessness and depression are attributes frequently associated with disease carriers. Prevention programs for patients with psoriasis should focus on self-motivation, prevention of addictive behaviour, knowledge transfer, and reduction of stress using different relaxation techniques. The prevention program for psoriasis patients and their relatives has been showing positive results.

#### PC01/30 POLYMORPHISMS IN TNFRSF13B ARE NOT ASSOCIATED WITH IDIOPATHIC THROMBOCYTOPENIC PURPURA

J. Birmelin<sup>1</sup>, U. Salzer<sup>2</sup>, B. Grimbacher<sup>1</sup>, J. Bussell<sup>3</sup>

<sup>1</sup>University College of London, Royal Free Campus, Dept. of Immunology, London, United Kingdom, <sup>2</sup>University Hospital Freiburg, Division of Rheumatology and Clinical Immunology, Freiburg, Germany, <sup>3</sup>Weill Medical College of Cornell University, New York, United States

TAC1 is an important factor for B cell survival and proliferation during immune responses and the development of the immune system. It was shown in TAC1<sup>-/-</sup> mice, that inactivation of TAC1 results in an impaired immune response upon vaccination. Furthermore TAC1<sup>-/-</sup> mice developed lymphoproliferative diseases and a lupus-like syndrome. In humans, TAC1 mutations were shown to be associated with common variable immunodeficiency (CVID). 20% of CVID patients develop secondary autoimmune complications such as idiopathic thrombocytopenic purpura (ITP) and autoimmune hemolytic anemia (AIHA). The incidence of ITP is sig-

nificantly higher in CVID patients with TACI mutations than in CVID patients with wild type TACI. In order to investigate a possible impact of TACI-mutations on the development of ITP, we sequenced the gene encoding TACI (*TNFRSF13B*) in a total of 95 patients suffering from ITP. We first focused on exons three and four since these exons reveal the most frequent genetic alterations in CVID patients.

In four patients, we detected a silent heterozygous single nucleotide polymorphism (SNP) at position 291 (T<sup>91</sup> G; P97P), whilst the other 91 patients did not harbour any SNP or other genetic variations.

In summary our data show that although ITP is increased in patients with CVID with mutations in TACI, there is no significant association between SNPs in exons three and four of *TNFRSF13B* and sporadic ITP without CVID.

#### PC01/31 GLUTATHIONE S-TRANSFERASE M1/T1 GENOTYPES AND ANKYLOSING SPONDYLITIS IN A POPULATION GROUP FROM ALGERIA

S. S. Salah<sup>1</sup>, H. Amroun<sup>1</sup>, N. Merad<sup>2</sup>, R. Allat<sup>3</sup>, H. Djoudi<sup>3</sup>, M. Busson<sup>4</sup>, A. Toubert<sup>4</sup>, R. Krishnamoorthy<sup>5</sup>, D. Charron<sup>4</sup>, M. C. Abbadi<sup>1</sup>, R. Tamouza<sup>4</sup>

<sup>1</sup>Institut Pasteur d'Algérie, Immunology, Algiers, Algeria, <sup>2</sup>Hôpital Douéra, Laboratoire central, Alger, Algeria, <sup>3</sup>Hôpital Douéra, Rhumatologie, Alger, Algeria, <sup>4</sup>Hôpital Saint Louis, Laboratoire d'Immunologie et d'histocompatibilité, Paris, France, <sup>5</sup>Hôpital Robert Debré, Inserm U763, Paris, France

**Objectives:** Glutathione S-transferase (GST) enzymes are involved in detoxification of many endo-xenobiotics and carcinogens. Genetic polymorphisms in these enzymes, showing an impaired enzyme activity, have been found to influence the inter-individual susceptibility to cancers, inflammatory and non-inflammatory diseases. Our study tries to determine the effects of genetic polymorphisms of glutathione S-transferase (GST) M1 and GSTT1, on risk and severity of ankylosing spondylitis (AS) in an Algeria population.

**Methods:** A total of 163 patients with AS [fulfilling the modified New York criteria] and 238 healthy non-related subjects were enrolled. DNA from peripheral white blood cells was obtained from all subjects. Deletion of GSTT1 and GSTM1 was assessed by multiplex PCR and phenotype/genotype frequencies were determined and, thus, compared between AS patients and healthy subjects.

**Results:** The OR for risk of AS with the GSTT1-null phenotype was 1.71 (95% CI 1.22- 2.02,  $P_c = 0.02$ ), while no association was observed for GSTM1 phenotypes and susceptibility to AS. When we analyze genotype distribution, taking into account both GSTM1 and T1 frequencies, we can show that : i) there was no statistical difference either in GST genotype 3 (M1-T1+) or 4 (M1-T1-) ; ii) however, a statistically significant difference has been observed in GST genotype 1 (M1+T1+) distribution (28,2% vs. 38,2% ;  $P_c = 0.04$ , OR = 0,64, 95% CI = 0,41 – 0,90) and, finally, iii) the frequency of genotype 2 (M1+T1-) was found 1,6-fold higher in AS patients comparing to healthy subjects (16,6% vs. 10,5%) but with no statistically significant difference ( $p = 0,07$ ).

**Conclusion:** These results suggest that the deletion polymorphism of GSTT1 is associated with increased susceptibility for AS.

#### PC01/32 NALP3 Q705K GENE POLYMORPHISM IN BEHCET'S DISEASE

F. Aydin<sup>1</sup>, T. Ergun<sup>2</sup>, M. Bicakcigi<sup>3</sup>, N. Sahin<sup>1</sup>, H. Direskeneli<sup>1</sup>, G. Saruhan-Direskeneli<sup>4</sup>

<sup>1</sup>Marmara University Medical Faculty, Rheumatology, Istanbul, Turkey, <sup>2</sup>Marmara University Medical Faculty, Dermatology, Istanbul, Turkey, <sup>3</sup>Yeditepe University Medical Faculty, Rheumatology, Istanbul, Turkey, <sup>4</sup>Istanbul University, Istanbul Medical Faculty, Physiology, Istanbul, Turkey

**Objective:** Behçet's disease (BD) is a systemic inflammatory disorder characterized by recurrent mucocutaneous, ocular, vascular, musculoskeletal, central nervous and gastrointestinal system involvement. Both genetic and environmental factors are implicated in BD pathogenesis. As BD has some common properties with auto-inflammatory syndromes, the gene encoding NALP3 inflammasome protein, which activates pro-inflammatory cytokines such as IL-1 and IL-18 may have a role in its pathogenesis. In this study, we investigated Q705K polymorphism of NALP3 gene (*NLRP3*), previously shown to be associated with auto-inflammatory disorders, in BD.

**Methods:** The study was designed as a case-control study with 157 BD patients (Male/Female: 86/71, mean age: 36.5 years) and 168 healthy controls (M/F: 92/76, mean age: 34.9 years). All BD patients had oral ulcers. Genital ulcers were present in 76%, folliculitis in 68.2%, vascular involvement in 48%, uveitis in 60.5% and neurological manifestations in 5% of the patients. The DNA samples from patients and the controls were genotyped by PCR-RFLP method for NALP3 Q705K (C/A, rs35829419) gene polymorphism. Polymorphic region was amplified by PCR and digested with Bsg I enzyme.

**Results:** In the control group the polymorphic allele was present as homozygous genotype (AA) in only 1.2% (2/168) and as heterozygous genotype (CA) in 11.3% (19/168). In BD group only one patient was homozygote for the polymorphism (0.6%) and 8.3% was heterozygote (CA) (13/157) without a statistically significant difference between the groups. Overall allele frequencies between two groups were also without a significant difference (8.6% and 12.5% respectively). Sub-group analysis according to ocular, mucocutaneous or vascular involvement did not reveal any significant differences either.

**Conclusion:** The distribution of NALP3 Q705K gene polymorphism in Turkey reveals no association of this polymorphism with the susceptibility to BD.

#### PC01/33 PEMPHIGUS ANTIBODIES INDUCE DESMOSOME FUSION LEADING TO THEIR INTERNALIZATION

A. A. Lysenko<sup>1</sup>, D. V. Dementieva<sup>1</sup>, E. V. Svirshchevskaya<sup>1</sup>

<sup>1</sup>Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry RAS, Immunology, Moscow, Russian Federation

Pemphigus vulgaris (PV) is induced by autoantibodies directed to adhesion molecule desmoglein 3 (DSG3) which is expressed by squamous epithelial cells within desmosomes. Desmosomes are formed by four type of adhesion molecules DSG 1,3 and desmocollins (DSC) 1,3. PVlgG penetrate epidermis, bind DSG3, and induce acantholysis – cell split, which occurs only in basal layer. Basal layer acantholysis is explained by the compensatory effect of DSG1 present in increased quantity in suprabasal layers. However PVlgG does not induce cell separation in monolayers of keratinocyte cell line HaCaT, which does not express DSG1/DSC1. This difference between keratinocytes in squamous epithelia and in monolayers is not clear. The aim of this work was to compare the effect of pemphigus sera on desmosome numbers on keratinocytes from epidermis and HaCaT which expressed DSG3 but not DSG1. Desmosomes were enumerated by monoclonal antibodies to DSG3 and DSC3 using confocal microscopy. HaCaT were cultivated on glass slides. Normal human skin was cryo-sectioned and stained in the same manner as HaCaT slides. HaCaT expressed 2-3 times more desmosomes than normal keratinocytes and DSG3 and DSC3 were present in comparable quantities. The expression of DSG3 and DSC3 in different layers of epidermis was inverse: in basal layers DSC3 expression was the highest while DSG3 the lowest and vice versa in suprabasal layers.

To estimate the effect of pemphigus sera skin biopsy or HaCaT confluent monolayer were incubated in vitro with sera from different patients overnight. After incubation both type of cells were paraformaldehyde fixed; skin was cryo-sectioned; and desmosomes were stained with MoAb. Pemphigus serum induced a 10-20% decrease in desmosome numbers in HaCaT without morphological modifications of desmosomes. On the contrary in human multilayer epidermis we found a significant decrease in desmosome numbers due to the formation of giant fused desmosomes which were steadily internalized. Desmosome split in the form of hemidesmosome formation was also found.

We concluded that acantholysis in normal skin is induced in immature desmosomes containing less DSG3. On the contrary HaCaT cells represent mature suprabasal cells expressing a balanced amount of both DSG3 and DSC3.

#### PC01/34 IMPAIRED INHIBITORY FC GAMMA RECEPTOR IIB EXPRESSION ON B CELLS IN CIDP

A. Bärenwaldt<sup>1</sup>, J. D. Lünemann<sup>2</sup>, F. Nimmerjahn<sup>1</sup>

<sup>1</sup>University Hospital Erlangen, Erlangen, Germany, <sup>2</sup>The Rockefeller University, New York, United States

The inhibitory Fc-gamma receptor FcγRIIB maintains peripheral tolerance in antigen-activated B lymphocytes by limiting the expansion of autoreactive memory B cells and IgG producing plasma cells. We investigated the expression profile and regulation of FcγRIIB on circulating blood cells in 23 untreated patients with chronic inflammatory demyelinating polyneuropathy (CIDP) and 17 demographically matched healthy controls. FcγRIIB expression levels on monocytes were unchanged in CIDP. In contrast, patients with CIDP showed consistently lower FcγRIIB expression levels on naïve B cells and failed to upregulate FcγRIIB during differentiation to memory cells. Moreover, we observed a significant increase in individuals heterozygous for a promoter polymorphism involved in regulating FcγRIIB expression levels. These data indicate that patients with CIDP show a selective deregulation of FcγRIIB expression on B cells and suggest that selective targeting of FcγRIIB levels might have a therapeutic merit in CIDP.

#### PC01/35 ANTI-β2-GLYCOPROTEIN I AUTOANTIBODY EXPRESSION AS A POTENTIAL BIOMARKER FOR STROKES IN PATIENTS WITH ANTI-PHOSPHOLIPID SYNDROME

H. Y. M. Ali<sup>1</sup>

<sup>1</sup>Dean, Technical & Health Institute-Zakho, Immunology, Zakho-Dohuk-Kurdistan Regional Government, Iraq

**Objective:** Anti-phospholipid syndrome (APS) is an autoimmune disease. Cerebral ischemia associated with APS occurs at a younger age than typical atherothrombotic cerebrovascular disease, is often recurrent, and is associated with high positive IgG anti-phospholipid (GPL) unit levels. This study sought to determine the frequency rates of anti-cardiolipin (aCL) dependent on the presence of β2-GPI, anti-β2-glycoprotein I (aβ2-GPI), and anti-phosphatidyl serine (aPS) IgG autoantibodies among stroke patients, and thus demonstrate the importance of testing for aβ2-GPI autoantibodies.

**Patients & methods:** For these study, stroke patients and control subjects recruited from Mosul, Erbil, and Dohuk provinces in Northern Iraq between March 2004 and March 2005 were evaluated. All cases were under 50 years-of-age and had no recognizable risk factors. Using ELISA to evaluate the presence of IgG isotype of aCL, aβ2-GPI, and aPS autoantibodies in their blood.

**Results:** The results indicated that the frequency of aβ2-GPI was 14/50 (28%), aCL was 11/50 (22%), and aPS was 9/50 (18%) among stroke patients. In contrast, aCL was detected in 2/30 (6.7%) of control subjects; each of the other anti-phospholipid antibodies (APLA) was never observed. Of all the aβ2-GPI+ cases, the incidence of stroke patients having the combined profile of aβ2-GPI + aCL was 11/14 (78.6%) and of aβ2-GPI + aPS was 9/14 (64.3%). Only 2/14 (14.3%)



of these  $\beta$  2-GPI+ patients also expressed aCL in the absence of aPS. The frequency of patients expressing all three markers was only 9/14 (64.3%). In none of the APS/stroke patients were aCL or aPS expressed in the absence of the  $\beta$  2-GPI. Conversely, IgG a  $\beta$  2-GPI as a sole marker was seen in 3/14 (21.4%) of these patients (i.e. in absence of either other marker).

**Discussion:** It can be concluded from these studies that the among the three major forms of APLA examined, the presence of IgG a  $\beta$  2-GPI autoantibodies appeared to correlate best with stroke in patients who were concurrently suffering APS.

#### PC01/36 TRAF1/C5 POLYMORPHISM IS NOT ASSOCIATED WITH PEMPHIGUS

K. Mejri<sup>1</sup>, H. Mbarek<sup>2</sup>, M. Kallel-Sellami<sup>1</sup>, L. Laadhar<sup>3</sup>, E. Petit-Teixeira<sup>2</sup>, Y. Zerzeri<sup>1</sup>, O. Abida<sup>4</sup>, M. Mokni<sup>5</sup>, B. Fezaa<sup>6</sup>, H. Turki<sup>7</sup>, F. Tron<sup>8</sup>, H. Masmoudi<sup>4</sup>, F. Cornelis<sup>2</sup>, S. Makni<sup>1</sup>

<sup>1</sup>La Rabta Hospital, Immunology, Tunis, Tunisia, <sup>2</sup>Genhotel-EA3886, Evry-Genopole, France, <sup>3</sup>La Rabta Hospital, Tunis, Tunisia, <sup>4</sup>Habib Bourguiba Hospital, Immunology, Sfax, Tunisia, <sup>5</sup>La Rabta Hospital, Dermatology, Tunis, Tunisia, <sup>6</sup>Charles Nicolle Hospital, Dermatology, Tunis, Tunisia, <sup>7</sup>Habib Bourguiba Hospital, Dermatology, Sfax, Tunisia, <sup>8</sup>Charles Nicolle Hospital, Immunology, Rouen, France

**Background:** Pemphigus is an autoimmune blistering disease characterized by the production of pathogen autoantibodies against desmosomal protein: antidesmoglein 1 (Dsg 1) in pemphigus foliaceus (PF) and anti-Dsg3 in pemphigus vulgaris (PV). Recently, a polymorphism into the intergenic region TRAF1/C5 was described as a risk factor for rheumatoid arthritis.

**Objectives:** Our aim was to investigate at a first step the polymorphism rs 10818488 G/A in PF and PV patients and to assess secondly the SNP's effect on the C5 sera level.

**Methods:** The genotyping of the SNP was carried out by a 5'allelic discrimination PCR TaqMan<sup>®</sup> assay. An ELISA test was performed to pinpoint the concentration of the complement component C5.

**Results and conclusion:** No association of the SNP with both forms of the pemphigus was observed. Serological assay revealed also that the SNP had no effect on the C5 concentration. Even though the polymorphism TRAF1/C5 seems to be not associated to pemphigus, its relevance to autoimmunity should be more analysed in autoimmune diseases other than rheumatoid arthritis where it was first reported.

#### PC01/37 THE ROLE OF BILE SALT EXPORT PUMP (BSEP) AND MULTIDRUG RESISTANCE PROTEIN2 (MRP2) POLYMORPHISMS IN DRUG INDUCED HEPATOTOXICITY

E. Crespo<sup>1</sup>, E. Ulzurrun<sup>2</sup>, P. Saenz-Lopez<sup>3</sup>, F. Ruiz-Cabello<sup>3</sup>, Y. Borraz<sup>2</sup>, R. Andrade<sup>4</sup>, M. Lucena<sup>2</sup>

<sup>1</sup>Facultad de Farmacia, Dpto. Farmacología, Granada, Spain, <sup>2</sup>Facultad de Medicina, Servicio de Farmacología Clínica, H. Virgen de la Victoria, Malaga, Spain, <sup>3</sup>Hospital Universitario Virgen de las Nieves, Analisis Clinicos, Granada, Spain, <sup>4</sup>Facultad de Medicina, Unidad de Hepatología, H. Virgen de la Victoria, Malaga, Spain

**Objectives:** A reduction in the level of expression of canalicular transporter genes could enhance the exposure to reactive metabolites in hepatocytes. Genetic polymorphisms in the bile salt export pump (BSEP, ABCB11) and the multidrug resistance protein 2 (MRP2, ABCC2) genes can lead to alteration in gene expression and protein function, which may increase the risk of developing idiosyncratic drug-induced liver injury (DILI).

**Material and methods:** Genotype distribution of polymorphisms in BSEP (ABCB11 1331T>C (V444A)) and MRP2 (ABCC2 -24C>T, 4581G>A (C1515Y), 3563 T>A (V1188E) y 1249G>A (V417L)) was analyzed in patients with liver injuries induced by pharmaceuticals present in the National Registry of Liver Toxicity (Andrade et al., Gastroenterology 2005) diagnosed as highly likely or probable according to the CIOMS scale and in controls consisting of healthy Caucasians age, gender and drug matched. The genotyping was performed using 5' allelic Taqman probes.

**Results:** The CC genotype frequency of ABCB11 1331T>C, which is associated with decreased expression, was augmented in patients with hepatocellular type of liver injury (49%) compared to controls (32%) (OR=2.1; 95% CI=1.4-2.9; Pc=0.008). A significant difference in allele frequency of this polymorphism was detected in patients with non-steroidal anti-inflammatory drug-induced liver injury (n=20) compared to controls (OR=2.9; 95% CI=1.6-5.4; Pc=0.002). No differences in ABCC2 C-24T, G1249A, G4581A and T3563A genotype distribution was found between DILI patients and controls. Significant ethnical differences in the ABCC2 C-24T genotype distribution was found between various control populations.

**Conclusions:** Our data suggest that the ABCB11 1331T>C polymorphism could be a risk factor for the development of drug-induced hepatocellular damage. In contrast, the polymorphisms analyzed in ABCC2 do not appear to confer susceptibility towards developing DILI. These data indicate that NSAIDs in conjunction with impairment of BSEP function caused by the existence of genetic polymorphisms could lead to toxic bile salts accumulation in hepatocytes. Hence, this BSEP transporter may represent a new site of drug interaction for NSAIDs.

### PC02 – ROLE OF MICROBES IN AUTOIMMUNITY

#### PC02/1 IMPACT OF PATHOGEN-SPECIFIC REGULATORY T-CELLS ON THE COURSE OF INFLUENZA INFECTION

H. Fraundorff<sup>1</sup>, M. Gereke<sup>1</sup>, S. Mauel<sup>2</sup>, A. Gruber<sup>2</sup>, D. Bruder<sup>1</sup>

<sup>1</sup>Helmholtz Center for Infection Research, Immune Regulation, Braunschweig, Germany, <sup>2</sup>Freie Universität Berlin, Institut für Tierpathologie, Berlin, Germany

**Objectives:** Regulatory T cells (Tregs) have been described to play a pivotal role in preventing autoimmune diseases or controlling overwhelming immune responses. However, there are significant gaps in our knowledge regarding the strength of pathogen-induced immune responses in Treg suppressed hosts. To investigate the impact of Tregs on pathogen specific immune responses, we examined the course of respiratory virus infections in individuals suffering from chronic pulmonary disease controlled by in vivo induced Tregs.

**Methods:** To dissect the immunological and molecular mechanisms underlying CD4<sup>+</sup> T cell dysregulation in the context of autoimmunity, influenza A hemagglutinin (HA) was expressed in the lung of SPC/HAXTCR/HA mice harboring CD4<sup>+</sup> T cells specific for HA which resulted in a severe immune-mediated interstitial lung disease in those SPC/HAXTCR/HA mice. Interestingly, pulmonary disease was controlled by induction of influenza HA-specific Tregs at the site of inflammation. Diseased SPC/HAXTCR/HA and healthy TCR/HA mice were infected with a sub-lethal dose of Influenza A and sacrificed at different time points after infection. The course of infection was monitored by weight loss and histological lung examinations. Moreover, activation status of HA-specific CD4<sup>+</sup> T cells and Treg distribution were assessed by FACS-analysis.

**Results:** Unlike TCR/HA mice SPC/HAXTCR/HA mice exhibit an increased number of in vivo induced Tregs in the lung. After influenza A infection SPC/HAXTCR/HA mice in comparison to TCR/HA mice neither lost weight nor did they display signs of influenza-specific pneumonia in the lung. Moreover, upon infection SPC/HAXTCR/HA mice exhibited impaired HA-specific CD4<sup>+</sup> T cell activation. Furthermore, we observed a transient decrease of HA-specific Foxp3<sup>+</sup> Tregs in the early phase of infection.

**Conclusion:** We could demonstrate that autoimmune-mediated interstitial lung disease in SPC/HAXTCR/HA mice induces influenza HA-specific Tregs, which were suspected to interfere with pathogen-specific immunity. Surprisingly, SPC/HAXTCR/HA mice showed neither influenza-related pneumonia nor weight loss upon infection, indicative for lack of pathogen-related immunopathology. Interestingly, despite a transient decrease in influenza-specific Tregs, no breakdown of self-tolerance in SPC/HAXTCR/HA mice was observed upon infection suggesting well balanced immunological mechanisms protecting SPC/HAXTCR/HA mice from influenza-mediated exacerbation of pulmonary autoimmune disease.

#### PC02/2 CCL3/MIP1-ALPHA, CCL1/I-309 AND CXCL9/MIG ARE THE MEDIATORS OF CELLULAR INFILTRATION OF MYOCARDIUM AND VALVULAR HEART LESIONS IN SEVERE RHEUMATIC CARDITIS PATIENTS

K.C. Fae<sup>1,2</sup>, S.A. Palacios<sup>1,2</sup>, A.C. Tanaka<sup>1</sup>, P.M.A. Pomerantzeff<sup>1</sup>, J. Kalil<sup>1,2,3</sup>, L. Guilherme<sup>1,2</sup>

<sup>1</sup>Heart Institute (InCor), School of Medicine, University of Sao Paulo, Sao Paulo, Brazil, <sup>2</sup>Institute for Immunology Investigation, Millenium Institute, Sao Paulo, Brazil, <sup>3</sup>Division of Clinical Immunology and Allergy, University of Sao Paulo, Sao Paulo, Brazil

**Objectives:** Rheumatic fever (RF) is a post-infectious autoimmune disease due to sequel of group A streptococcus pharyngitis. Rheumatic heart disease (RHD) the major manifestation of RF is characterized by inflammation of myocardium/heart valves. Intense inflammatory infiltrate, with predominance of T cells, macrophages and B cells in cardiac rheumatic lesions was described. The *in situ* analysis of cytokines showed predominance of IFN $\gamma$ , TNF $\alpha$ -producing cells in the myocardium and valvular tissue with scarce IL-4<sup>+</sup> cells in the valves suggesting that inflammatory cytokines (Th1) mediated rheumatic lesions and the lack of regulatory cytokines (Th2) contribute to the severity of valve lesions. The mechanism involved in the recruitment/maintenance of these cells is not completely understood. In this study we examined the gene expression of several chemokines and their receptors (CCL3/MIP1 $\alpha$ , CCL4/MIP1 $\beta$ , CCL5/RANTES and CCR5; CXCL10/IP10, CXCL9/Mig and CXCR3; CCL22/MDC, CCL17/TARC and CCR4; CCL1/I-309 and CCR8) involved in the recruitment of cells.

**Methods:** Gene expression was studied by qRT-PCR using mRNA from myocardium (N=12) and valvular (N=5) biopsies from RHD patients that underwent to surgery for valve replacement. Relative gene expression was calculated using as control mRNA post-mortem tissue samples from individuals with non-rheumatic etiology.

**Results:** Our results showed high expression of CCL3/MIP1- $\alpha$  in myocardium lesions (p=0.01 vs valves). In contrast CCL1/I-309, CXCL9/Mig, CCR5 and CCR8 were highly expressed in valvular lesions (p=0.03 vs myocardium).

**Conclusion:** These results suggested that cellular infiltration of myocardium tissue is mediated essentially by CCL3/MIP1- $\alpha$ . On the other hand, the chemokines CCL1/I-309 and CXCL9/Mig and the receptors CCR5 and CCR8 must be the main molecules involved in cellular recruitment and infiltration in the valves. In addition CCL1/I-309 could act in the angiogenesis induction via endothelial cells. Therapeutic interventions aiming to block locally the chemokines would be of interest to control the development of RHD lesions.

**Financial support:** CNPq and FAPESP, from Brazil.

# PC02/3 TRYPANOSOMA CRUZI INFECTION IN NOD MICE INDUCES A PREFERENTIAL CD4+ T CELL IMMUNE RESPONSE IN ADDITION TO PROTECT THESE MICE FROM TYPE I DIABETES

D. Pessina<sup>1</sup>, J. Nihei<sup>1</sup>, F. Cardillo<sup>1</sup>, J. Mengel<sup>2</sup><sup>1</sup>CPqGM/Fiocruz, Salvador, Brazil, <sup>2</sup>Fiocruz, Rio de Janeiro, Brazil

**Objectives:** This study aims to characterize peripheral tissue- and lymphoid-immune responses to the *T. cruzi* (Tulahuen strain) parasite in an autoimmune strain such as the NOD mouse. At the same time to verify a possible autoimmune disease related to the parasite itself as well as to the NOD genetic background (type I diabetes).

**Methods:** NOD and BALB/c mice were used. Mice were sacrificed at different days after infection. FACS analysis of splenic, lymph nodes and mononuclear cells that infiltrates skeletal muscle and heart were analyzed for different markers and cytokines. Parasitemia, mortality and blood glucose levels were determined.

**Results:** NOD mice are more susceptible than BALB/c mice to the *T. cruzi* infection. Parasitemia levels in NOD mice were higher than in Balb/c mice. The mortality of NOD mice reached up to 20% during the acute disease, whereas 100% of BALB/c mice survived. The resistance in BALB/c mice correlates with a rapid and huge expansion of CD8+ T cells and a long lasting induction of CD8+ effector memory T cells that could not be detected in NOD mice. CD8+ T cells predominate as inflammatory lymphocytes in skeletal muscle from BALB/c mice. In contrast, CD4+ T cells preponderate in NOD mice skeletal muscle. The absolute splenic cell numbers of IFN-gamma producing T cells in BALB/c mice were higher than the numbers found for infected NOD mice. The opposite situation was found in inflammatory cells that infiltrate skeletal and heart muscles, where the frequency of T cells producing IFN-gamma was higher in NOD mice tissues. In addition, we have observed that *T. cruzi* infection protects NOD mice from diabetes.

**Conclusion:** NOD and BALB/c mice have different response patterns to the same antigenic stimulation. CD4+ T cells dominate in NOD mice, whereas BALB/c mice presented a long-lasting CD8+ T cell response. This situation may favor the appearance of a possible CD4+ T cell-related autoimmunity to the *T. cruzi* infection in NOD mice and at the same time, inducing protection to type I diabetes. Studies are in progress to better characterize this model and clarify the mechanisms responsible for diabetes protection induced by *T. cruzi*.

# PC02/4 CHARACTERISING THE IMPACT OF THE EARLY LIFE MICROFLORA ON THE MATURATION OF THE REGULATORY IMMUNE SYSTEM

C. H. F. Hansen<sup>1</sup>, M. R. Hufeldt<sup>2</sup>, B. Aasted<sup>1</sup>, A. K. Hansen<sup>3</sup><sup>1</sup>Faculty of Life Sciences, Copenhagen University, Immunology, Department of Veterinary Pathology, Frederiksberg C, Denmark, <sup>2</sup>Scanbur A/S, Karlslunde, Denmark, <sup>3</sup>Faculty of Life Sciences, Copenhagen University, Biomedicine, Department of Veterinary Pathology, Frederiksberg C, Denmark

Life style diseases are becoming an increasing societal problem. Much focus has been upon obesity, but many of these diseases are inflammatory by nature and the causal factors are among other thing to be found in a combination of genetics, early life incidents and the food we eat. Diseases, inflammatory by nature, are such as type 1 and type 2 diabetes mellitus, multiple sclerosis, atherosclerosis and colitis. Common for all these disease models are that a state of subacute or chronic inflammation is induced by involvement of the adaptive immune response based upon T helper cell. The involved adaptive immune response can be downregulated by a regulatory immune response. The balance between the regulatory and the adaptive immune response, and the balance between different parts of the adaptive immune response is crucial for characteristics of the diseases, such as severity, onset time and recovery.

The balance between the regulatory and the adaptive immune response is heavily influenced by early life bacterial stimulation and the commensal microflora represent a critical factor for the development and maturation of the gut mucosal immune system.

In a period after birth contact with microorganisms is more likely to induce the formation of regulatory T cells rather than T effector cells. There is good reason to assume that this priming window in rodents is rather short, maybe even less than a week. So the regulatory immune response can be prevented or induced in laboratory rodents in the early phase of life.

In this study, mice were born under germ-free conditions and inoculated with a standardized flora at different young ages. The impact of inoculation on intestinal immunology was characterized primarily based upon sampling cells from the Peyer patches. The amount of regulatory T cells, the balance between Th1 and Th2 and the activity of dendritic and NKT cells was monitored and the window for priming of a regulatory immune response was characterized.

# PC02/5 IRRESPECTIVE OF THE CONTEXT OF HIGH DOSES TETANUS TOXOID APPLICATION MOLECULAR MIMICRY COULD NOT BE NEGLECTED AS A CAUSE OF $\beta_2$ -GLYCOPROTEIN I-SPECIFIC ANTIBODIES PRODUCTION

M. M. Stojanović<sup>1</sup>, A. B. Inić-Kanada<sup>1</sup>, I. P. Živković<sup>1</sup>, V. Ž. Petrušić<sup>1</sup>, L. A. Dimitrijević<sup>1</sup><sup>1</sup>Institute of Virology, Vaccine and Sera – Torlak, Belgrade, Research and Development, Belgrade, Serbia

**Objectives:** We already reported that immunization with tetanus toxoid (TTd) in high doses induced rise in anti- $\beta_2$ -glycoprotein I ( $\beta_2$ GPI) antibodies (Abs) with pathogenic potential dependant on context of TTd application. The rise of anti- $\beta_2$ GPI Abs could be induced due to structural similarity of TTd and  $\beta_2$ GPI (molecular mimicry) or could be a consequence of polyclonal/bystander  $\beta_2$ GPI-specific B cells stimulation. This study evaluated the contribution of those mechanisms to anti- $\beta_2$ GPI Abs production in our model system.

**Methods:** Non- or CFA-pretreated Balb/c mice were immunized with TTd (3x100  $\mu$ g/dose; two-week intervals) mixed with aluminiumhydroxide or 2.5M glycerol (5 mice/group). We analyzed by ELISA: 1) inhibition of sera IgG binding to  $\beta_2$ GPI adsorbed directly onto MaxiSorp plates ( $\beta_2$ GPI system) or to PolySorp plates coated with phosphatidylserine (PS+ $\beta_2$ GPI system) in the presence of TTd; 2) abundance of TTd-,  $\beta_2$ GPI- and PS+ $\beta_2$ GPI- specific cells within splenic B cell pool; 3) levels of TTd- or  $\beta_2$ GPI-specific IgG in supernatants of non-, LPS- or ConA-stimulated immunized mice' splenocytes (48h).

**Results:** TTd inhibited binding of immunized mice' IgG to  $\beta_2$ GPI in both systems while its influence on normal controls (nc) was negligible. In all groups inhibition was more pronounced in PS+ $\beta_2$ GPI system (8-20% vs. 24-40%). Comparing to nc, non-pretreated mice'splenic IgM<sup>+</sup> B cell pool contained significantly higher portion of TTd- and  $\beta_2$ GPI-specific cells while in CFA-pretreated mice abundance of cells possessing those specificity were significantly elevated within IgG<sup>+</sup> pool. Abundance of PS+ $\beta_2$ GPI-specific cells were significantly higher within IgM<sup>+</sup> B cell pools, irrespective of immunization protocol, but in CFA-pretreated groups PS+ $\beta_2$ GPI-specific cells were also significantly abundant within IgG<sup>+</sup> pool. The rise in anti-TTd IgG production was observed following both ConA and LPS splenocytes stimulation while only ConA augmented secretion of anti- $\beta_2$ GPI IgG in all groups.

**Conclusion:** CFA pretreatment stimulated IgM to IgG switch of both TTd- and  $\beta_2$ GPI- specific immune response. Glycerol more efficiently stimulated differentiation of IgG<sup>+</sup> cells specific for  $\beta_2$ GPI cryptic epitopes. In all groups, majority of anti- $\beta_2$ GPI IgG were secreted by polyclonally/bystander activated LPS-low responding B cells but portion of  $\beta_2$ GPI-specific IgG, especially those recognizing cryptic epitopes, were produced due to molecular mimicry.

# PC02/6 BORRELIA BURGDORFERI INFECTION IN IRANIAN PATIENTS WITH MULTIPLE SCLEROSIS

G. Mosayebi<sup>1</sup>, M. Khakei<sup>2</sup>, A. Ghazavi<sup>2</sup>, K. Ghasami<sup>2</sup>, M. Rafei<sup>2</sup><sup>1</sup>Arak University of Medical Sciences, Immunology and Microbiology, Arak, Iran, Islamic Republic of, <sup>2</sup>Arak University of Medical Sciences, Arak, Iran, Islamic Republic of

**Background:** Multiple sclerosis is an autoimmune disease characterized by the inflammatory demyelination of central nervous system (CNS).

Epidemiological studies have been shown that many exogenous factors (bacteria- viruses) and endogenous criteria (genetically fields) are affected in MS.

**Materials and Methods:** In this descriptive case-control study, 31 new cases of MS patients and 65 healthy controls were studied with matched for sex, age and same geographic region. Antibodies titers (IgM and IgG) to *Borrelia burgdorferi* were detected in sera by ELISA method.

**Results:** The results show that there was significant difference between case and control groups in IgM titer against borrelia burgdorferi (p=0.001). According to logistic regression test, probability of MS is increased by acute infection of borrelia (ORs=4.324, p=0.006).

**Conclusion:** *Borrelia burgdorferi* is one of the most infectious agents that accounted as exogenous factor in immunopathogenesis of MS.

**Keywords:** multiple sclerosis, borrelia burgdorferi, antibody

# PC02/7 THE ROLE OF MYCOPLASMAL INFECTION AND ANTICARDIOLIPIN ANTIBODIES AS AUTOIMMUNE PARAMETERS IN PREGNANCY LOSS

F. S. Bayoumi<sup>1</sup>, I. M. Ramzi<sup>2</sup>, H. M. Rashad<sup>3</sup>, I. M. R. Hussein<sup>4</sup>, M. G. Hind<sup>4</sup><sup>1</sup>National Research Center, Immunogenetics Dep., Cairo, Egypt, <sup>2</sup>National Research Center, Molecular Genetics and Enzymology Dept, Cairo, Egypt, <sup>3</sup>National Research Center, Environmental & Occupational Medicine Dept, Cairo, Egypt, <sup>4</sup>National Research Centre, Cairo, Egypt

**Aim:** We aimed to find a relationship between repeated abortions of unknown etiology and autoimmune disease either caused by *Mycoplasmas* infection and/or Anticardiolipin antibodies (IgG & IgM).

**Subjects:** 23 women (21-41 years) with history of recurrent abortion, intrauterine fetal death and/ or neonatal death (after exclusion of other factors as cause abortion) & ten women with normal pregnancy outcome with the same age were chosen as controls.

**Methods:** *Mycoplasma hominis* & *Ureaplasma urealyticum* were detected in blood by PCR. Anticardiolipin antibodies (IgG & IgM) were detected in blood using ELISA technique.

**Results:** *Mycoplasma hominis* could be detected by PCR in 7/23 (30.4%) in women with pregnancy losses, but was not detected in control group. *Ureaplasma urealyticum* could not be detected in the two groups. No relation was observed between infection with *Mycoplasma* and number of pregnancy losses. High levels of Anticardiolipin antibodies (IgM and IgG) were observed in 10/23 (43.5%) and 4/23 (17.4%) of cases respectively. Six cases (26.1%) showed high levels of both IgG & IgM. No significant differences in their mean values was observed compared to the control group, however, significant difference was observed in patients with four abortions or patients with adverse outcome compared to controls.

**Conclusion:** The role of autoimmune abnormalities induced by *Mycoplasma* infection in the etiology of pregnancy losses was proposed; therefore we recommend that all women with poor pregnancy outcome, before planning a subsequent pregnancy, should test for the presence of antiphospholipid antibodies and/or bacterial infections.

## PC02/8 ANTIBODIES TITERS TO MUMPS, MEASLES, HUMAN HERPES VIRUS-6, PARA-INFLUENZA AND EB VIRUSES IN IRANIAN PATIENTS WITH MULTIPLE SCLEROSIS

M. Khakei<sup>1</sup>, G. Mosayebi<sup>1</sup>, A. Ghazavi<sup>2</sup>, K. Ghasami<sup>2</sup>, M. Rafei<sup>2</sup>

<sup>1</sup>Arak University of Medical Sciences, Immunology and Microbiology, Arak, Iran, Islamic Republic of, <sup>2</sup>Arak University of Medical Sciences, Arak, Iran, Islamic Republic of

**Background:** Multiple Sclerosis (MS) is a demyelination, auto-immune disease which affects the central nervous system. The etiology of MS is unknown, but some evidences indicated that environmental factors such as viruses are implicated in the development of MS.

**Materials and Methods:** In this descriptive case-control study, 31 new cases of MS patients and 65 healthy controls were studied with matched for sex, age and same geographic region. Antibodies titers (IgM and IgG) against mumps, measles, human herpes virus-6, para-influenza and EB viruses were detected in sera by ELISA method.

**Results:** Antibodies titers (Special IgM) against mumps, measles, human herpes virus-6 and para-influenza virus were significantly higher in patients with MS compared to controls. There is no significant difference at the level of Anti-EBV antibodies in the patient and control groups.

**Conclusion:** In contrary to the most similar studies, EBV dose not exert a direct role in the pathophysiology of MS in this geographic area.

**Keywords:** multiple sclerosis, viruses, antibody

## PC03 – ANTIBODY DEFICIENCIES

### PC03/1 OUTCOMES IN PATIENTS WITH COMMON VARIABLE IMMUNODEFICIENCY DISORDERS (CVIDs)

H. Chapel<sup>1</sup>, M. Lucas<sup>1,2</sup>

<sup>1</sup>University of Oxford, Oxford, United Kingdom, <sup>2</sup>Oxford Radcliffe Hospitals, Oxford, United Kingdom

**Aims:** To determine whether the division of CVIDs into five distinct clinical phenotypes results in differing survival figures and whether the bacterial infection rate is modified by replacement immunoglobulin therapies.

**Methods:** Analysis of European CVIDs database (334 patients over 10 years) as well as the Oxford PID database (115 patients over 25 years).

**Results:** Analysis of mortality in patients with CVIDs has shown that survival has improved since previous data was published in 1999. Analysis by CVID phenotype shows that those without disease-related complications have an almost normal life expectancy whereas survival has not changed substantially since 1999 for those with such complications.

The Oxford infection analysis (90 patients over 22 years) shows that IgG trough levels have increased over three decades, in keeping with a reduction of serious and moderate infections. This is reflected by the finding that an increasing proportion of patients remain free of bacterial infection, as well as a decreasing number of patients who suffer from more than 4 infections pa.

The data also confirms the clinical suspicion that each patient has a unique IgG trough level [5-17 g/l] at which bacterial infections can be prevented. Furthermore this level is hugely variable amongst patients with a CVID.

Comparison of this data with infection data from 15 patients with XLA is currently underway.

The development of bronchiectasis is related to previous serious infections [p=0.03] and results in a higher rate of mortality in patients with CVIDs [p=0.0004]. However, if patients are treated with adequate doses of replacement immunoglobulin, there is no difference in the rate of breakthrough infections between those with pre-existing bronchiectasis and those without.

**Conclusions:** This study provides some data to support the "expert opinions" that form the basis of many national guidelines for treatment of these patients.

### PC03/2 REPLICATIVE SENESECE PREVENTS THE DIFFERENTIATION OF B-CELLS TO ANTIBODY PRODUCING CELLS IN COMMON VARIABLE IMMUNODEFICIENCY

M. Visentini<sup>1</sup>, V. Conti<sup>1</sup>, M. Cagliuso<sup>1</sup>, G. Siciliano<sup>1</sup>, C. Milito<sup>1</sup>, I. Quinti<sup>1</sup>, M. Fiorilli<sup>1</sup>

<sup>1</sup>Sapienza – University of Rome, Rome, Italy

**Objective:** A reduction of memory B-cells associated with the loss of serological memory and impaired secondary antibody responses represents the hallmark feature of CVID. Recent data evidenced the expansion of an abnormal B cell subset identified as CD21<sup>low</sup> B-cells in a large subgroup of CVID patients. The aim of our work is to contribute to understand their functional properties and to identify their possible pathogenic role.

**Methods:** Sixty-two CVID subjects were enrolled in this study. All patients fulfilled the criteria for CVID based on the ESID/PAGID definition and were classified in accord with the EUROclass classification. Patient's B cells were characterized by phenotypic analysis. Their capacity to proliferate and differentiate to antibody producing plasmablasts in response to Toll-like receptor 9 (TLR9) stimulation with CpG DNA was determined by the CFSE dilution method. Telomere length was analysed by Flow-FISH.

**Results:** A large subset (~70% of cases) of patients with CVID have an increased proportions of B-cells with reduced expression of CD21. The immunophenotype of CD21<sup>low</sup> B-cells demonstrated that they largely (50% on average) expressed the inhibitory receptor FCRL4, found in a subset of tonsillar CD27<sup>+</sup> B-cells, called tissue-like memory cells. The analysis of the CFSE dilution profile revealed a poor proliferative capacity of CD21<sup>low</sup> B-cells, in that few of these cells entered division (precursor cohort) as shown by the inverse correlation between the frequency of CD21<sup>low</sup> B cells and the precursor cohort of total B cells. Overall, B-cells from CVID patients had a poor "division destiny", reflected by a reduced capacity to achieve the 4<sup>th</sup> cell division resulting in an inability to differentiate into plasmablasts upon stimulation with CpG. Moreover, telomere attrition was found in CVID purified B cells and the analysis of the mean fluorescence intensities of the telomere probe in CVID was inversely proportional to the percentages of CD21<sup>low</sup> B-cells.

**Conclusion:** The functional defect of CD21<sup>low</sup> B-cells of CVID patients appears to consist of an inability to undergo the number of cell divisions necessary for differentiation to the plasmablast stage. This reduced proliferative potential appears, in turn, related to a state of replicative senescence associated with telomere loss.

### PC03/3 IG LEVELS AND B CELL DIFFERENTIATIVE CAPACITY PREDICT THE RISK OF ACUTE INFECTIONS IN CVID

I. Quinti<sup>1</sup>, M. Visentini<sup>1</sup>, M. Cagliuso<sup>1</sup>, V. Conti<sup>1</sup>, C. Agostini<sup>2</sup>, G. Spadaro<sup>3</sup>, A.C. Trombetta<sup>1</sup>, A. Soresina<sup>4</sup>, C. Milito<sup>1</sup>, M. Fiorilli<sup>1</sup>

<sup>1</sup>Sapienza – University of Rome, Rome, Italy, <sup>2</sup>University of Padua, Padua, Italy, <sup>3</sup>University of Naples, Naples, Italy, <sup>4</sup>University of Brescia, Brescia, Italy

**Aims:** The increased survival of patients with PID is the result of improved detection of infectious diseases, the use of antibiotics and immunoglobulins, the prophylaxis against infections, the availability of new antimicrobial agents, and definitive treatment of their diseases. Despite immunoglobulin substitutive therapy has been proved to be definitely beneficial (evidence category IIB), Ig level to be maintained over time has never been defined in prospective studies. Moreover, biological correlates of the risk to develop associated clinical conditions are poorly understood.

**Methods:** In 2000, because of the increasing complexity of PID management, a collaborative group involving both pediatric and adult immunological centers has been established with the aim to increase the awareness of these disorders among physicians and to provide the best clinical assistance to all patients on the national territory. All centers were invited to register the clinical data of patients with a diagnosis of CVID, in a national database. Clinical data have been correlated with the results obtained in the in vitro study aimed to analyse the proliferative and differentiative capacity of patients B cells.

**Results and conclusions:** The multicenter prospective study on a cohort of 201 patients with Common Variable Immunodeficiency provided information on the spectrum of illnesses which occurred over a long time period of follow-up, and information on the effects of long-term immunoglobulin treatment. The multivariate analysis demonstrated that despite the IgG replacement treatment, a progressive increase in the prevalence of patients with chronic diseases, mainly sinusitis and chronic lung disease, was observed in all age groups, including the pediatric population. Proliferative and differentiative response to TLR 9 and TLR 7 stimulation in CVID patients correlated with in vivo and in vitro IgM and IgA secretion, and with a clinical history positive for pneumonia and CLD. The following risk factors for pneumonia have been identified:

- 1) in patients with CLD, pneumonias developed more frequently than in patients without CLD;
- 2) in patients without CLD, the risk of pneumonias correlates with the residual IgA levels;
- 3) the reduced capacity of B cell to differentiate into plasma cells correlates to the risk to develop pneumonias and CLD.

### PC03/4 THE DEFI CLASSIFICATION, BASED ON B AND T CELL PHENOTYPES IN CVID PATIENTS, CORRELATES THE CLINICAL PHENOTYPE OF THE DISEASE

G. Mouillot<sup>1</sup>, M. Carmagnat<sup>2</sup>, L. Gérard<sup>3</sup>, C. Fieschi<sup>3</sup>, P. Debré<sup>1</sup>, C. Rabin<sup>2</sup>, E. Oksenhendler<sup>3</sup>, DEFI study group

<sup>1</sup>Assistance Publique-Hôpitaux de Paris, Laboratoire d'immunologie cellulaire et tissulaire, INSERM UMR-S945, GH Pitié-Salpêtrière, Paris, France, <sup>2</sup>Assistance Publique-Hôpitaux de Paris, Laboratoire d'immunologie et histocompatibilité, hôpital Saint-Louis, Paris, France, <sup>3</sup>Assistance Publique-Hôpitaux de Paris, Immunopathologie clinique, Hôpital Saint Louis, Paris, France

Common variable immunodeficiency (CVID) is a heterogeneous disorder characterized by recurrent infections and defective immunoglobulin production. We investigated the peripheral T and B cell compartments in 313 CVID patients enrolled in the DEFI French national study and in 50 healthy controls by whole blood



flow cytometry. The major B cell abnormalities were a defect in switched memory B (smB) cells and an expansion of CD21low B cells. The defect in naive CD4+ T-cells was the main T cell defect; it was associated with an increase of activated CD95+ (Fas) T-CD4+ cells. We combined B and T cell analyses into a common simple T/B classification based on the percentages of naive CD4+ T-cells (< or ≥ 20% of CD4+ T-cells), of B cells (≤ or > 1% of total lymphocytes) and of smB cells (≤ or > 2% of B cells) and showed that splenomegaly and lymphoma were associated with a defect in both T and B cells; granulomatous disease was linked to a T cell defect whereas bronchiectasis was linked to a B cell defect. No correlation could be demonstrated for autoimmune complications.

PC03/5

**EVALUATION OF CARMA1 AS A CANDIDATE GENE FOR THE PATHOGENESIS OF CVID**G. Tampella<sup>1</sup>, M. Baronio<sup>1</sup>, M. Vitali<sup>1</sup>, F. Morandini<sup>1</sup>, S. Bolognini<sup>1</sup>, A. Soresina<sup>1</sup>, V. Lougaris<sup>1</sup>, A. Plebani<sup>1</sup><sup>1</sup>Department of Pediatrics and Laboratory for Molecular Medicine 'A. Nocivelli', Brescia, Italy

**Objectives:** Ligation of antigen receptors (TCR, BCR) on T and B lymphocytes leads to the activation of new transcriptional programs and cell cycle progression. CARMA1 (originally called CARD11) is a membrane-associated guanylate kinase family member that is required for T cell receptor (TCR)-induced NF-kappa B activation in T cell leukemia lines. It uses its N-terminal caspase activation and recruitment domain (CARD) to interact with the CARD in the downstream adaptor Bcl-10. CARMA1 deficient animal models showed development disruption of CD5(+) peritoneal B cells; in addition, B cell proliferation in response to both BCR and CD40 ligation was severely compromised. Finally, serum immunoglobulin levels were also markedly reduced in the mutant mice. CVID is the most frequent primary humoral immunodeficiency characterized by low Ig serum levels and defective formation of plasma cells and memory B cells. The phenotype observed in the knock-out animal model for CARMA1 resembles the phenotype of CVID. Considering the above, we decided to investigate whether mutations in the gene encoding CARMA1 may be associated with the pathogenesis of this disorder.

**Methods:** Sixty five patients with CVID diagnosed according to the ESID criteria were included in this study. Direct gene sequencing was performed for the exons and flanking regions of the CARMA1 gene using the ABI PRISM 310 Genetic Analyzer sequencer.

**Results:** CARMA1 analysis performed showed the presence of wild type genotype in all of them, with the exception of the exons 2,4,7,10,14,15,16,18,19. The majority of the mutations are synonymous and were identified in exon 2 (A68A), exon 4 (I222I), exon 7 (D408), exon 10 (D526D), exon 14 (A680A), exon 15 (T741T), exon 16 (L775), exon 18 (P867), exon 19 (N909) and exon 23 (R1085). Despite this, our analysis identified a novel missense mutation in exon 4 (L276V).

**Conclusion:** Although the animal knock out model for CARMA1 resembles the CVID phenotype, no disease causing mutations were identified within our cohort of patients. The incidence of the novel L276V mutation is currently under investigation in more than 150 healthy controls.

PC03/6

**GENETIC ANALYSIS OF IMMUNE-RELATED MICRORNAS IN COMMON VARIABLE IMMUNODEFICIENCY PATIENTS**S. Jennings<sup>1</sup>, M. Grudzien<sup>1</sup>, H.-H. Peter<sup>1</sup>, U. Salzer<sup>1</sup><sup>1</sup>University Hospital of Freiburg, Rheumatology and Clinical Immunology, Freiburg, Germany

**Background:** Common variable immunodeficiency (CVID) comprises a heterogeneous group of patients and is mainly characterized by an impaired terminal B-cell differentiation, hypogammaglobulinemia and recurrent respiratory tract infections. Although CVID is the most common form of immunodeficiency in adults, the etiology of CVID is largely unknown.

MicroRNAs have recently been described as a new group of posttranscriptional regulators of gene expression critically involved in the development and function of the mammalian immune system. Some of the microRNAs show some restriction to immune cells with regard to their expression and function.

**Rationale:** We asked if genetic changes in miRNA genes, which have shown to be relevant for B- cell differentiation and/or the immune response, could be associated with the CVID phenotype.

**Methods:** We analyzed the pre-miRNA hairpins and 100 bp 5' and 3' flanking regions of miR-146a, miR-150, miR-155 and miR-223 by direct sequencing of genomic DNA.

**Results:** We sequenced 120 CVID patients for miR-155, 99 patients for miR-150, 103 patient samples for miR-146a, and 135 CVID patients for the miR-223 gene. In miR-150 and miR-155 no genetic alteration could be detected. In miR146a we found the previously described single nucleotide polymorphism (SNP) rs2910164, but there was no significant difference of genotype frequencies when compared to ethnically matched healthy controls. Sequencing of the x-chromosome located miR-223 gene, however, revealed one novel G to A change at position 22 of the miR-223 precursor molecule in three patients (2 hemizygotes and one heterozygote) and one patient carrying a heterozygous T to C change at position 63 of the miR-223 hairpin precursor.

**Conclusion:** We did find the sequences of miRNA genes 150 and 155 to be highly conserved in the CVID patient cohort studies as we were unable to identify any sequence variant in the samples studied. There is no association of the miR-146a SNP rs2910164 with CVID. A novel rare variant was found in the miR-223 gene in two CVID patients.

**Grant Acknowledgments:** Wissenschaftliche Gesellschaft, Freiburg; SFB620/C2 by the Deutsche Forschungsgemeinschaft.

PC03/7

**CD21LOW B CELLS DEMONSTRATE SIGNS OF INTERFERON TYPE I EXPOSURE**B. Keller<sup>1</sup>, S. Gutenberger<sup>1</sup>, M. Rakhmanov<sup>1</sup>, D. Holzinger<sup>2</sup>, H. Eibel<sup>1</sup>, H.-H. Peter<sup>1</sup>, K. Warnatz<sup>1</sup><sup>1</sup>University Medical Center Freiburg, Rheumatology and Clinical Immunology, Freiburg, Germany, <sup>2</sup>University Medical Center Freiburg, Virology, Freiburg, Germany

**Objectives:** Common variable immunodeficiency (CVID) Ia patients exhibit an abnormal expansion of an unusual B cell population in peripheral blood, which was first characterized by low expression of CD21, therefore called CD21low B cells. These cells represent an activated phenotype with increased cell size and elevated levels of CD86. Increased expression of Interferon (IFN) response genes MxA and 2'-5' Oligoadenylatesynthetase 1 suggests recent IFN Type I exposure. Additionally, since an accumulation of this cell type has also been reported in viral infection (HIV) and SLE, a possible role of IFN Type I in the induction of CD21low B cells was investigated.

**Methods:** In order to examine a direct role of IFN in the induction of CD21low B cells, PBMC of healthy donors were stimulated for different time points with either Multiferon, a synthetic IFN Type I, CpG, trigger of IFN Type I and potent B cell activator or serum from patients with HIV or CVID, presenting with expanded CD21low B cells. B cell phenotype was determined by flowcytometry. Further, owing to the IFN Type I gene expression pattern, we examined the phosphorylation of Signal Transducer of Activation 1 (STAT1) downstream of the activation of the IFN Type I receptor by intracellular flowcytometry using phosphospecific antibodies to STAT1. Cells were analysed directly following isolation or PBMC were stimulated with Multiferon for 15min.

**Results:** None of the different stimuli was capable of inducing the CD21low B cell phenotype *in-vitro*. Preliminary results suggest enhanced levels of phosphorylated STAT1 in CVID Ia patients' B cells compared to healthy controls and CD21low B cells exhibit the highest levels of phosphoSTAT1 *ex vivo*. Currently we are investigating the *in vitro* response of B cells to stimulation of the Interferon Alpha Receptor using MFN.

**Conclusion:** The findings indicate an increased exposure of CD21low B cells from CVID Ia patients to IFN Type I *in vivo*. Elevated IFN response gene expression is corroborated by enhanced levels of STAT1 phosphorylation of freshly isolated cells. IFN Type I is not sufficient to induce the phenotype of CD21low B cells but it might play a role in their differentiation and activation.

PC03/8

**PHENOTYPICALLY AND FUNCTIONAL ANALYSIS OF B CELLS IN PERSISTENT POLYCLONAL B CELL LYMPHOCYTOSIS (PPBL)**N. Voelxen<sup>1</sup>, S. Gutenberger<sup>1</sup>, M. Rakhmanov<sup>1</sup>, C. Förster<sup>1</sup>, S. Goldacker<sup>1</sup>, H.-H. Peter<sup>1</sup>, K. Warnatz<sup>1</sup><sup>1</sup>University Medical Center; University Hospital Freiburg, Rheumatology/ Clinical Immunology, Freiburg, Germany

Persistent Polyclonal B cell Lymphocytosis (PPBL) is a disorder which mainly affects female smokers in their 4<sup>th</sup> to 6<sup>th</sup> decade of life. PPBL is often associated with a polyclonal rise of serum IgM.

In this trial we examined phenotype and function of B cells of 6 PPBL patients (all female, age: 35-63 years) by flow cytometry as well as by *in vitro* activation. The B lymphocytosis in PPBL is due to a significant polyclonal expansion of CD27<sup>+</sup>IgD<sup>+</sup>IgM<sup>+</sup> marginal zone like B cells (relatively 85.02 ± 9.75% of CD19<sup>+</sup> B cells; normal range: 7.8 – 36%) [p< 0.001] (absolutely 781.44 ± 552.75/μl; normal range: 13.52 ± 9.25/μl) [p< 0.00001] and of CD21<sup>low</sup> B cells (relatively 33.46 ± 18.72% of CD19<sup>+</sup> B cells; normal range: 1.1-6.9%) [p=0.03] (absolutely: 387.86 ± 264.09/μl; normal range: 9.68 ± 1.70/μl), while all other populations showed absolute numbers within the normal range. There were no detectable aberrations within the T cell compartment.

Functional analysis revealed an abnormal response to B cell receptor as well as CD40 stimulation. Thus the upregulation of CD86 after stimulation for one to three days with anti-IgM as well as anti-CD40 was significantly decreased (MFI: 53.87 ± 12.19 compared to 226.9 ± 33.93 in healthy donor) [p=0.01]. Proliferation at day 6 after stimulation with different stimuli was also decreased as demonstrated in CFSE and <sup>3</sup>H-Thymidine uptake assays. The reduced proliferative response was associated with a significantly enhanced cell death revealed by staining for propidium iodide (PI) after stimulation (% of PI positive B cells: 49.0% ± 18.02% compared to 85.63% ± 3.70% in healthy donor) [p=0.00002] as well as an enhanced apoptosis revealed by staining for annexin V after two to three days of stimulation (% of annexin V B cells 17.20% ± 2.05% versus 3.95 ± 1.67 in healthy donor) [p=0.001].

The analysis of early signalling events as tyrosine phosphorylation and Ca<sup>2+</sup> influx revealed normal responses in the CD19<sup>+</sup> B cells of PPBL patients compared to age matched controls.

PC03/9

**SAFETY PROFILE OF SUBCUVIA FROM POST-AUTHORISATION SAFETY SURVEILLANCE (PASS)**H. Permin<sup>1</sup>, T. Herlin<sup>2</sup>, H. Gajek<sup>3</sup>, M. Mattauch<sup>3</sup>, R. Gustafson<sup>4</sup><sup>1</sup>Bispebjerg Hospital, Copenhagen, Denmark, <sup>2</sup>Hospital – Paediatric Department, Arhus, Denmark, <sup>3</sup>Baxter Innovations GmbH, Vienna, Austria, <sup>4</sup>Baxter AB, Kista, Sweden

**Objectives:** Assess safety, tolerance and efficacy of SUBCUVIA in patients with immunodeficiency receiving SUBCUVIA subcutaneously. Additionally, clinically diagnosed infections, IgG trough levels and dosage regimens were recorded. Data on healthcare utilisation were also collected.

**Method:** Prospective, multi-centre, uncontrolled, open-label, post-authorisation safety surveillance of SUBCUVIA (16% liquid IgG for subcutaneous administration). Planned observation period for each subject was 6 months.

**Results:** As of 31<sup>st</sup> December 2007, 79 patients (46 male, 33 female) had been enrolled, and observations from 78 of 79 total patients, (0 to ≤12 years (34), 13 to 18 years (10), >18years (34)), were recorded in the database. Of these 78 patients, 72 had completed the study according to the observational plan. Analysis of safety included incidence of non-serious and serious adverse events in general, and related to SUBCUVIA. Analysis of efficacy included incidence of clinically documented bacterial infections for the observation period. The majority of drug-related adverse events represented local injection site reactions. A single serious adverse event was reported (hospitalisation for bronchoscopy), assessed as unrelated to the drug. Otherwise, the spectrum of adverse events reflected the known AE pattern of IgG, and what is expected in the study population. Overall, tolerance was assessed by attending physicians, either as very good, good, moderate, poor or not assessable (n=60, 11, 2, 2, 3). Overall efficacy as determined by attending physicians, was rated very good, good, moderate or not assessable (n=42, 29, 3, 4). Patients' preference was evaluated at study exit. Sixty patients, or parents resp., preferred subcutaneous therapy. The remaining either preferred IVIG, had no preference or it was not known (n=2, 4, 12).

**Conclusion:** Results indicate that SUBCUVIA is well tolerated and effective in management of patients with immunodeficiency syndrome observed under routine conditions.

**Keywords:** Primary immunodeficiency, IgG replacement therapy, SUBCUVIA, subcutaneous immunoglobulin, adverse events, bacterial infections.

#### PC03/10 VACCINATION AGAINST TICK-BORNE ENCEPHALITIS VIRUS TESTS SPECIFIC IGG PRODUCTION ABILITY IN PATIENTS UNDER IMMUNOGLOBULIN SUBSTITUTION THERAPY

M.G. Seidel<sup>1</sup>, E. Grohmann<sup>2</sup>, K. Sadeghi<sup>3</sup>, A. Heitger<sup>4</sup>, E. Förster-Wald<sup>1,3</sup>

<sup>1</sup>St. Anna Children's Hospital, Immunology Outpatient Clinics, Vienna, Austria, <sup>2</sup>Landeskinder- und Frauenklinik Linz, Immunology Outpatient Clinics, Linz, Austria, <sup>3</sup>General Hospital, Medical University of Vienna, MUW, Pediatric Immunology Outpatient Clinics, Vienna, Austria, <sup>4</sup>St. Anna Children's Hospital, Children's Cancer Research Institute (CCRI), Immunology Outpatient Clinics, Vienna, Austria

Patients with primary (PID) or secondary immunodeficiency syndromes and antibody deficiency may require immunoglobulin (IgG) replacement therapy. To assess B-cell function during ongoing Ig-replacement therapy might become necessary in order to decide about possible discontinuation of therapy. Up to now, the bacteriophage (phi x174) protein has been used as an artificial neoantigen to test residual B-cell function *in vivo*. However, the experience with the non-licensed product phi x174 is limited, and the quantification of specific antibodies is established in few laboratories only. Active prophylactic immunization against tick-borne encephalitis (TBE) is performed in few European countries where TBE occurs as an endemic disease and not in the U.S.. Hence, immunoglobulin preparations pooled from international sources are not expected to contain substantial levels of TBE-specific IgG. We postulated, that this background might offer the opportunity to assess specific B cell function in immunodeficient individuals under Ig-substitution therapy by applying a licensed TBE vaccine. Thus, TBE-IgG levels were analyzed in 12 patients with at least two-years of continuous intravenous or subcutaneous IgG substitution therapy and in parallel in the administered IgG products. Although TBE-specific IgG were detectable in IgG concentrates at highly variable levels, serum levels in patients were below the threshold which is considered as protective at all occasions in all individuals (n=28). Our data demonstrate that TBE vaccination might offer the opportunity to test antibody production capacity in patients during IgG replacement therapy.

#### PC03/11 LYMPHOCYTE ACTIVATION DEFECTS IN WHIM SYNDROME PATIENTS

M. Kallikourdis<sup>1</sup>, L. Tassone<sup>2</sup>, R. Badolato<sup>2</sup>, A. Viola<sup>1</sup>

<sup>1</sup>Istituto Clinico Humanitas, Rozzano, Italy, <sup>2</sup>Clinica Pediatrica, University of Brescia, c/o Spedali Civili, Brescia, Italy

The WHIM syndrome is a rare disease characterized by symptoms including neutropenia, hypogammaglobulinemia, recurring infections and warts. It is caused by C-terminal truncating, dominant mutations in the chemokine receptor CXCR4. This has been shown to impair the intracellular trafficking of the receptor in neutrophils, leading to increased responsiveness to chemokine and retention of neutrophils in the bone marrow. Yet the defects in adaptive immunity remain unexplained. Having previously demonstrated that chemokine receptors CXCR4 and CCR5, in addition to their function in migration, have a role in increasing the stability of immunological synapses (ISs) between T cells and antigen presenting cells, we hypothesized that the WHIM-associated mutations may be affecting the role of CXCR4 in the stabilization of the IS, leading to the observed adaptive response-related symptoms. We show that mutant CXCR4 on T cells, whilst unimpaired in its ability to be recruited to the IS and to enhance T cell costimulation, leads to significantly compromised stability of the T cell-antigen presenting cell conjugates in the presence of competing external chemokine signals, which may explain the defective adaptive responses in WHIM patients.

#### PC03/12 A FULL-BLOWN ANTIBODY DEFICIENCY SYNDROME IN A PATIENT WITH CARTILAGE HAIR HYPOPLASIA

J. Horn<sup>1</sup>, M. Schlesier<sup>1</sup>, K. Warnatz<sup>1</sup>, A. Prasse<sup>2</sup>, A. Superti-Furga<sup>3</sup>, H.-H. Peter<sup>1</sup>, U. Salzer<sup>1</sup>

<sup>1</sup>University Hospital of Freiburg, Rheumatology and Clinical Immunology, Freiburg, Germany, <sup>2</sup>University Hospital of Freiburg, Department of Pneumology, Freiburg, Germany, <sup>3</sup>University Hospital of Freiburg, Centre for Pediatrics and Adolescent Medicine, Department of Pediatrics, Freiburg, Germany

Cartilage hair hypoplasia (CHH, OMIM #250250) is an autosomal recessive disorder caused by mutations in the *RMRP* gene. While its most constant feature is metaphyseal dysplasia with short stature, CHH is associated with extraskelletal defects such as thin hair, anemia, immunodeficiency, and increased incidence of lymphomas. The spectrum of immunologic phenotypes in CHH translates into clinical severity. Whereas T cell deficiency may remain subclinical or result in severe combined immunodeficiency or Omenn syndrome, humoral immunodeficiency (mainly IgG subclass and IgA deficiency) has only rarely been noted in these patients. Here we report on the diagnosis of CHH in a 29 year old woman who presented with severe short stature and a full-blown antibody deficiency, clinically resembling common variable immunodeficiency (CVID). The patient suffered from recurrent and chronic respiratory tract infections, in particular sinusitis, bronchitis, and 3 episodes of pneumonia. We found pronounced hypogammaglobulinemia of all isotypes, lack of specific antibody responses to pneumococcal polysaccharides, and very low numbers of class-switched memory B cells. Although CD4<sup>+</sup> and CD8<sup>+</sup> cells were reduced in absolute numbers, there were no clinical signs of overt T cell deficiency. In addition, the patient had bronchiectasis, mild splenomegaly, a moderate expansion of CD21<sup>low</sup> B cells, and transient macrocytic anemia. Mutation analysis of the *RMRP* gene revealed compound heterozygosity for two novel compound heterozygous mutations (g.68–69delinsTT and g.76C>T). In conclusion we report here on the diagnosis of CHH in an adult patient presenting with short stature, recurrent respiratory infections, and an unusual CVID-like phenotype.

#### PC03/13 STUDY OF *PRDM1* AND *XBPI* POLYMORPHISMS IN SELECTIVE IMMUNOGLOBULIN A DEFICIENCY

N. Del Pozo Rodríguez<sup>1</sup>, R. López-Mejías<sup>1</sup>, A. Ferreira<sup>2</sup>, M. C. García-Rodríguez<sup>2</sup>, M. Fernández-Arquero<sup>1</sup>, G. Fontán<sup>1</sup>, C. Núñez<sup>1</sup>

<sup>1</sup>Hospital Clínico San Carlos, Clinical Immunology, Madrid, Spain, <sup>2</sup>Hospital Universitario La Paz, Immunology, Madrid, Spain

**Objective:** Selective immunoglobulin A deficiency (IgAD) is the most common immunodeficiency in Caucasian individuals. It is a multifactorial disease with genetic factors involved, but association with certain *HLA* alleles is the only firmly established genetic contribution. The pathogenesis of IgAD is not well understood, but different processes can be altered during B-cell differentiation into immunoglobulin-secreting plasma cells. Genes encoding products involved in this maturation process are good candidate genes for disease development, as *Prdm1* and *XBPI*, which encode two transcription factors, BLIMP1 and XBPI, respectively. Moreover, a single nucleotide polymorphism (SNP) (rs2269577) located in the promoter of *XBPI* has been previously associated with IgAD in the Asian population. Our aim was to assess the role of *Prdm1* and *XBPI* polymorphisms on IgAD susceptibility in the Spanish population.

**Methods:** We performed a case-control study including 303 Caucasian Spanish IgAD patients defined by ESID criteria and recruited from a single centre, and 717 ethnically matched healthy controls obtained from blood donors and laboratory staff. We studied five polymorphisms: rs2269577 (-116C/G), located in the promoter of *XBPI*; and rs4946722, rs1340064, rs6924807 and rs811925, located in *Prdm1*, the first three located in the promoter and the last one in an exon. All SNPs were genotyped using TaqMan technology. The frequency of the haplotypes conformed by the SNPs studied in the *Prdm1* gene were estimated with the Expectation-Maximization algorithm implemented in the Haploview software. Genetic frequencies were compared between patients and controls with the chi-square test.

**Results:** All polymorphisms conformed to Hardy-Weinberg expectations in controls. No statistically significant differences were obtained when comparing the frequency of individual polymorphisms or *Prdm1* haplotypes between patients and controls. We also analyzed the association between *XBPI* and *Prdm1* polymorphisms and IgAD susceptibility after stratifying by sex and by the HLA susceptibility alleles *HLA-DRB\*0102*, *HLA-DR3* and *HLA-DR7*, but no statistically significant differences were obtained in any case.

**Conclusions:** The genetic polymorphisms studied in the *Prdm1* and *XBPI* genes do not seem to be influencing on IgAD susceptibility in the Spanish population. Additional genes involved in B-cell maturation could be investigated to progress in the genetic basis of this common immunodeficiency.

#### PC03/14 COMMON VARIABLE IMMUNODEFICIENCY: RELATIONSHIP BETWEEN IL-17-PRODUCING CD4 T CELLS AND THE IMBALANCES OF THE B- AND T-CELL POPULATIONS

R. Barbosa<sup>1,2</sup>, S.P. Silva<sup>1,2</sup>, S.L. Silva<sup>1,2</sup>, A. Melo<sup>1,2</sup>, M.C. Santos<sup>1,2</sup>, E. Pedro<sup>2</sup>, M.P. Barbosa<sup>2</sup>, R.M. Victorino<sup>1,2</sup>, A.E. Sousa<sup>1,2</sup>

<sup>1</sup>Unidade de Imunologia Clínica, Instituto de Medicina Molecular, Faculdade de Medicina, Universidade de Lisboa, Lisboa, Portugal, <sup>2</sup>Centro de Imunodeficiências Primárias, Hospital de Santa Maria, Centro Hospitalar Lisboa Norte, Instituto de Medicina Molecular, Faculdade de Medicina, Universidade de Lisboa, Lisboa, Portugal

**Objectives:** Common Variable Immunodeficiency (CVID) is a heterogeneous disease defined by defective antibody production. However, T-cell imbalances are frequently present and have been associated with autoimmune, inflammatory and granulomatous manifestations. Here, we asked whether IL-17, a major pro-inflammatory cytokine, is related to the major alterations of the B- and T-cell subsets found in CVID.

**Methods:** IL-17-producing CD4 T cells were characterized at the single cell level by flow cytometry, after short-term stimulation of freshly isolated peripheral blood mononuclear cells with PMA plus ionomycin. Phenotypic analysis was performed by direct staining of whole blood samples. Spearman correlations and Mann-Whitney t tests were conducted using Prism 5.0 Software.

**Results:** The CVID cohort exhibited a significantly increased frequency of IFN-γ and TNF-α-producing CD4 T cells as compared to healthy controls, as expected, and these increases were positively correlated with the up-regulation of T-cell activation markers. However, no increase in the frequency of IL-17-producing CD4 T cells or of the populations that simultaneously produce IL-17 and IFN-γ or TNF-α was found. Moreover, in contrast to the inverse correlation of the frequencies

of IFN- $\gamma^+$  and TNF- $\alpha^+$  cells with naïve CD4 T cells found in CVID patients, IL-17-producing cell frequency was unrelated to naïve CD4 T-cell depletion. Importantly, we found that the frequency of IL-17-producing CD4 T cells was negatively correlated with the levels of CD4 T-cell activation as well as with the B-cell disturbances characteristic of CVID patients, namely the expansion of the activated CD21<sup>low</sup> B-cell subset.

**Conclusion:** No increase in the frequency of CD4 T cells able to produce IL-17 was found in CVID patients even after stratification for clinical presentation, including autoimmune manifestations. In spite of the inflammatory processes frequently observed in CVID that share similarities with the pathological findings of Crohn's disease and sarcoidosis, IL-17 production seems to be regulated differently in these patients. Our data further suggest a direct association between the impairment of B-cell differentiation and IL-17 production by CD4 T cells in CVID.

#### PC03/15 RECESSIVE INHERITANCE LESS COMMON IN THE HUMAN POPULATION THAN IN THE OUTCOME OF ENU MUTAGENESIS, THUS IMPLICATING NATURAL SELECTION

N.A. Nicholas Mitchison<sup>1</sup>, Immunodeficiency

<sup>1</sup>University College London, Institute of Ophthalmology, London, United Kingdom

The monofactorial diseases of man provide a rich source of information bearing on human evolution. Within a panel of monofactorial genetically heterogeneous diseases we collect (i) the relative frequency of the recessive form of the disease (compared with dominant/XL forms), and (ii) the relative disease frequencies per gene. A significant shortage of the recessive forms and a smaller frequency per recessive gene becomes evident, in marked contrast to the outcome of ENU mutagenesis. This we attribute to natural selection in the human population, operating through demographic factors and/or negative selection of heterozygotes.

#### PC03/16 ROLE OF CHEMOKINE SIGNALING IN THE PATHOGENESIS OF GOOD'S SYNDROME – A PRELIMINARY STUDY

P. Zdziarski<sup>1</sup>, K. Suchnicki<sup>1</sup>, A. Lange<sup>1</sup>

<sup>1</sup>Lower Silesian Center for Cellular Transplantation & National Bone Marrow Donor Registry, Institute of Immunology & Experimental Therapy, Clin. Immunol, Wrocław, Poland

Good's syndrome (GS), a rare coincidence of thymoma and hypogammaglobulinemia is marked by reduction (or absence) of B cells. Sometimes a B and T cell immunodeficiency in thymoma patients is referred to as GS, but it rather represents a different etiology and pathogenesis, which remains undescribed.

The coincidence of involvement of central immune organs (bone marrow and thymus) may indicate that lymphocyte circulation in GS is defective. Enlargement of thymus and atrophy of B-cell compartment in bone marrow direct to the hypothesis that GS is the effect of defective mobilization of common lymphocyte precursors (CLP) to thymus and insufficient homing of cells to bone marrow (BM). Cell homing is occurring in response to higher levels of SDF-1 in the BM. Previous study indicate that low CXCR4 membrane expression on CD34(+) cells characterizes cells mobilized to blood. The same phenomenon, may be true in the pathogenesis of GS.

We describe two patients with mild and severe grade Good syndrome, who were tested for expression of CXCR4, a SDF-1 receptor, on cells in blood and bone marrow. First patient presented with:

- (1) mild hypogammaglobulinemia, sporadically demanding intravenous immunoglobulin substitution,
- (2) 1,4% CD20 positive lymphocytes,
- (3) thymic enlargement of moderate degree.

The second patient suffered from:

- (1) severe hypogammaglobulinemia (IgG  $\approx$  60mg/dl)
- (2) absence of B cells in peripheral blood (CD20+ = 0.4% lymphocytes) (3) severe thymic enlargement.

The first patient had CXCR4 expression on 82.6% of lymphocytes in BM the other patient: 46.3%. Interestingly, patient with severe form of GS (thymic enlargement) had in BM more CD10-positive cells than the first patient (1.0% vs 0.1% cells).

CD10 (endopeptidase, enkephalinase), which is capable of cleaving many small molecular weight peptides, may limit activity of chemokines, for example SDF-1. This data indicate that SDF-1 may promote homing of lymphoid precursors to BM, and protect from migration to the vascular niche and of CLPs to the thymus. In contrast weak expression of CXCR4 and SDF-1 signal stimulate vigorous CLP migration to the thymus, and thymoma development (especially predominantly lymphocytic) in Good's syndrome.

#### PC03/17 PATIENTS WITH CSR DEFECT (WITHOUT CD40L, CD40, AID AND UNG DEFECT) MAY RECOVER SPONTANEOUSLY DURING LATE CHILDHOOD

N.E. Karaca<sup>1</sup>, G. Aksu<sup>1</sup>, N. Gulez<sup>1</sup>, A. Durandy<sup>2</sup>, N. Kutukculer<sup>1</sup>

<sup>1</sup>Ege University Faculty of Medicine, Department of Pediatric Immunology, Izmir, Turkey, <sup>2</sup>Hopital Necker-Enfants Malades, INSERM U768, Paris, France

Hyper IGM syndrome (HIGM) is a heterogeneous condition characterized by impaired Ig class switch recombination (CSR). There are some HIGM patients with defective CSR who do not have CD40L, CD40, AID and UNG defects. Some of the patients present with a nonidentified molecular defect, associating defective CSR but normal somatic hypermutation (SHM) generation in the variable region of the Ig locus. Herein, we present 8 patients who exhibited an *in vivo* and *in vitro* transient CSR defect which spontaneously recovered.

A total of 8 patients (7 male, 1 female) (age during the study: 78.3  $\pm$  14.0 months) with initial low serum IgG (n:8) (546  $\pm$  117 mg/dl) and IgA (n:8) (36  $\pm$  17 mg/dl) and high (n:4) or normal (n:4) IgM (82  $\pm$  49 mg/dl) levels were included into the study. All of them had normal percentages of CD40 on B cells and CD40 induced B cell proliferations were normal. CD40L on activated T cells were also found to be in normal ranges. CSR was absent and SHM was normal in all cases. AID and UNG genes did not show any abnormality while mean  $\pm$  SD of CD19+CD27+memory B cell percentage was 14.5  $\pm$  7.8. Lymphocyte subsets were normal in all cases. Specific antibodies against tetanus and Haemophilus Influenza type B were not in protective levels in only one case.

These patients were followed-up 43.6  $\pm$  7.1 months. Serum Ig's were normalized in all cases. Ages for normalization of IgG and IgA were 68.6  $\pm$  8.1, 52.7  $\pm$  25.4 months, respectively. All patients with high IgM levels at admission recovered spontaneously (60.5  $\pm$  30.4 months) and mean IgM level decreased to 67  $\pm$  12 mg/dl. Fifty percent of cases received intravenous immunoglobulin while the others used OM-85BV (bacterial lysate) and/or low dose antibiotics (amoxicillin) for infection prophylaxis. The total number of infections per year decreased from 8.0  $\pm$  3.8 to 2.5  $\pm$  0.5. The cause of this *in vivo* transient CSR defect is up to now unknown. These patients will be examined for their latest ability for CSR and for their IgG subset production.

In conclusion; detection of a non AID or UNG associated *in vivo* and *in vitro* CSR defect in infancy should be confirmed later on since spontaneous recovery can occur.

#### PC03/18 LACK OF ASSOCIATION OF IL12B AND EBF1 POLYMORPHISMS WITH IGAD IN THE SPANISH POPULATION

N. Del Pozo Rodríguez<sup>1</sup>, R. Lopez-Mejías<sup>1</sup>, M. C. García-Rodríguez<sup>2</sup>, A. Ferreira<sup>2</sup>, M. Fernández-Arquero<sup>1</sup>, C. Núñez<sup>1</sup>

<sup>1</sup>Hospital Clínico San Carlos, Clinical Immunology, Madrid, Spain, <sup>2</sup>Hospital Universitario La Paz, Immunology, Madrid, Spain

**Background:** Immunoglobulin A deficiency (IgAD) is the most common immunodeficiency in Caucasians. This is a complex disease with genetic factors involved, but only association with certain HLA alleles has been firmly established. Interleukin 12 (IL-12) is a cytokine with an important role in the innate and adaptive immune response, comprising two subunits: p35 and p40, with a constitutive and inducible expression, respectively. High levels of IL-12 p40 have been described in patients showing a different but related immunodeficiency, common variable immunodeficiency. Genetic polymorphisms affecting IL12-p40 levels and then potentially affecting regulation of the immune response have been described in the gene encoding this subunit, the *IL12B* gene. In addition, also in the chromosome 5 and adjacent to the *IL12B* gene, the *EBF1* gene is located, which codes for the early B-cell factor, a transcription factor essential for B-cell development.

**Objective:** To study the role of several polymorphisms in the 5q33 genetic region, involving the *IL12B* and *EBF1* genes, in IgAD susceptibility in the Spanish population.

**Methods:** We performed a case-control study including 332 Caucasian Spanish IgAD patients and 557 ethnically matched healthy controls; and a familial study with 128 IgAD trios (the affected individual and both parents). All samples were genotyped for the microsatellite D5S1352, 116 kb upstream of *IL12*; and for three single nucleotide polymorphisms (SNPs): rs3212227 (+1188A/C), rs6887695 and rs1368297. Haplotypic frequencies were estimated by the Expectation-Maximization algorithm implemented in the software Arlequin. Case-control comparisons were performed using the  $\chi^2$  test, and the transmission disequilibrium test (TDT) was used for the family study.

**Results:** No significant differences between patients and controls were observed when allelic frequencies for any of the studied polymorphisms were considered. Frequencies of haplotypes conformed by all the studied markers did not differ between both groups either. TDT analysis did not show significant results. Stratification by the susceptibility alleles *HLA-DRB\*0102*, *HLA-DR3* and *HLA-DR7* did not show statistically significant differences.

**Conclusions:** The genetic polymorphisms studied in the 5q33 region, involving the *IL12B* and *EBF1* genes, do not show association with IgAD susceptibility.

#### PC03/19 TACI GENE MUTATIONAL ANALYSIS IN SELECTED CHILDREN WITH HYPOGAMMAGLOBULINEMIA

A.B. Barroeta Seijas<sup>1,2</sup>, G. Di Matteo<sup>1,3</sup>, S. Ferrari<sup>1</sup>, S. Di Cesare<sup>1,3</sup>, M. La Rocca<sup>1</sup>, S. Graziani<sup>1</sup>, E. Freda<sup>1,3</sup>, E. Risquez<sup>2</sup>, I. Blanca<sup>2</sup>, A. Finocchi<sup>1,3</sup>, C. Cancrini<sup>1,3</sup>, V. Moschese<sup>1</sup>

<sup>1</sup>University of Rome Tor Vergata, Pediatrics, Rome, Italy, <sup>2</sup>Immunology Institute of the Central University of Venezuela, Caracas, Venezuela, <sup>3</sup>Children's Hospital Bambino Gesù, Immunoinfectology, Rome, Italy, <sup>4</sup>S. Orsola-Malpighi University Hospital, Medical Genetics Unit, Bologna, Italy

Common variable immunodeficiency disorder (CVID) is the most prevalent primary immunodeficiency in adults with a frequency of about 1:25,000 to 1:66,000. Low serum immunoglobulin values of one or more isotypes, defective specific antibody response together with increased susceptibility to infections feature this condition. Moreover, autoimmunity, non malignant lymphoproliferation and malignancies frequently occur. Symptoms may begin at any time of life, including the extremes of both young and old age. Mutations in four genes have been reported, TNFRSF13C (BAFF-R), CD19, ICOS and TNFRSF13B (encoding TACI), together associated to 10-15% of CVID cases.



**Objective:** To provide a preliminary outlook of the identification of TACI mutations in selected children with hypogammaglobulinemia where the main primary immunodeficiencies were excluded, i.e. XLA, HIGM syndrome and XLP.  
**Methods:** We analyzed 15 pediatric patients (age range 1-12 ys) with hypogammaglobulinemia by direct sequencing of the five exons of TACI (TNFRSF13B).  
**Results:** Of the 15 patients studied, six carried monoallelic mutations in TACI gene. The mutations were found in two exons: In exon 3 the C104R in 3 patients (two related) and the I87N in one patient, and in exon 5 the P251L was found in two unrelated patients.  
**Conclusions:** The identification of TACI mutations in 6/15 children with hypogammaglobulinemia supports as these gene alterations influence the risk for development of CVID and the role of molecular diagnosis in dissecting children with primary immunodeficiencies. Early recognition of these children and their clinical and immunological monitoring will allow prediction of disease prognosis and improvements in our knowledge to develop optimal therapeutical strategies.

**PC03/20 TACI EXPRESSION AND MUTATIONAL STATUS IN GREEK PATIENTS WITH COMMON VARIABLE IMMUNODEFICIENCY AND SELECTIVE IGA DEFICIENCY**  
**M. Speletas<sup>1</sup>, A. Mamara<sup>1</sup>, G. Iordanakis<sup>1</sup>, E. Papadopoulou-Alataki<sup>2</sup>, E. Tsitsami<sup>1</sup>, A.E. Germenis<sup>1</sup>**  
<sup>1</sup>University of Thessaly, Department of Immunology and Histocompatibility, Larissa, Greece, <sup>2</sup>Aristotle University of Thessaloniki, Pediatric Clinic, Papageorgiou General Hospital, Thessaloniki, Greece

**Purpose:** TACI (Transmembrane Activator, calcium modulator and Cyclophilin ligand Interactor) is a transmembrane receptor, which mediates isotype switching in B cells. Recently, mutations in *TNFRSF13B/TACI* gene were found in 10-20% of patients with common variable immunodeficiency (CVID), implying their contribution in the disease pathogenesis and/or phenotypic expression. The purpose of this study was to analyze the mutational status and the expression of *TNFRSF13B/TACI* in Greek patients with CVID and selective IgA deficiency (sIgAD).  
**Methods:** Thirty-two unrelated patients (M/F: 16/17), with a mean age at diagnosis 13.7 years (range: 1-64) were enrolled. Amongst them, 14 suffered from CVID, 13 from sIgAD, 3 from sIgG4D and 2 from transient hypogammaglobulinemia. DNA was extracted from peripheral blood by standard protocol. Amplification of all exons of *TNFRSF13B/TACI* gene was followed and the purified PCR products were directly sequenced. The expression levels of TACI were measured by flow cytometry using an anti-CD267 monoclonal antibody (Abcam, clone: 1a1). 108 healthy controls (M/F: 44/64, mean age: 42.6 years, range: 7-93) were also analyzed for TACI expression and for the presence of the two most common missense polymorphisms (V220A, P251L – detected by PCR-RFLP).  
**Results:** Only the two common *TNFRSF13B* polymorphisms (V220A and P251L) were detected amongst patients, with prevalence, however, that did not differ from that detected in normal controls (allele frequencies V220A: 3.3% vs 4.7%, p=0.79 and P251L: 11.7% vs 10.3%, p=0.6). In immunodeficient patients, B cell TACI expression was unrelated with the presence of *TNFRSF13B/TACI* polymorphisms but its levels were found significantly lower than that detected in normal controls (mean±SDEV: 18.9±20.8 vs 34.8±17.2, p=0.018). In normal controls, the presence of polymorphisms was not associated with hypogammaglobulinemia. In the later group, however, a negative correlation was observed between the P251L polymorphism and TACI expression.  
**Conclusions:** The *TNFRSF13B/TACI* coding region does not seem to dispose disease-associated mutations in Greek patients with primary immunodeficiencies. Differences, however, in TACI expression levels uncovered in this study indicate a possible functional role of common *TNFRSF13B/TACI* polymorphisms that remains to be elucidated.

**PC03/21 CD22, A B LYMPHOCYTE-SPECIFIC ADHESION MOLECULE THAT REGULATES ANTIGEN RECEPTOR SIGNALLING**  
**S. Ceccarelli<sup>1</sup>, A. Aranburu<sup>1</sup>, F. Capolunghi<sup>1</sup>, M.M. Rosado<sup>1</sup>, E. Giorda<sup>1</sup>, S. Cascioli<sup>1</sup>, R. Carsetti<sup>1</sup>**  
<sup>1</sup>Research Center, Bambino Gesù Children's Hospital (IRCCS), Rome, Italy

Lymphocytes respond to extracellular microenvironment for development, activation, proliferation, differentiation and survival. The BCR signalling, critical for the B cell fate, is fine-tuned by different surface molecules that mediate the intracellular signalling. One of these molecule is CD22, a B-cell specific lectin-like member of the Ig superfamily that binds sialylated cell surfaces and soluble ligands. CD22 acts predominantly as an inhibitory coreceptor of the BCR, even if there are many controversial data about the mechanisms that modulate its function, and the role of  $\alpha 2,6$ Sia-binding activity. However, it is clear that CD22 plays a key role in establishing the BCR signalling threshold, and alterations in CD22 activity have implications for the generation of B-cell hypo- or hyper-activity. Altered BCR signalling in CD22-deficient mice might also predispose to autoimmunity by promoting the generation of B-1 B cells, which appear to require strong BCR signals for their development. B-1 B cells have been implicated in the pathogenesis of a number of autoimmune diseases, and are found in increased numbers in two lines of CD22-deficient mice. Again, changes in CD22 function appear to influence predisposition to autoimmunity. It has been shown that a number of autoimmune-prone strains of mice possess CD22 polymorphisms, and that several studies have associated the chromosomal region that contains CD22 with the development of autoantibodies, lupus-like glomerulonephritis and autoimmune haemolytic anaemia. Until now there is no convincing link between CD22 polymorphisms in humans, and the development of autoimmunity. We are currently studying a patient that shows Common Variable Immunodeficiency (CVID) symptoms and an abnormal B cell phenotype. This patient has been shown to exhibit low levels of CD22 by cytofluorimetric analysis. In addition we have been able to confirm a dysregulation in CD22 expression. Because of the impossibility to generate CD22-deficient human model we are trying to understand how the lack of CD22 could affect normal B-cell development in a given individual. It would be interesting to extend our study into possible CD22 polymorphisms occurring in this patient.

**PC03/22 GOOD'S SYNDROME (THYMOMA AND IMMUNODEFICIENCY): REPORT OF TWO CASES**  
**M. Sáenz Cuesta<sup>1</sup>, M. Alonso<sup>1</sup>, P. Echaniz<sup>1</sup>, R. Sesplugues<sup>2</sup>, A. Prada<sup>1</sup>, M.D. De Juan<sup>1</sup>, E. Cuadrado<sup>1</sup>**  
<sup>1</sup>Laboratorio Unificado Donostia, Laboratorio de Inmunología, San Sebastián – Donostia, Spain, <sup>2</sup>Hospital Donostia, Neumología, San Sebastián – Donostia, Spain

Good's Syndrome (GS) is a rare association between thymoma and combined immunodeficiency. Its presentation, late in life, usually occurs as recurrent sinopulmonary infections. The main immunological defects are hypogammaglobulinaemia, few or absent B cells, abnormal CD4:CD8+ T-cell ratio and impaired T-cell mitogenic responses. Haematological disorders and associated autoimmune diseases are frequently found. Some authors described cases documenting reduced specific antibodies values. The pathogenesis remains unknown.  
**Objective:** To report two cases of GS.

FEATURES	CASE 1 80-year-old woman	CASE 2 76-year-old man
Thymoma	Spindle, encapsulated	Spindle, encapsulated
Infections:		
Recurrent pneumonia	Yes	Yes
Chronic Diarrhoea	Yes	No
Others	Ureitis, oral candidiasis	Tetanus, TBC
Haematological disorders:		
Anaemia	Yes (FBCA)*	No
Leucopenia	Yes	Yes
Thrombocytopenia	Yes	No
Neutropenia	Yes	Yes
Eosinopenia	No	Yes
Presenting features	Recurrent infections	Mediastinal mass in EX
Immunological characteristics:		
Hypogammaglobulinaemia	Yes	Yes
Absence of B cells	Yes	Yes
Inverted CD4:CD8+ T-cell ratio	Yes	Yes
Low CD4+ T-cell counts	Yes	Yes
Functional responses:		
Proliferation response to PHA	Not tested	+ diminished
IFN $\gamma$ in response to PPD	Not tested	+ weak
Specific antibodies responses to:		
<i>Streptococcus pneumoniae</i>	+++	+
<i>Haemophilus influenzae</i>	+	+
<i>Toxoplasma gondii</i>	+	
CMV	+	
<i>Plasmodium falciparum</i>	-	
<i>Entamoeba histolytica</i>	-	
<i>Clostridium tetani</i>	-	+
Autoantibodies	Not found	Not found

[Characteristics of two patients with GS]  
\* Pure Red Cell Aplasia. \*\* + Positive; – Negative.

**Conclusion:** In both cases we found specific antibody responses despite the recurrence of pulmonary infections and the absence of circulating B cells.

**PC03/23 TACI GENE MUTATIONS IN PATIENTS FROM VENEZUELA: REPORT OF TWO CASES**A. B. Barroeta Seijas<sup>1,2</sup>, G. Di Matteo<sup>1</sup>, M. Fortes<sup>2</sup>, E. Ríquez<sup>2</sup>, L. Deibis<sup>2</sup>, V. Moschese<sup>1</sup>, I. Blanca<sup>2</sup><sup>1</sup>University of Rome Tor Vergata, Pediatrics, Rome, Italy, <sup>2</sup>Immunology Institute of the Central University of Venezuela, Caracas, Venezuela

TACI mutations have been associated to the risk of development of CVID. CVID is a heterogeneous disorder characterized by hypogammaglobulinemia and low response to vaccines. Major clinical manifestations are recurrent bacterial infections, autoimmunity and lymphoproliferative disease.

**Objective:** To provide a preliminary report of CVID patients from Venezuela and to identify associated gene mutations by molecular diagnosis.

**Methods:** Samples from three female unrelated Venezuelan hypogammaglobulinemic patients have been investigated by direct sequencing of the 5 exons of TACI. The first one was a 6 ys old child, the second a 44 ys old and the third a 45 ys old, all of them with a clinical history suggestive of CVID.

**Results:** Of the three patients studied, two had the same P251L mutation in the exon 5 in heterozygous state. This mutation has been previously reported and consensus amongst 18 bioinformatics methods predicts that this mutation causes deleterious changes in the protein. However, the same mutation in heterozygous state has not been proven yet to be associated to an increased risk of CVID as reported for C104R.

**Conclusions:** This is the first report of the identification of TACI mutations in CVID patients from Venezuela. Dissemination of the importance of molecular genetic testing as a valuable tool for definitive and early diagnosis of primary immunodeficiencies is mandatory for an appropriate clinical management of these patients. The establishment of a national network for the study of primary immunodeficiencies is ongoing. Extended studies in this country would be rewarded for the benefit of the affected patients and their families.

**PC03/24 USE OF COMPLEMENTARY AND ALTERNATIVE MEDICINE IN PATIENTS WITH HUMORAL IMMUNE DEFICIENCY**S. S. Kilic<sup>1</sup>, Z. Karali<sup>1</sup>, Y. Karali<sup>1</sup><sup>1</sup>Uludag University, Faculty of Medicine, Bursa, Turkey

**Background:** Complementary and alternative medicine (CAM) is a broad domain of healing resources that encompasses all health systems, modalities, and practices and their accompanying theories and beliefs, other than those intrinsic to the politically dominant health system of particular society or culture in a given historical period.

**Methods:** A total of 43 patients (29 boys and 14 girls) with the diagnosis of humoral immune deficiency attending outpatient clinics of Pediatric Immunology and receiving IVIG every three weeks were included. Data were collected through a questionnaire completed by the parents.

**Results:** The mean age at diagnosis was 7.56±9.44 years (6 months to 44 years) and the mean IVIG treatment duration was 6.02±3.84 years (1 to 20). It was found that 36 of 43 (83.7%) patients had used one or more of CAM approaches. The most frequently used method was herbal medicines in 28 (65.1%) patients (table 1). Twenty seven (62.8%) patients used dietary approaches (table 2). There are 20 (46.5%) patients using vitamin tablets. There were 15 (34.9%) patients using religious methods such as praying. Ten patients sought help from other institutions besides health centers. Of them 4 went to sellers of herb, three to a tomb, two to a prayer leader and one to a bio-energy center. The mean duration of CAM usage was 19.9±26.9 (1 to 120) months. Only 4 of 36 patients informed their doctor about CAM usage. The most common reason of CAM usage was reported as improving body resistance. Eighteen (50%) patients claimed that they benefited from CAM usage. The most common benefit that was declared by patients was the improvement in body resistance (27 patient; %75), followed by decrease in frequency in infection per year (16 patient; %44.4). No adverse effect was reported. All patients were on regular IVIG therapy during the study period. Non of the patients quit IVIG therapy when they have had ongoing CAM therapy. There was no statistical relation between CAM therapy and educational status of mother and father, socioeconomic status, income per month.

**PC03/25 CLINICAL MANIFESTATIONS IN GREEK PATIENTS WITH SELECTIVE IGA-DEFICIENCY AND SELECTIVE IGA-DEFICIENCY WITH IGG SUBCLASS DEFICIENCY**E. Angelopoulos<sup>1</sup>, D. Chrysosvergi<sup>1</sup>, K. Pesiridou<sup>1</sup>, V. Tsiamas<sup>1</sup>, S. Nikolakopoulos<sup>1</sup>, G. Mousoulis<sup>1</sup><sup>1</sup>Evangelismos General Hospital, Third Department of Internal Medicine, Athens, Greece

**Objective:** To investigate the distribution and clinical spectrum of IgA-D and IgA-D with IgGSc-D.

**Patients and methods:** The patients were hospitalised cases, identified during routine investigation of serum immunoglobulin levels (Igs). Measurement of serum IgG subclasses, detection of various autoantibodies and investigation for malabsorption and small intestinal biopsies were also performed.

**Results:** A total of 70 cases were identified: 56 cases (80%) of IgA-D and 14 cases (20%) of IgA-D with IgGSc-D. The distribution of the patients according to their age at diagnosis showed that 24% were diagnosed at the age between 15-20 years, 26% between 21-40 years, 40% between 41-60 years, whereas 10% were > 60 years of age at diagnosis. The incidence of autoimmune disease was significantly higher in IgA-D with IgGSc-D cases than in cases with IgA-D (50% vs 30%). The occurrence of infections was not significantly higher in patients with IgA-D than in IgA-D with IgGSc-D patients (36% vs 28%). Malignancies were also higher in cases with IgA-D than in IgA-D with IgGSc-D patients (11% vs 7%) whereas chronic diarrhoea or malabsorption was equally observed in the two groups (18% vs 21%).

**Conclusion:** Adult cases with IgA-D and IgA-D with IgGSc-D present increased morbidity and a delay in the diagnosis, pointing to the need for a wider information of the medical community in Greece.

**PC04 – T CELL DEFICIENCIES****PC04/2 A NOVEL HUMAN CD3D GENE MUTATION ASSOCIATED WITH LOW NUMBERS OF T-CELLS**C. Chean<sup>1</sup>, J. Gil<sup>1</sup>, J.R. Regueiro<sup>2</sup>, C. Beléndez<sup>3</sup>, J. Navarro<sup>1</sup>, E. Martínez-Busto<sup>2</sup>, A. Diaz-Alderete<sup>1</sup>, E. Cela<sup>3</sup>, D. Gurbindo<sup>3</sup>, V. Pérez-Flores<sup>2</sup>, J. Reiné<sup>2</sup>, I. Pilar<sup>1</sup>, A. Crespo-Guardo<sup>2</sup>, R. Rodríguez<sup>3</sup>, E. Fernández-Cruz<sup>1</sup>, M.J. Recio<sup>2</sup><sup>1</sup>Hospital General Universitario 'Gregorio Marañón', Immunology, Madrid, Spain, <sup>2</sup>Universidad Complutense de Madrid, Immunology, Madrid, Spain, <sup>3</sup>Hospital General Universitario 'Gregorio Marañón', Pediatrics, Madrid, Spain

**Objectives:** Genetic and functional characterization of a T-/B+NK+ primary immunodeficiency.

**Patient and methods:** A 14-month-old boy presented with failure to thrive, muguet, pneumonia, absence epilepsy, diarrhea caused by *Cryptosporidium* species, and sclerosing cholangitis. Complete peripheral blood or CFSE-labelled PBMC incubated with fluorochrome-conjugated mAbs were analysed by flow cytometry. Serum immunoglobulins were tested using nephelometry, ELISA, or hemagglutination. Genomic DNA and RNA were obtained from PBMC and, following CD3δ-specific reverse rtPCR, PCR products were purified, cloned, and sequenced.

**Results:** Immunological analysis revealed a normal peripheral lymphocyte count (3300 cells/μL), low T-cell numbers (14%, 532 cells/μL), and decreased CD3 expression. T-cells included CD4+, CD8+, TCRαβ+, and TCRγδ+ subsets. These cells were autologous with memory phenotype and a limited Vβ repertoire. The thymus was present but recent thymic emigrant T cells remained low throughout follow-up. T-cells were able to proliferate following PHA or anti-CD3 stimulation, as measured by CFSE dye dilution in gated CD3+ lymphocytes. Normal TCR downregulation after PMA stimulation and relatively preserved BMA031 (framework TCRαβ-specific mAb) binding excluded CD3γ deficiency. Intracellular binding of a CD3δ cytosolic domain-specific mAb (APA1/2) in T-cells was comparable to that of controls. A homozygous substitution (IVS2+5G>A) at the 5' splice site of intron 2 of the CD3D gene was detected, and both parents were heterozygous carriers for the mutation. IVS2+5G>A led to skipping of exon 2 in most of the patient's CD3δ mRNA. However, sizing of cloned PCR products indicated that normal transcripts were also present in +/- samples (14%), compared to carriers (28%) and controls (100%). Although levels of circulating B cells, IgG, IgM, and IgA were normal, no humoral responses were elicited after tetanus toxoid, influenza, or HBV vaccination. Elevated IgE (2141-4525 KU/L) and eosinophilia (800-5200 cells/μL) were consistently found.

**Conclusion:** We describe a new mutation causing CD3δ human immunodeficiency. This is the first clinical CD3δ case showing a limited repertoire of functional T-cells associated with late clinical onset and TH2 features. Differential criteria for CD3 deficiency diagnosis are defined. The role of a putative truncated CD3δ chain in T-cell selection and function remains to be established.

**PC04/4 MOLECULAR ANALYSIS OF THE MUTANT CD8 MOLECULE RESPONSIBLE FOR FAMILIAL CD8 DEFICIENCY**C. Gonzalez-Santesteban<sup>1</sup>, L. Martinez-Martinez<sup>1</sup>, N. Casamitjana<sup>1</sup>, M. Hernández<sup>2</sup>, C. Poppi<sup>1</sup>, O. de la Calle-Martin<sup>1</sup><sup>1</sup>Hospital Sant Pau – Universitat Autònoma Barcelona, Immunology, Barcelona, Spain, <sup>2</sup>Hospital Vall d'Hebron, Immunology, Barcelona, Spain

**Objectives:** Familial CD8 Deficiency is a primary immunodeficiency caused by homozygous mutations in the CD8A gene. The patients lack CD8 glycoproteins in peripheral blood lymphocytes, either T lymphocytes or NK cells. The only described mutation is a single aminoacid substitution affecting the V-like immunoglobulin domain of the CD8 alpha chain (Gly111Ser, CD8<sup>ser</sup>). Our aim was to elucidate the biochemical basis of this defect.

**Methods:** Chimeric proteins and site-directed mutants of the CD8 alpha molecule were generated and their stable transfectants were established. Biochemical analysis of the different CD8 constructs was performed. Since the missense mutation creates a consensus site for N-glycosylation in CD8, digestion with endoglycosidases and treatment of the transfectants with inhibitors of glycosylation were also conducted.

**Results:** Whereas CD8 is not detected in lymphocytes from the patients, we demonstrated that chimeric CD8 molecules were present at cell surface in transfected cells, even they were not recognized by any CD8 antibody. Mutant CD8 molecules have higher molecular weight than wild-type CD8, and an additional N-glycosylation site was established to be responsible for this phenomenon. Moreover, N-glycosylated mutant CD8 molecules showed an altered O-glycosylation, suggesting that mutant CD8 suffers an alteration in their biogenesis and maturation. Treatment with glycosidases and inhibitors of N-glycosylation was not able to restore the CD8 detection. Site-directed mutagenesis was used to generate CD8<sup>ser</sup> molecules without the N-glycosylation, but they were not detected either, suggesting that the mutation per se was responsible for the lack of CD8 detection. Cotransfection with the CD8 beta chain and biochemical analysis showed that

mutated CD8<sup>ser</sup> is unable to generate stable dimers. Experiments conducted with CD7 (the glycoprotein most related to CD8) and chimeric CD7/CD8 proteins also emphasize the importance of lack of dimerization.

**Conclusion:** Mutant CD8 molecules have an abnormally high molecular weight originated in an extra N-glycosylation site of the Ig-V domain. Nevertheless, this gain of glycosylation is not responsible either for the altered maturation, or the difficulties for a proper dimerization or the abnormal routing of the mutant molecule to the cell surface.

#### PC04/5 A SPONTANEOUS MUTATION IN BN RATS IS RESPONSIBLE FOR PARTIAL CD4 T CELL LYMPHOPENIA AND INFLAMMATORY BOWEL DISEASE DEVELOPMENT

M. Chabod<sup>1</sup>, A. S. Dejean<sup>1</sup>, D. Lagrange<sup>1</sup>, N. Vergnolle<sup>1</sup>, A. Saoudi<sup>1</sup>, G. Fournié<sup>1</sup>

<sup>1</sup>INSERM – U563, Toulouse, France

Partial T-cell immunodeficiencies are frequently associated with inflammatory or autoimmune disorders. The identification of the genes and mechanisms underlying these lymphopenic phenotypes are clue to a better understanding of the pathogenesis of these diseases. Recently, we have serendipitously discovered in our BN rat colony, a spontaneous recessive mutation that is responsible for a T cell lymphopenia. To further study this phenotype, we bred a new sub-strain bearing this mutation. Phenotypic analysis of peripheral lymphoid organs revealed that this lymphopenic syndrome affected specifically the CD4 T cell population, while the CD8 and B lymphocyte populations were not affected. We further showed that this CD4 lymphopenia originated in the thymus and resulted from a defect in selection from double positive to CD4 single positive stage. By using bone marrow chimeras, we showed that this CD4 lymphopenia was intrinsic to haematopoietic cells. In this sub-strain, macroscopic analysis revealed patchy multi-focused erythema and wall thickening all along the intestine. Microscopically, sub-mucosal oedema and severe transmural granulocyte infiltration were observed indicating that the CD4 lymphopenia is associated with the development of inflammatory bowel disease (IBD). In addition, we showed that peripheral CD4 T cells from lymphopenic rats exhibited an activated phenotype and mesenteric lymph node cells of these rats, upon adoptive transfer, induced IBD in naive BN recipients. We therefore named the new substrain BNLaibd (Laibd for Lymphopenia-associated IBD). Through linkage analyses in a (BNLaibd × DA) × BNLaibd backcross, we identified a new quantitative trait locus named Laibd1 localized in a 5.6 Mb region on chromosome 1. Genetic dissection of the locus is in progress to identify the Laibd1 gene(s). The identification of the gene(s) within Laibd1 that control(s) the CD4 T cell development and the susceptibility to IBD should further advance our basic knowledge into the mechanisms that support T cell homeostasis during normal immune response and that lead to breakdown of tolerance when IBD develops.

#### PC04/6 ARTEMIS SPLICE-DEFECT CAUSES NON-CLASSICAL SCID AND CAN BE RESTORED IN VITRO BY AN ANTISENSE OLIGONUCLEOTIDE

H. JSpeert<sup>1</sup>, C. M. R. Weemaes<sup>2</sup>, A. Warris<sup>2</sup>, A. C. Lankester<sup>3</sup>, W. W. Wiegant<sup>4</sup>, M. C. van Zelm<sup>1</sup>, Q. Pan-Hammarström<sup>5</sup>, M. J. D. van Tol<sup>3</sup>, D. C. van Gent<sup>6</sup>, J. J. M. van Dongen<sup>1</sup>, M. van der Burg<sup>1</sup>

<sup>1</sup>Erasmus MC, Dept. of Immunology, Rotterdam, Netherlands, <sup>2</sup>UMC-St Radboud, Dept. of Pediatrics, Nijmegen, Netherlands, <sup>3</sup>Leiden University Medical Center, Dept. of Pediatrics, Leiden, Netherlands, <sup>4</sup>Leiden University Medical Center, Dept. of Toxicogenetics, Leiden, Netherlands, <sup>5</sup>Karolinska University Hospital Huddinge, Dept. of Laboratory Medicine, Stockholm, Sweden, <sup>6</sup>Erasmus MC, Dept. of Cell Biology and Genetics, Rotterdam, Netherlands

**Objectives:** Severe Combined Immunodeficiency (SCID) is a life-threatening disease that is characterized by absence of functional T-lymphocytes. One of the subtypes, T-B-SCID, is caused by defective V(D)J recombination. Part of these patients is sensitive for ionizing radiation (RS-SCID), because of a defect in the non-homologous end joining (NHEJ) pathway that is essential for proper V(D)J recombination. Here we report a 10-yr old girl with a non-classical RS-SCID resulting in severe hypogammaglobulinemia and reduced absolute numbers of B- and T-lymphocytes, particularly in the naïve compartment. Clinical symptoms include bronchiectasis and progressive localized granulomatous skin lesions. Notably, she spontaneously recovered from an EBV and an Influenza A infection.

**Methods:** Several assays were performed to characterize the V(D)J recombination defect, including analysis of the precursor B-cell compartment, analysis of DH-JH coding joints and sequencing of candidate genes. Artemis transcripts were determined by quantitative PCR (RQ-PCR) in fibroblasts and lymphocyte subsets.

**Results:** Sequence analysis of the NHEJ factors known to cause RS-SCID (*Artemis*, *LIG4*, *XRCC4*, *Cernunnos*) did not show a defect in the coding exons and splice-sites. Analysis of DH-JH junctions amplified from bone marrow showed elevated numbers of palindromic nucleotides, which is characteristic for an Artemis defect. Therefore, *Artemis* was reconsidered as candidate gene and transcripts were analyzed. This revealed the inclusion of 190 nucleotides of intronic DNA between exon 11 and 12 due to a homozygous intronic mutation that created a new splice-site. RQ-PCR showed over 1,000 fold reduction of the normally spliced Artemis transcripts in patient's fibroblasts, which were radiosensitive. The splice defect in fibroblasts could be restored by transfection with an antisense oligonucleotide masking the new splice site. In contrast to fibroblasts, sorted B- and T-lymphocytes showed only a 10-fold reduction of normally spliced Artemis transcripts. Moreover, lymphocytes were not radiosensitive, and memory B-cells were present with normal features of somatic hypermutation and class switch recombination.

**Conclusion:** An Artemis splice-site mutation can lead to a non-classical form of RS-SCID. Moreover, this study provides new insights in the concept of the diagnostic strategy for RS-SCID and in splicing differences between tissues and differentiation stages.

#### PC04/7 DEFECT OF REGULATORY T CELLS IN PATIENTS WITH OMENN SYNDROME

B. Cassani<sup>1</sup>, L. Poliani<sup>2</sup>, D. Moratto<sup>3</sup>, V. Marrella<sup>1,4</sup>, L. Imperadori<sup>3</sup>, A. Plebani<sup>3</sup>, S. Giliani<sup>3</sup>, F. Facchetti<sup>3</sup>, F. Porta<sup>3</sup>, L. D. Notarangelo<sup>5</sup>, A. Villa<sup>4,6</sup>, R. Badolato<sup>3</sup>

<sup>1</sup>IRCCS Istituto Clinico Humanitas, Rozzano (MI), Italy, <sup>2</sup>University of Brescia, Department of Pathology, Brescia, Italy, <sup>3</sup>Istituto di Medicina Molecolare "Angelo Nocielli", University of Brescia, Department of Pediatrics, Brescia, Italy, <sup>4</sup>CNR-Istituto Tecnologie Biomediche, Milano, Italy, <sup>5</sup>Children's Hospital, Harvard Medical School, Division of Immunology, Boston, United States, <sup>6</sup>San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET), Milano, Italy

Omenn Syndrome (OS) is an autosomal recessive disorder characterized by severe immunodeficiency and T-cell-mediated autoimmunity. The disease is caused by hypomorphic mutations in recombination-activating genes that hamper the process of VDJ recombination, leading to the generation of autoreactive T cells with a highly restricted receptor repertoire. We have previously shown that in OS the expression of AIRE, a key factor in mediating central tolerance, is markedly reduced. In order to address the role of peripheral tolerance in the development of autoimmune manifestations of this genetic disease, we have investigated the expression of FOXP3 and the function of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells in OS patients.

An individual variability in the fraction of circulating FOXP3<sup>+</sup> CD4<sup>+</sup> cells was observed among OS patients, with an increased number detected in subjects displaying an activated/memory T cell phenotype. CD4<sup>+</sup>CD25<sup>high</sup> T cells isolated ex vivo from patients consistently failed to suppress proliferation of autologous or allogeneic CD4<sup>+</sup> effector T cells.

In spite of peripheral blood variability of FOXP3 levels, the immunohistochemical and qPCR analysis of its expression in lymph nodes and thymus of OS patients demonstrated a severe reduction of this cell subset, likely related to the defective thymic development of naturally occurring Tregs and to their impaired migration to lymph nodes due to lack of CCR7. Overall, these results suggest that in Omenn Syndrome reduced positive selection and breakdown of peripheral tolerance may actively concur to the development of autoimmune manifestations.

#### PC04/8 APPEARANCE OF LYMPHOMA IN TWO SIBLINGS FROM A FAMILY WITH CLINICAL FEATURES OF IPEX SYNDROME BUT NORMAL FOXP3 PROTEIN EXPRESSION

C. Sabelli<sup>1</sup>, G. Rossi<sup>2</sup>, D. Moratto<sup>1</sup>, F. Bono<sup>1</sup>, C. Mazza<sup>1</sup>, F. Facchetti<sup>3</sup>, L. Imperadori<sup>1</sup>, L. Passerini<sup>4</sup>, R. Bacchetta<sup>4</sup>, A. Plebani<sup>1</sup>, F. Porta<sup>1</sup>, H. D. Ochs<sup>5</sup>, L. D. Notarangelo<sup>6</sup>, L. Imberti<sup>7</sup>, R. Badolato<sup>1</sup>

<sup>1</sup>Brescia University Hospital, Nocielli Institute of Molecular Medicine, Oncohematology and BMT Unit and Pediatric Clinic, Brescia, Italy, <sup>2</sup>Spedali Civili, Hematology Department, Brescia, Italy, <sup>3</sup>University of Brescia, Spedali Civili Hospital, Department of Pathology, Brescia, Italy, <sup>4</sup>San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET), Division of Regenerative Medicine, Stem Cells and Gene Therapy, Milan, Italy, <sup>5</sup>Children's Hospital, Seattle, United States, <sup>6</sup>Children's Hospital Boston, Harvard Medical School, Boston, United States, <sup>7</sup>Spedali Civili of Brescia, Department of Diagnostic, Terzo Laboratorio, Brescia, Italy

Immunodysregulation, Polyendocrinopathy, Enteropathy, X-linked (IPEX) syndrome is a rare recessive disorder of immune system caused by mutation of the transcription factor forkhead box protein 3 gene (*FOXP3*), required for the function of CD4<sup>+</sup>/CD25<sup>+</sup> T regulatory cells. *FOXP3* is expressed as two different isoforms in humans: the full-length isoform and an alternative-splicing product lacking the exon 2 (*FOXP3ΔE2*).

We describe a family composed of three males who presented a mild form of the disease. Two of them, at the age of 25 and 15, developed a B-cell and a T-cell lymphoma respectively, suggesting an increased risk of hematopoietic tumors in IPEX patients surviving to adulthood.

Immunophenotyping of peripheral blood, performed by cytofluorimetric assay, showed in our patients, compared with healthy donors, a reduced number of T cells, while B and NK cells level were normal. We observed a depletion of both T naïve CD4<sup>+</sup> and CD8<sup>+</sup>, with relatively high proportion of memory T cells, and a depletion of memory B cells, while naïve and transitional B cells were present at normal levels. Expression of FOXP3 protein in our patients was detectable at normal levels on CD4<sup>+</sup>/CD25<sup>+</sup> T cells. FOXP3 gene studies revealed that all three patients and their mother carried a previously reported single-base substitution (C543T) at the intron 4/exon 5 boundary which result in a silent mutation (S181S). FOXP3 mRNA sequencing don't reveal aberrant splicing isoforms. In a preliminary experiment, performed in IL-2 stimulated lymphoblasts derived from patients, we failed to detect significant differences of FOXP3 mRNA expression levels, and of the expression ratio between the full-length and the FOXP3ΔE2 isoforms between patients and control subjects.

Analysis of Treg suppressive activity against allogeneic responder cells from healthy controls has shown normal suppression of proliferation and IFN-γ secretion of allogeneic responder T cells.

On the basis of this single observation, it is not possible to determine whether the clinical phenotype observed in these patients is related to abnormal regulation of FOXP3 expression or it constitutes a distinct genetic disease.



# PC04/9 THE INFLUENCE OF IRON LOADING AND IRON CHELATION ON THE PROLIFERATION AND TELOMERASE ACTIVITY OF LYMPHOCYTES

B. Bagherpour<sup>1</sup><sup>1</sup>Medical University of Isfahan, Molecular Biology and Genetic, Isfahan, Iran, Islamic Republic of

**Background:** Iron is an essential trace element in cell proliferation. Several researches demonstrate that iron deprivation inhibits cell proliferation. However, the impact of iron on telomerase activity of activated lymphocytes remains unexplained to date.

**Aims:** In this study, the effect of iron on the proliferation and telomerase activity of lymphocytes stimulated by phytohemagglutinin (PHA) were investigated.

**Methods:** Iron loading was performed by incubating peripheral blood mononuclear cells in 500 µM FeSO<sub>4</sub>·7H<sub>2</sub>O for 24 hours and iron chelation was done by exposing cells to desferrioxamine, a potent iron chelator. The effects of silymarin, a flavonoid with both antioxidant and iron chelating activities, on the proliferation and telomerase activity of PHA-activated lymphocytes were also compared with desferrioxamine. Proliferation and telomerase activity were assessed using BrdU incorporation assay and Telomeric Repeat Amplification Protocol (TRAP) respectively.

**Results:** The proliferations of lymphocytes were significantly inhibited by 10 and 20 µg/ml desferrioxamine in a dose dependent manner, while iron loading recovered suppressed cell proliferation to the normal level. Silymarin at 20 µg/ml significantly increased the proliferation of lymphocytes in both normal and iron-treated condition. Telomerase activity of lymphocytes markedly increased by iron treatment and suppressed by desferrioxamine. Conversely, iron treatment had no effect on the telomerase activity of lymphocytes incubated with silymarin.

**Conclusion:** Iron plays a significant role in the proliferation and telomerase activity of lymphocytes. The effects of silymarin on the proliferation and telomerase activity of lymphocytes were completely different from those of desferrioxamine, suggesting that the immunomodulatory effect of silymarin is probably not associated with its iron chelating activity.

# PC04/10 COMPARTMENTALIZATION OF IMMUNE RESPONSES IN HUMAN TUBERCULOSIS: FEW CD8+ EFFECTOR T CELLS BUT ELEVATED LEVELS OF FOXP3+ REGULATORY T CELLS IN THE GRANULOMATOUS LESIONS

S. Rahman<sup>1</sup>, B. Gudetta<sup>2,3</sup>, J. Fink<sup>1</sup>, A. Granath<sup>4,5</sup>, S. Ashenafi<sup>1,3,6</sup>, A. Aseffa<sup>7</sup>, M. Derbew<sup>3,8</sup>, M. Svensson<sup>1,9</sup>, J. Andersson<sup>1,9</sup>, S. G. Brighenti<sup>1</sup>

<sup>1</sup>Karolinska Institute, Center for Infectious Medicine, Stockholm, Sweden, <sup>2</sup>Faculty of Medicine, Addis Ababa University, Department of Paediatrics, Addis Ababa, Ethiopia, <sup>3</sup>Tikur Anbessa Hospital, Addis Ababa, Ethiopia, <sup>4</sup>Karolinska Institute, Ear- Nose and Throat Clinic, Stockholm, Sweden, <sup>5</sup>Karolinska University Hospital Huddinge, CLINTEC, Stockholm, Sweden, <sup>6</sup>Faculty of Medicine, Addis Ababa University, Department of Pathology, Addis Ababa, Ethiopia, <sup>7</sup>Armauer Hansen Research Institute, Addis Ababa, Ethiopia, <sup>8</sup>Faculty of Medicine, Addis Ababa University, Department of Surgery, Addis Ababa, Ethiopia, <sup>9</sup>Karolinska Institute, Dept. of Medicine, Stockholm, Sweden

Immune responses were assessed at the single cell level in the lymph nodes from children with tuberculous lymphadenitis. Tuberculosis infection was associated with tissue remodeling of lymph nodes as well as altered cellular composition. Granulomas were significantly enriched with CD68+ macrophages expressing the *Mycobacterium tuberculosis* complex specific protein antigen MPT64 and the inducible nitric oxide synthase. There was a significant increase of CD8+ cytolytic T cells surrounding the granuloma; however, CD8+ T cells expressed low levels of the cytolytic and antimicrobial effector molecules, perforin and granzyme, in the granulomatous lesions. Quantitative real-time mRNA analysis revealed that IFN-γ, TNF-α and IL-17 were not up-regulated in infected lymph nodes while there was a significant induction of TGF-β and IL-13. In addition, granulomas contained an increased level of CD4+FoxP3+ T cells co-expressing the immunoregulatory CTLA-4 and GITR molecules. Low numbers of CD8+ T cells in the granulomatous lesions correlated with a high expression of TGF-β and FoxP3+ regulatory T cells, suggesting active immunosuppression at the local site of infection. Compartmentalization and skewing of the immune response toward a regulatory phenotype may result in an uncoordinated effector T cell response that reduce granuloma-mediated killing of *Mycobacterium tuberculosis* infected cells and subsequent disease control.

# PC04/11 DIFFERENT X CHROMOSOME INACTIVATION PATTERN IN TWO WAS MUTATED FEMALES (MOTHER AND DAUGHTER) RESULTS IN A DISTINCT CLINICAL PHENOTYPE EXPRESSION

V. Daza<sup>1</sup>, A. M. García-Alonso<sup>2</sup>, N. Martínez Pomar<sup>1</sup>, D. Heine-Suñer<sup>3</sup>, G. Zaldívar<sup>4</sup>, S. Torres<sup>4</sup>, M. Bermudez<sup>5</sup>, R. Alvarez-Lopez<sup>2</sup>, I. Molina<sup>4</sup>, N. Matamoros<sup>1</sup>

<sup>1</sup>Hospital Son Dureta, Servicio de Inmunología, Palma de Mallorca, Spain, <sup>2</sup>Hospital Universitario Virgen de la Arrixaca, Servicio de Inmunología, Murcia, Spain, <sup>3</sup>Hospital Son Dureta, Servicio de Genética, Palma de Mallorca, Spain, <sup>4</sup>Parque Tecnológico de Ciencias de la Salud, Centro de Investigación Biomédica, Granada, Spain, <sup>5</sup>Hospital Universitario Virgen de la Arrixaca, Unidad Oncohematología Pediátrica, Murcia, Spain

**Introduction:** Wiskott-Aldrich syndrome (WAS) is an X-linked recessive disorder characterized by thrombocytopenia, eczema and various degrees of immune deficiency. WAS gene mutations are responsible for WAS syndromes and a genotype-phenotype correlation has been reported. Mild allelic variants were associated to as X-linked thrombocytopenia (XLT), intermittent thrombocytopenia or congenital X-linked neutropenia. In general, female carriers are asymptomatic as a consequence of a positive selection of cells with an active normal X-chromosome.

**Results:** We report two females, mother and daughter, that present in heterozygote state, the already described mutation, p.V332A, normally associated to XLT. The daughter was born to non-consanguineous parents and presented from birth severe thrombocytopenia, repetitive upper respiratory infections and otitis. Western blot analysis showed a normal size and protein expression, and X-chromosome inactivation pattern analysis showed a total inactivation of the non mutated paternal X chromosome in peripheral blood cells. The mother was a healthy female and presented a random X chromosome inactivation pattern.

**Conclusions:** In contrast to other studies that describe the presence of positive selection of normal X chromosome in healthy female carriers, our healthy mother's patient presented random X inactivation pattern. Our results show that milder mutations together with a total inactivation of non mutated X chromosome are required for the presence of clinical manifestation in female carriers. These results confirm the genotype-phenotype correlation already described for WAS disease and supports that the presence of clinical manifestations in female carriers is strongly dependent of mutation characteristics and the X-chromosome inactivation pattern. Further studies are needed to provide a clearer insight in to the molecular basis of the non-inactivation process of the mutated X chromosome.

# PC04/12 DIVERSE PHENOTYPIC AND GENOTYPIC PRESENTATION OF RAG1 MUTATIONS

G. Aksu<sup>1</sup>, N. E. Karaca<sup>1</sup>, N. Gulez<sup>1</sup>, K. Emin<sup>2</sup>, S. Can<sup>1</sup>, S. Aksoylar<sup>1</sup>, S. Kansoy<sup>1</sup>, C. Balkan<sup>1</sup>, Y. Aydinok<sup>1</sup>, N. Kutukculer<sup>1</sup>

<sup>1</sup>Ege University Faculty of Medicine, Department of Pediatrics, Izmir, Turkey, <sup>2</sup>Ege University Faculty of Medicine, Department of Medical Genetics, Izmir, Turkey

Severe combined immunodeficiencies (SCID) comprise a spectrum of genetic defects that involve both humoral and cellular immunity. Defects in recombining activating gene 1 (RAG1), RAG2, Artemis or LIG4 can disrupt V(D)J recombination. Defective V(D)J recombination of the T and B cell receptors is responsible for TB<sup>+</sup> NK<sup>+</sup> SCID. Amorphic mutations in RAG1 and RAG2 cause TB<sup>+</sup> NK<sup>+</sup> SCID, whereas hypomorphic mutations cause an immunodeficiency characterized by oligoclonal expansion of TCRγδ T cells, severe CMV infection and autoimmunity. In the majority of cases carrying these mutations, the symptoms start within the second or third month after birth. SCIDs are commonly fatal early in life. Adequate diagnosis and rapid institution of treatment, such as allogeneic stem cell transplantation is essential. In this report, two distinct clinical spectrums associated with RAG1 mutations are presented. First patient had a typical clinical and laboratory proven TB<sup>+</sup> NK<sup>+</sup> SCID. Second was atypical with normal levels of immunoglobulins, and nearly normal CD3+ T cell, CD19+ B cell and CD56+ NK cell ratios but persistent CMV infection and overt autoimmune findings. Clinical and immunologic phenotypes of patients bearing RAG mutation are diverse as previously reported and this is related to the specific type of RAG mutation that abolishes T cell function leading to autoreactive T cells in the absence of other classical laboratory findings of SCID. Villartay et al. reported four unrelated patients, with hypomorphic RAG1 mutations. The hypomorphic del A368/A369 mutation demonstrated in their fourth patient (*this patient was also Turkish*) was the same as our second patient's. We think that this is the fifth patient with SCID associated with hypomorphic RAG1 mutation and persistent CMV infection with autoimmune findings. If severe combined immunodeficiency is suspected and atypical clinical findings like autoimmunity and persistent CMV infection, it is necessary to investigate for RAG1 mutations.

# PC04/13 THE EFFECTS OF ABNORMAL PURINE NUCLEOSIDE PHOSPHORYLASE FUNCTION ON T LINEAGE DEVELOPMENT

T. Papinazath<sup>1,2</sup>, W. Min<sup>1</sup>, C. M. Roifman<sup>3</sup>, E. Grunebaum<sup>1,3</sup>

<sup>1</sup>Hospital for Sick Children, Developmental and Stem Cell Biology, Toronto, Canada, <sup>2</sup>University of Toronto, Institute of Medical Science, Toronto, Canada, <sup>3</sup>Hospital for Sick Children, Immunology, Toronto, Canada

**Background:** Purine nucleoside phosphorylase (PNP) is a ubiquitous enzyme crucial for degradation and salvage of purine metabolites. Patients with inherited defects in the function of PNP suffer from severe T lineage immune deficiency. Difficulties in obtaining patients tissue samples have significantly hindered our ability to study the precise cause of the immune abnormalities, which is also crucial for development of appropriate treatments. We used an animal model (PNP<sup>-/-</sup> mice), which closely resembles the human disease, and the OP9-DL1 co-culture system that allows expansion of bone marrow (BM) cells into T lineage cells in PNP-deficient conditions to better characterize the effects of abnormal PNP metabolism on thymocyte development.

**Methods:** Cells from thymic of 6-week old PNP<sup>-/-</sup> mice and normal littermates were characterized by analyzing surface expression of differentiation markers. Proliferation and apoptosis of thymocytes were determined by BrdU incorporation and Annexin-V labeling, respectively. BM cells purified from PNP<sup>-/-</sup> mice were co-cultured on OP9-DL1 stroma cells in the presence of the PNP inhibitor (BCX-177) to inhibit PNP in stroma cells with and deoxyguanosine (dGuo) to simulate conditions of PNP deficiency.

**Results:** Thymic of PNP<sup>-/-</sup> mice contained significantly (p=0.012) more CD117<sup>+</sup>CD25<sup>+</sup> double negative (DN) 3 cells but significantly decreased DN4 and double positive (DP) cells. Proliferation of thymocytes was similar in PNP<sup>-/-</sup> and normal mice and normal controls. In the first two weeks of BM co-culture, when most cells were in stages equivalent to DN1 and DN2, there were no differences between BM cells grown in PNP-deficient versus PNP-proficient conditions. In contrast, by the 3rd week of culture, more cells remained in the stages equivalent to DN3 and DN4 in PNP-deficient cultures, with a significant (p=0.001) reduction in the

development of cells with characteristics of DP. PNP deficiency was also associated with increased apoptosis of T lineage cells. The abnormal T lineage development from BM was dependent on the presence of dGuo, which supports the role of dGuo in mediating the effects of PNP deficiency.

**Conclusions:** Our models demonstrate abnormal maturation of thymocytes in PNP deficiency, likely related to accumulation of dGuo.

#### PC04/14 P.V131F MUTATION IN CD3G AS A RISK FACTOR FOR IMMUNODEFICIENCY

E. M. Busto<sup>1</sup>, J. Reine<sup>1</sup>, A. Crespo<sup>1</sup>, V. Pérez<sup>1</sup>, L. M. Allende<sup>2</sup>, M. Á. Moreno-Pelayo<sup>3</sup>, J. M. van Montfrans<sup>4</sup>, G. Lefranc<sup>5</sup>, J. R. Regueiro<sup>1</sup>, M. J. Recio<sup>1</sup>

<sup>1</sup>Universidad Complutense de Madrid. F. Medicina, Madrid, Spain, <sup>2</sup>Hospital 12 de Octubre, Madrid, Spain, <sup>3</sup>Hospital Ramón y Cajal, Madrid, Spain, <sup>4</sup>Wilhelmina Children's Hospital, Utrecht, Netherlands, <sup>5</sup>Institut de Génétique Humaine, Montpellier, France

The p.V131F mutation is located at the transmembrane region of the CD3g protein within a highly conserved region and is crucial for the assembly of the TCR/CD3 complex. It has been identified in three immunodeficient patients from two unrelated families in which no other pathogenic mutations were found.

**Objective:** To study the impact of this mutation in the assembly of the TCR/CD3 complex and address if this mutation is pathogenic.

**Cases report:** Patients 1 and 2.- The first proband (patient 1) was the first child of consanguineous parents and homozygous for this mutation. He displayed SCID symptoms with normal levels of T and B cells, low serum IgG levels, normal proliferation response to PHA but decreased to antigens and OKT3. He died at age 7 years. His sister (patient 2) was heterozygous for this mutation and showed the same clinical symptoms and lab findings. She died at age 12 years. Both parents are healthy and carry the p.V131F mutation in heterozygous state.

Patient 3.- A two-years old boy, heterozygous for this mutation, showed a severe episode of HHV-6 pneumonia without SCID symptoms. He presented mild TCD4 lymphopenia, low serum immunoglobulins levels and normal response to PHA and decreased to antigens. There is no consanguinity or familial history of immunodeficiency.

**Material and Methods:** Peripheral blood samples from controls and patients were incubated with fluorochrome-conjugated monoclonal anti-CD3 antibodies and analysed by flow cytometry. Searching of the p.V131F mutation in 114 healthy Spanish controls was performed by a RFLP screening test using the HpyCH4III restriction enzyme.

**Results:** Immunophenotype: peripheral blood T cells from both heterozygous patients showed a slight decreased expression of CD3 as detected by flow cytometry assays. CD3 expression was normal in all the healthy controls (homozygous, heterozygous) examined. Population study: the results obtained revealed that in the cohort of controls examined the p.V131F mutation was in heterozygous and homozygous state in 22% and 6% of individuals, respectively.

**Conclusions:** The data presented here show that the p.V131F mutation is frequent in healthy population. Therefore, although it cannot be pathogenic by itself, it cannot be excluded as a risk factor for immunodeficiency.

#### PC04/15 DEFECTS ALONG THE TH17 DIFFERENTIATION PATHWAY UNDERLIE GENETICALLY DISTINCT FORMS OF THE HYPER IGE SYNDROME

S. Al Khatib<sup>1</sup>, S. Keles<sup>2</sup>, M. Garcia-Lloret<sup>1</sup>, E. Karakoc-Aydiner<sup>2</sup>, I. Reisli<sup>3</sup>, H. Artac<sup>3</sup>, Y. Camcioglu<sup>4</sup>, H. Cokugras<sup>4</sup>, A. Somer<sup>5</sup>, N. Kutukculer<sup>6</sup>, M. Yilmaz<sup>7</sup>, A. Ikinciogullari<sup>8</sup>, O. Yegin<sup>9</sup>, M. Yuksek<sup>10</sup>, F. Genel<sup>11</sup>, E. Kucukosmanoglu<sup>12</sup>, A. Baki<sup>13</sup>, N. Nadir Bahceciiler<sup>2</sup>, A. Rambhatla<sup>1</sup>, D. W Nickerson<sup>1</sup>, S. Mc Ghee<sup>1</sup>, I. B Barlan<sup>1</sup>, T. Chatila<sup>1</sup>

<sup>1</sup>David Geffen School of Medicine at the University of California at Los Angeles, Division of Immunology, Allergy and Rheumatology, Department of Pediatrics, Los Angeles, United States, <sup>2</sup>Marmara Medical Faculty, Division of Pediatric Allergy and Immunology, Istanbul, Turkey, <sup>3</sup>Selcuk University, Faculty of Medicine, Division of Pediatric Allergy and Immunology, Konya, Turkey, <sup>4</sup>Istanbul University, Cerrahpasa Faculty of Medicine, Division of Pediatric Immunology, Istanbul, Turkey, <sup>5</sup>Istanbul University Faculty of Medicine, Division of Pediatric Allergy-Immunology and Infectious Diseases, Istanbul, Turkey, <sup>6</sup>Ege University Faculty of Medicine, Department of Pediatrics, Izmir, Turkey, <sup>7</sup>Cukurova Medical Faculty, Division of Pediatric Allergy and Immunology, Adana, Turkey, <sup>8</sup>Ankara University School of Medicine, Division of Pediatric Immunology, Istanbul, Turkey, <sup>9</sup>Akdeniz University School of Medicine, Department of Pediatric Immunology, Antalya, Turkey, <sup>10</sup>Zeynep Kamil State Hospital, Division of Pediatric Immunology, Istanbul, Turkey, <sup>11</sup>Behcet Uz State Hospital, Division of Pediatric Immunology, Istanbul, Turkey, <sup>12</sup>Gaziantep University Faculty of Medicine, Division of Pediatric Allergy, Gaziantep, Turkey, <sup>13</sup>Karadeniz Technical University, Faculty of Medicine, Division of Pediatric Allergy, Trabzon, Turkey

**Objective:** To elucidate mechanisms underlying different forms of HIES.

**Methods:** A cohort of 25 Turkish children diagnosed with HIES were examined for STAT3 mutations by DNA sequencing. Activation of STAT3 by IL-6 and IL-21 and STAT1 by interferon alpha (IFN $\alpha$ ) was assessed by intracellular staining with anti-phospho (p)STAT3 and pSTAT1 antibodies. Th17 and Th1 cell differentiation was assessed by measuring the production of IL-17 and IFN $\gamma$ , respectively.

**Results:** Six subjects had STAT3 mutations affecting the DNA binding, SH2 and transactivation domains, including 3 novel ones. Mutation-positive but not mutation-negative HIES subjects exhibited reduced phosphorylation of STAT3 in response to cytokine stimulation, while pSTAT1 activation was unaffected. Both patient groups exhibited impaired Th17 responses, but whereas STAT3 mutations abrogated early steps in Th17 differentiation, the defect(s) in HIES patients with normal STAT3 affected more distal steps.

**Conclusion:** In this cohort, a majority children with HIES had normal STAT3, implicating other targets in disease pathogenesis. Impaired Th17 responses were evident irrespective of the STAT3 mutation status, indicating that different genetic forms of HIES share a common functional outcome.

#### PC04/16 CD8 T CELLS EXHAUSTION-LIKE IN CHRONIC LEISHMANIASIS AND RESTAURATION BY TLR2 LIGANDS

J. Hernández-Ruiz<sup>1</sup>, N. Salaiza<sup>1</sup>, A. Ruiz<sup>1</sup>, Y. Rosenstein<sup>2</sup>, A. Zentella<sup>1</sup>, I. Becker<sup>1</sup>

<sup>1</sup>Universidad Nacional Autónoma de México, Medicina Experimental Facultad de Medicina, México DF, Mexico, <sup>2</sup>Universidad Nacional Autónoma de México, Instituto de Biotecnología, Cuernavaca, Mexico, <sup>3</sup>Universidad Nacional Autónoma de México, Instituto de Investigaciones Biomedicas, Mexico DF, Mexico

In México, human cutaneous leishmaniasis (CL) is caused by the infection with the protozoan parasite *Leishmania mexicana* (Lm), which can produce two clinically opposed forms: the localized CL (LCL), a more benign form of the disease characterized by a protective cellular immune response circumscribing the parasite to the inoculation site, and diffuse CL (DCL), characterized by a poor cellular immune response permitting the spread of the parasite throughout the skin leading to chronicity. It has been shown that CD8 T cells (CD8) play a crucial role in infection clearance; it is feasible that their participation in disease control could be diminished in DCL. **Objective.** Determine the participation of CD8 in control of Lm in LCL y DCL. **Methodology.** TUNEL and CD68 were realized in skin biopsies of LCL and DCL patients. CD8 were purified from peripheral blood of LCL (10) and DCL (4) patients and flow cytometry-based cytotoxicity assay against autologous macrophages infected by Lm (MOi) was carried out in addition to proliferation and IFN $\gamma$  production studies done. Higher expression of TUNEL+ was observed in LCL as compare to DCL patients. Double-staining revealed TUNEL+CD68+ in high proportion. DCL CD8 cells exhibited low or null response in cytotoxicity, proliferation and IFN $\gamma$  production, when were stimulated with MOi. Additionally, we found PD-1 expression in a DCL CD8 subgroup. These characteristics are similar to cellular exhaustion condition described in chronic infections like HCV and LCMV where cellular restoration has been reported. Thus, we intended to restore functional ability of DCL CD8 by preincubation with Lm lipophosphoglycan or Pam3Cys, two TLR2 ligands. Cytotoxicity as well as proliferation and IFN $\gamma$  production were restored with both stimulus, although to a lesser degree than those of LCL patients. **Conclusions.** Our work suggests that CD8 cytotoxicity is associated with Lm infection control. This is the first report suggesting that exhausted CD8 cells can be rescued through TLR2 stimulation. This work was supported by CONACyT No 47256. Joséln Hernández-Ruiz was recipient of a DGEP-UNAM and PAPIIT fellowship, and is indebted to Posgrado en Ciencias Biológicas – UNAM.

#### PC04/17 SEVERE COMBINED IMMUNODEFICIENCY: CLINICAL; IMMUNOLOGICAL FEATURES AND OUTCOME IN 25 CASES

F. Dogu<sup>1</sup>, F. Cipe<sup>1</sup>, C. Aytekin<sup>2</sup>, D. Guloglu<sup>1</sup>, A. Yildiran<sup>1</sup>, M. Yuksek<sup>3</sup>, G. Bozdogan<sup>4</sup>, I. Reisli<sup>5</sup>, A. Ikinciogullari<sup>1</sup>

<sup>1</sup>Ankara University School of Medicine, Department of Pediatric Immunology and Allergy, Ankara, Turkey, <sup>2</sup>Dr. Sami Ulus Children's Hospital, Division of Pediatric Immunology, Ankara, Turkey, <sup>3</sup>Zeynep Kamil Hospital, Istanbul, Turkey, <sup>4</sup>Acibadem Hospital, Istanbul, Turkey, <sup>5</sup>Selcuk University, Meram School of Medicine, Department of Pediatric Immunology and Allergy, Konya, Turkey

Severe combined immunodeficiency (SCID) comprises a collection of genetic defects that involve both humoral and cellular immunity. A profound lack of immune function leads to infections that are generally fatal in infancy unless the immune system can be reconstituted. SCID can be phenotypically and genotypically categorized according to the presence or absence of lymphocyte subtypes. T-B+NK- SCID, accounting for up to 50% SCID cases in USA and most of the Europe, seem to be less frequent in Turkey. In the present study clinical, immunological features and outcome of 25 SCID cases who were admitted to Ankara University, Department of Pediatric Immunology and Allergy between 1997-2008 were retrospectively analyzed. Twelve out of 25 cases (48%) were girls and the rest were boys. Median age at diagnosis was 4 months (range:1-12months). The median age of beginning of symptoms was 1 month of age. (range: 10 days-6 months). Seventeen patients have T-B-NK+ (68%) phenotype while the others have T-B+NK+ ( 24%), T-B-NK- (4%), T-B+NK- (1%). 24 hematopoietic stem cell transplantation (HSCT) was performed in 16 cases. Two patients received 3, 4 patients received two transplantations. Ten patients received haploidentical parental CD34+ selected peripheral blood while the others had fully matched family donor bone marrow. The percentage of survival based on the type of transplantations was 80% and 66% respectively. Nine patients who were either not accepted transplantation or have severe infections died before transplantation. In conclusion early diagnosis and HSCT was the life saving treatment for SCID patients even in the absence of fully matched sibling donor.

**PC04/18 REGISTER OF COMBINED IMMUNODEFICIENCY IN UKRAINE: PROBLEMS AND SOLUTIONS**L. Chernyshova<sup>1</sup>, A. Bondarenko<sup>1</sup>, L. Kostuchenko<sup>2</sup><sup>1</sup>National Medical Academy for Post-graduate Education, Department of Pediatric Infectious Diseases and Clinical Immunology, Kiev, Ukraine, <sup>2</sup>Child Regional Hospital, Lviv, Ukraine**Objectives:** Up to 1997 the statistics on combined immunodeficiency in Ukraine practically was absent. Since the 1997 the work on detection of CID has been started in Ukraine. The aim of the study is to make the register of the primary combined immunodeficiency.**Methods:** Data about primary immunodeficiency were collected on the basis of active centralized system of the reporting according to the developed form from regional pediatric immunologists. Diagnosis of CID was done on clinical criteria and immunophenotyping in our clinics. Cases when the diagnosis was first established on the basis of autopsy also were included into register.**Results:** Currently, there have been registered 30 cases of Nijmegen breakage syndrome (NBS), 24 patients with ataxia-telegenectasia (AT), 11 patients with Wiskott-Aldrich syndrome (WAS), 16 children with severe combined immunodeficiency (SCID), 1 patient with CD4 lymphopenia, 10 patients with Di George's syndrome, 8 children with mucocutaneous candidiasis and 2 with Netherton syndrome. In some cases, diagnosis was confirmed by genetic analysis. Thus, in all patients with both NBS, homozygote "Slavic" mutation 657del5 and in 15 patients with AT the ATM was detected in Ukraine. 6 patients with WAS were confirmed by genetic analysis, 2 cases of RAG1/RAG2 deficiency, IL-7R, IL-2R deficiency and ADA deficiency and 4 cases of Di George's syndrome were also confirmed in other countries. High mortality among the patients with CID in Ukraine is primarily caused by the absence of immunoreconstructive treatment. Thus, 15 out of 16 patients with SCID died from infectious complications. Only one child received HSCT (Brescia, Italy). Several patients died from oncologic disease: 6 patients with NBS and 1 with AT (Lymphomas).**Conclusion:** In our country lower occurrence of CID in comparison with other countries, obviously, is connected with insufficient detecting ability. The death of patient before an establishment of the diagnosis and carrying out of dull treatment at the primary combined immunodeficiency remains the frequent phenomenon in our country. The second problem of untimely receiving the adequate help by patients is absence of immunoreconstructive treatment in our country, and necessity of sending the patients for treatment to foreign clinics.**PC04/19 TUBERCULOUS ENTERITIS IN A MALE WITH IDIOPATHIC CD3+CD4+ LYMPHOPOENIA**E. Angelopoulos<sup>1</sup>, D. Chrysosvergi<sup>1</sup>, K. Pesiridou<sup>1</sup>, A. Farris<sup>1</sup>, V. Tsiamas<sup>1</sup>, D. Rontoyianni<sup>2</sup>, G. Mousoulis<sup>1</sup><sup>1</sup>Evangelismos General Hospital, Third Department of Internal Medicine, Athens, Greece, <sup>2</sup>Evangelismos General Hospital, Department of Pathology, Athens, Greece

This is the case of a 26-year-old HIV-negative student evaluated for microcytic anaemia of one month's duration.

**History:** Hashimoto's thyroiditis, reactive axillary and inguinal lymphadenopathy, and CD3+CD4+ lymphopenia, the investigation of which had revealed no underlying disease. At admission the patient presented mild tenderness of the right lower abdomen and palpable axillary and inguinal lymph nodes bilaterally. Laboratory testing showed microcytic anaemia, CD4 lymphopenia, eosinophilia, iron deficiency, positive occult fecal blood test, and a negative Mantoux test.

Upper GI endoscopy showed diffusely oedematous mucosa of the gastric fundus and body, and multiple, small ulcerations in the first portion of the duodenum. Colonoscopy showed an oedematous ileocecal valve, with diffuse oedema and redness in the mucosa of the terminal ileum, small, shallow ulcers and polyps.

**Chest CT:** One lymph node of 2 cm diameter in the left axilla and one of 1 cm diameter in the right.**Abdominal CT:** Segmental thickening of the intestinal wall, mostly of the ileum, and marginally enlarged mesenteric lymph nodes.**Histology:** lymphonodal specimens showed reactive nodular lymphadenitis. Upper GI specimens showed ulcerations compatible with malabsorption syndrome of grade I to II, and chronic lymphocytic gastritis of grade II. Terminal ileum lesions indicated chronic granulomatous non necrotic ileitis. Ziehl-Neelsen staining was negative.Molecular testing from the terminal ileus with PCR was positive for *M. tuberculosis* complex infection.**Conclusion:** Cellular immunity is crucial in Mycobacterial infections. In patients presenting with T-cell abnormalities, a thorough search for such infections is warranted.**PC05 – DEFICIENCIES OF THE INNATE IMMUNE SYSTEM****PC05/1 TLR HYPORESPONSIVENESS OF HUMAN NK CELLS IS ASSOCIATED WITH RECURRENT HERPES LABIALIS**C.-A. Yang<sup>1</sup>, N. Unterwiesing<sup>1</sup>, M. Guerreiro<sup>1</sup>, G. Grütz<sup>1</sup>, R. Schumann<sup>2</sup>, G. Schönrich<sup>3</sup>, H.-D. Volk<sup>1</sup>, C. Scheibenbogen<sup>1</sup><sup>1</sup>Charité – Universitätsmedizin Berlin Campus Mitte, Institute of Medical Immunology, Berlin, Germany, <sup>2</sup>Charité – Universitätsmedizin Berlin Campus Mitte, Institute of Microbiology and Hygiene, Berlin, Germany, <sup>3</sup>Charité – Universitätsmedizin Berlin Campus Mitte, Institute of Virology, Berlin, Germany**Objectives:** Herpes simplex virus-1 persists life-long after primary infection. However, only 30% of the infected individuals suffer from recurrent herpes labialis. Natural killer cells (NK) are major components in antiviral innate immunity and express a broad range of toll-like receptors (TLR). Recently, impaired NK cell response to TLR3 agonist poly(I:C) was reported in two patients with herpes simplex encephalitis. Therefore, we hypothesized that impaired TLR responses of the NK cells would be associated with the predisposition to recurrent herpes labialis.**Methods:** Specific IFN- $\gamma$  productions of NK cells in response to the stimulation of TLR2/1, 3, 4, 7 and 8 ligands Pam3CSK4, poly(I:C), LPS and R-848 were analyzed by intracellular cytokine cytometry. TLR-responses in NK cells of presumably HSV1-infected individuals (seropositivity of 90% in our community) with and without histories of recurrent herpes labialis were evaluated. Mann-Whitney U tests were performed to determine significant differences between the two groups.**Results:** IL-12 primed isolated NK cells showed TLR-specific IFN- $\gamma$ -production with considerable inter-individual variance; with a median of 21.5% of IFN- $\gamma$  producing CD56<sup>bright</sup> NK cells to Pam3CSK4, 1.7% to poly(I:C), 34.4% to LPS, and 39% to R-848. In comparison to asymptomatic healthy donors, individuals with recurrent herpes labialis demonstrated significantly lower responses to poly(I:C), LPS and R-848 in their CD56<sup>bright</sup> isolated NK cells ( $P=0.003$ ,  $P=0.013$ ,  $P=0.010$ ). Interestingly, in whole blood mononuclear cells the NK cell response was not different between patients and controls. Most subjects showed neither a general TLR hyporesponsiveness of NK cells to all ligands nor a TLR hyporesponsiveness in other cell types. Neither TLR expression level nor NK cell cytotoxic activity were associated with the lower TLR-induced IFN- $\gamma$  secretion in NK cells.**Conclusion:** People who suffer from recurrent herpes labialis have a reduced NK response to TLR stimulation. Our findings suggest that this hyporesponsiveness can be compensated by costimulatory signals from other immune cells and is multifactorial. Taken together, they provide a model to explain why only a subset of HSV-1 infected subjects suffer from herpes labialis and why recurrence could be triggered by factors disturbing the costimulatory function of innate immune cells.**PC05/2 STRUCTURE/FUNCTION ANALYSIS OF THE PYRIN PRYSPLY DOMAIN, A HOT-SPOT FOR MUTATIONS ASSOCIATED WITH FAMILIAL MEDITERRANEAN FEVER**P. Mittl<sup>1</sup>, C. Weinert<sup>1</sup>, M. Grütter<sup>1</sup><sup>1</sup>University Zürich, Zürich, Switzerland**Introduction:** The systemic auto-inflammatory disease Familial Mediterranean Fever (FMF) is characterized by recurrent episodes of inflammation and fever without signs of bacterial infection or antigen-specific T-cells. Patients suffering from this disease carry mutations in the gene that codes for pyrin (Marenostrin, TRIM20). More than 20 FMF-associated mutations cluster in the C-terminal Prysply domain of pyrin.**Objectives:** To decipher the structural consequences of FMF-associated mutations the three-dimensional structure of the pyrin Prysply domain was determined by X-ray crystallography.**Results:** The crystal structure of the pyrin Prysply domain was refined at 1.35 Å resolution and revealed a shallow cavity, which seems to be involved in binding the pyrin binding partner. FMF-associated mutations cluster around this ligand binding site and around the N-terminus of the Prysply domain. The distribution of FMF-associated mutations suggests that either

(i) the Prysply ligand must be very large to contact both hot-spots simultaneously,

(ii) Prysply binds more than one ligand, or

(iii) the relative spatial orientation of the Prysply and BBCC domains are crucial for the biological function of pyrin.

**Conclusion:** The comparison with other Prysply domain structures suggests, that pyrin recognizes its ligand through hydrophobic interactions and surface complementarities. The structure serves as a basis for an accurate modelling of FMF-associated mutations.**PC05/3 NEW TOOLS FOR A RAPID DIAGNOSIS OF IMMUNODEFICIENCIES WITH CYTOLYTIC DEFECTS AND SEARCH FOR NEW GENE RELATED DISEASE**S. Marcenaro<sup>1</sup>, S. Martini<sup>2</sup>, V. Cetica<sup>3</sup>, S. Grieve<sup>4</sup>, G.M. Griffiths<sup>4</sup>, M. Arico<sup>3</sup>, L. Moretta<sup>1,5,6</sup>, D. Pende<sup>2</sup><sup>1</sup>Università di Genova, DIMES, Genova, Italy, <sup>2</sup>Istituto Nazionale per la Ricerca sul Cancro, Genova, Italy, <sup>3</sup>Azienda Ospedaliero-Universitaria Meyer, Firenze, Italy, <sup>4</sup>Cambridge Institute for Medical Research, Cambridge, United Kingdom, <sup>5</sup>Istituto Giannina Gaslini, Genova, Italy, <sup>6</sup>Università di Genova, Centro di Eccellenza per la Ricerca Biomedica, Genova, Italy**Objectives:** Hemophagocytic lymphohistiocytosis (HLH) is a rare, heterogeneous fatal disease of early infancy. The primary form (FHL) has a genetic origin; disease-causing mutations encode for perforin (PRF1, FHL2), Munc13-4 (UNC13D, FHL3), and syntaxin-11 (STX11, FHL4). Our aim was to set appropriate assays to discriminate between the different genetic subtypes, applying our expertise on NK cells to underline the cytotoxic defects typical of these patients.



**Methods:** Intracytoplasmic staining of freshly derived NK cells with anti-perforin mAb allows to detect a possible lack of this protein, typical of FHL2 patients. We optimized the CD107a assay, which we first demonstrated to be a potent tool to detect defects in degranulation. Chromium-release assays were performed to reveal certain cytolytic defects. Production of cytokines was tested. Western blot could assess the protein expression of Munc13-4 and perforin in selected cases. Genetic analyses were performed.

**Results:** A defect of CD107a expression on the NK cell surface after co-culture with K562 target cells heralds a defect of Munc13-4 protein and direct analysis of UNC13D gene in search of mutations. Defective CD107a only in resting but not in activated NK cells can address to syntaxin-11 defect. A dysfunction of 2B4 receptor which exerts inhibitory instead of activating function, typical of XLP, can be assessed by an appropriate redirected killing assay using anti-2B4 mAb; this allows to direct analysis of SAP gene. Western blot analyses in selected cases can further demonstrate defects of Munc13-4, perforin and SAP protein expression. Cytokine production can be tested upon stimulation of triggering receptors or after co-culture with appropriate target cells. Excessive pro-inflammatory cytokine production is typical in these patients and play a role in the pathogenesis of the disease. Finally, a major effort is the identification of cases with a clear familial origin, characterized by a well defined cytotoxic defect without any association to a known mutated gene in order to possibly find novel gene(s) implicated in the disease.

**Conclusions:** We can address to a particular gene analysis searching for mutations. These researches will greatly impact in the diagnosis and care of patients with immunodeficiency with defects of cytolytic activity.

PC05/4

#### PROTECTIVE IMMUNITY TO SYSTEMIC INFECTION WITH SALMONELLA ENTERICA IN THE ABSENCE OF IL-12 IS ASSOCIATED WITH IL-23-DEPENDENT IL-22 BUT NOT IL-17

S.M. Schulz<sup>1</sup>, G. Koehler<sup>2</sup>, N. Schuetze<sup>1</sup>, J. Knauer<sup>1</sup>, R.K. Straubinger<sup>1</sup>, A.A. Chackerian<sup>3</sup>, E. Witte<sup>4</sup>, K. Wolk<sup>4</sup>, R. Sabat<sup>4</sup>, Y. Iwakura<sup>5</sup>, C. Holscher<sup>6</sup>, U. Mueller<sup>1</sup>, R.A. Kastelein<sup>7</sup>, G. Alber<sup>7</sup>

<sup>1</sup>University of Leipzig, Institute of Immunology, College of Veterinary Medicine, Leipzig, Germany, <sup>2</sup>University of Münster, Gerhard Domagk Institute of Pathology, Münster, Germany, <sup>3</sup>Schering-Plough Biopharma, Discovery Research, Palo Alto, United States, <sup>4</sup>University Hospital Charité, Interdisciplinary Group of Molecular Immunopathology, Dermatology/Medical Immunology, Berlin, Germany, <sup>5</sup>University of Tokyo, Tokyo, Japan, <sup>6</sup>Research Center Borstel, Borstel, Germany, <sup>7</sup>University of Leipzig, Institute of Immunology, College of Veterinary Medicine, Berlin, Germany

IL-12 is essential for protective T cell-mediated immunity against Salmonella infection. To characterize the role of the IL-12-related cytokine IL-23, wild-type C57BL/6 (WT) and p19<sup>-/-</sup> mice were infected systemically with an attenuated strain of Salmonella enterica serovar Enteritidis (S. Enteritidis). In the absence of IL-23, infected animals showed strongly reduced IL-17A and IL-22 but similar IFN- $\gamma$  production, and had a compromised delayed-type hypersensitivity reactivity compared to WT mice. Nevertheless, IL-23-deficient mice controlled S. Enteritidis infection similarly to WT mice. Hence, although IL-23 was required for T-cell effector responses, it was not essential for the protection against systemic S. Enteritidis infection when IL-12 was present. To analyze the role of IL-23 in the absence of IL-12, low doses of S. Enteritidis were administered to p35<sup>-/-</sup> mice (lacking IL-12), p35/19<sup>-/-</sup> mice (lacking IL-12 and IL-23), p35/40<sup>-/-</sup> mice (lacking IL-12, IL-23, and homodimeric p40), and p35/IL-17A<sup>-/-</sup> mice (lacking IL-12 and IL-17A). We found survival of p35<sup>-/-</sup> and p35/IL-17A<sup>-/-</sup> mice, whereas p35/19<sup>-/-</sup> and p35/p40<sup>-/-</sup> mice died within 3–6 weeks and developed liver necrosis. This indicates that IL-23 but not homodimeric IL-12p40 is required for protection which, surprisingly, is independent of IL-17A. Moreover, protection was associated with IL-22 but not IL-17F or IL-21 expression or with neutrophil recruitment. Finally, anti-IL-22 treatment of S. Enteritidis-infected p35<sup>-/-</sup> mice resulted in liver damage. In conclusion, simultaneous abrogation of IL-12 and IL-23 causes mice to become hypersensitive to systemic infections with S. Enteritidis and IL-23-dependent IL-22 but not IL-17 production is associated with protection against systemic infection with S. Enteritidis in the absence of IL-12.

PC05/5

#### NOVEL MUTATIONS IN STAT3 ASSOCIATED WITH HYPER IGE SYNDROME

C. Woellner<sup>1</sup>, E.M. Gertz<sup>2</sup>, A.A. Schäffer<sup>2</sup>, M. Lagos<sup>3</sup>, M. Perro<sup>1</sup>, M.C. Pietrogrande<sup>4</sup>, F. Cosu<sup>5</sup>, J.L. Franco<sup>6</sup>, N. Matamoros<sup>7</sup>, B. Pietrucha<sup>8</sup>, E. Heropolitańska-Pliszka<sup>8</sup>, M. Yeganeh<sup>9</sup>, M. Moir<sup>9</sup>, T. Español<sup>10</sup>, S. Ehl<sup>11</sup>, A.R. Gennery<sup>12</sup>, M. Abinun<sup>12</sup>, A. Breborowicz<sup>13</sup>, T. Niehues<sup>14</sup>, S.S. Kilic<sup>15</sup>, A. Junker<sup>16</sup>, S.E. Turvey<sup>16</sup>, A. Plebani<sup>17</sup>, B. Sánchez<sup>18</sup>, B.-Z. Garty<sup>19</sup>, C. Pignata<sup>20</sup>, C. Cancrini<sup>21</sup>, J. Litzman<sup>22</sup>, Ö. Sanal<sup>23</sup>, U. Baumann<sup>24</sup>, R. Bacchetta<sup>25</sup>, A.P. Hsu<sup>26</sup>, J.N. Davis<sup>26</sup>, L. Hammarström<sup>27</sup>, E.G. Davies<sup>28</sup>, E. Eren<sup>29</sup>, P.D. Arkwright<sup>30</sup>, J.S. Moilanen<sup>31</sup>, D. Viemann<sup>32</sup>, S. Khan<sup>33</sup>, L. Maródi<sup>34</sup>, A.J. Cant<sup>12</sup>, A.F. Freeman<sup>35</sup>, J.M. Puck<sup>36</sup>, S.M. Holland<sup>36</sup>, B. Grimbacher<sup>37</sup>

<sup>1</sup>University College London, Royal Free Campus, Department of Immunology and Molecular Pathology, London, United Kingdom, <sup>2</sup>National Institutes of Health, National Center for Biotechnology Information, Bethesda, United States, <sup>3</sup>Universidad de Valparaíso, Cátedra de Inmunología Escuela de Medicina, Valparaíso, Chile, <sup>4</sup>University of Milan, Department of Pediatrics, Milan, Italy, <sup>5</sup>Ospedale Microcitico, Bone Marrow Transplant Unit, Cagliari, Italy, <sup>6</sup>University of Antioquia, Group of Primary Immunodeficiencies, Medellín, Colombia, <sup>7</sup>Son Dureta Hospital, Immunology Service, Palma de Mallorca, Spain, <sup>8</sup>Children's Memorial Health Institute, Gastroenterology, Hepatology and Immunology Clinic, Warsaw, Poland, <sup>9</sup>Tehran University of Medical Sciences, Immunology, Asthma and Allergy Research Institute, Children Medical Centre, Tehran, Iran, Islamic Republic of, <sup>10</sup>Hospital Vall d'Hebron, School of Medicine, Immunology Unit, Barcelona, Spain, <sup>11</sup>University Hospital Freiburg, Department of Pediatrics and Adolescent Medicine, Freiburg, Germany, <sup>12</sup>University of Newcastle upon Tyne, Children's Bone Marrow Transplant Unit, Newcastle upon Tyne, United Kingdom, <sup>13</sup>Poznan University of Medical Sciences, Department of Pediatric Pulmonology, Allergy and Clinical Immunology, Poznan, Poland, <sup>14</sup>HELIOS Klinikum Krefeld, Heinrich Heine University of Düsseldorf, Immunodeficiency and Pediatric Rheumatology Centre, Düsseldorf, Germany, <sup>15</sup>Uludağ University, Faculty of Medicine, Department of Pediatric Immunology, Bursa, Turkey, <sup>16</sup>British Columbia's Children's Hospital and University of British Columbia, Department of Pediatrics, Vancouver, Canada, <sup>17</sup>University of Brescia, Department of Pediatrics and Institute of Molecular Medicine A. Novicelli, Brescia, Italy, <sup>18</sup>University Hospital, Virgen del Rocio, Immunology Service, Sevilla, Spain, <sup>19</sup>Schneider Children's Medical Center, Department of Pediatrics, Petah Tiqva, Israel, <sup>20</sup>University of Naples, Federico II, Department of Pediatrics, Naples, Italy, <sup>21</sup>Bambino Gesù Children's Hospital, University of Rome Tor Vergata, Division of Immunology and Infectious Disease, Rome, Italy, <sup>22</sup>Masaryk University, St. Anne's University Hospital, Department of Clinical Immunology and Allergology, Brno, Czech Republic, <sup>23</sup>Hacettepe University Children's Hospital, Immunology Division, Ankara, Turkey, <sup>24</sup>Medical School Hannover, Department of Pediatric Pulmonology and Neonatology, Hannover, Germany, <sup>25</sup>Raffaello Telethon Institute for Gene Therapy (HSR-TIGET), Milan, Italy, <sup>26</sup>National Institutes of Health, National Institute of Allergy and Infectious Diseases, Bethesda, United States, <sup>27</sup>Karolinska Institute at the Karolinska University Hospital, Department of Laboratory Medicine, Stockholm, Sweden, <sup>28</sup>Great Ormond Street Hospital, Department of Immunology, London, United Kingdom, <sup>29</sup>Southampton General Hospital, Immunology Department, Southampton, United Kingdom, <sup>30</sup>University of Manchester, Paediatric Immunology, Manchester, United Kingdom, <sup>31</sup>University of Oulu, Department of Clinical Genetics, Oulu, Finland, <sup>32</sup>University of Münster, Institute of Immunology and Department of Pediatrics, Münster, Germany, <sup>33</sup>Scunthorpe General Hospital, Path Links Immunology, Scunthorpe, United Kingdom, <sup>34</sup>University of Debrecen, Medical and Health Science Centre, Department of Infectious and Pediatric Immunology, Debrecen, Hungary, <sup>35</sup>NCI-Frederick, SAIC-Frederick, Inc., Frederick, United States, <sup>36</sup>University of California, Department of Pediatrics, San Francisco, United States, <sup>37</sup>University College London, Royal Free Hospital, Department of Immunology and Molecular Pathology, London, United Kingdom

**Objectives:** Autosomal-dominant Hyper-IgE syndrome (AD-HIES) is a rare primary immunodeficiency characterized by recurrent infections of the skin, elevated serum IgE-levels, recurrent pneumonia, and involvement of the soft and bony tissues. Heterozygous mutations of the signal transducer and activator of transcription 3 (STAT3) have recently been found to account for the majority of patients with sporadic or autosomal-dominant HIES.

**Methods:** In a worldwide collaboration we collected DNA samples of 101 unrelated sporadic patients with the clinical diagnosis of AD-HIES and sequenced both the coding exons and splice sites of STAT3.

**Results:** In 64 patients we identified 31 different STAT3 mutations, 18 of which were previously unreported. These included mutations both at splice sites and outside the previously implicated DNA-binding- and SH2-domains. Interestingly, 37 out of the 101 patients did not show any mutation in the coding regions or splice sites in STAT3.

Of the 18 yet unreported mutations, seven affected the DNA-binding domain, six the SH2 domain, four the transactivation domain and one the coiled-coil domain. 14 of the new mutations found were missense mutations, one was a duplication of nine amino acids in exon 10. One patient revealed a heterozygous mutation at the 3' splice site prior to exon 12, one patient had a mutation at the 5' splice site following exon 12. These mutations result in exon 12 skipping, suggesting an in-frame deletion of 10 amino acids in the DNA-binding domain.

Another patient harboured a mutation after exon 22 that caused the exon to be skipped, whereas one patient carried a missense mutation in the coiled-coil domain (H58Y). Since none of 100 healthy controls carried any of the yet unreported mutations functional studies investigating the STAT3 pathway are required and ongoing.

**Conclusions:** Although the majority of new mutations we found in our cohort affected the DNA-binding and the SH2 domain, STAT3 mutations may also be detected in other domains of the protein.

PC05/6

#### DIET-INDUCED OBESE MICE HAVE INCREASED MORTALITY AND IMPAIRED IMMUNE RESPONSE TO STAPHYLOCOCCUS AUREUS-INDUCED SEPSIS

L. Strandberg<sup>1</sup>, M. Verdrengh<sup>2</sup>, M. Enge<sup>1</sup>, N. Andersson<sup>1</sup>, S. Amu<sup>2</sup>, K. Önnheim<sup>2</sup>, A. Benrick<sup>1</sup>, M. Brissler<sup>2</sup>, J. Bylund<sup>2</sup>, M. Bokarewa<sup>2</sup>, S. Nilsson<sup>3</sup>, J.-O. Jansson<sup>1</sup>

<sup>1</sup>University of Göteborg, Gothenburg, Sweden, <sup>2</sup>University of Göteborg and Sahlgrenska University Hospital, Gothenburg, Sweden, <sup>3</sup>Chalmers University of Technology, Gothenburg, Sweden

It is well established that obesity is followed by macrophage infiltration into fat tissue and a chronic low-grade inflammation. The fat tissue of obese individuals produces both inflammatory and anti-inflammatory cytokines. Much less is known about how these metabolic changes affect the ability of the immune system to perform its main task, to defend against infections. The aim of the present study was to investigate if the life threatening *Staphylococcus aureus* (*S. aureus*) sepsis is affected by diet-induced obesity (DIO).

We found that obese C57BL/6 mice with DIO after being fed high fat diet for 8 weeks before bacterial inoculation had markedly increased mortality following intravenous challenge with *S. aureus*. Mice with DIO had decreased proportion of phagocytosing granulocytes, as well as decreased capacity of granulocytes to ingest staphylococci before inoculation. The proportion of phagocytosing monocytes before inoculation and the capacity of phagocytes to produce reactive oxygen species

after inoculation were decreased in DIO mice. The number of bacteria in the kidneys 5–7 days after the inoculation was more than 10-fold higher in DIO mice. These mice also had highly increased mRNA expression of the anti-inflammatory cytokines interleukin-10 (IL-10) and IL-1 receptor antagonist (IL-1Ra) day 5–7 after inoculation in gonadal adipose tissue, but not in liver or spleen. At this time point, the DIO mice also had increased serum levels of IL-10 and IL-1Ra. Our findings imply that long-term exposure to western diet, associated with obesity, suppresses several immune functions in mice, and impairs their ability to combat *S. aureus* and thereby survive sepsis.

This work was supported by EC FP6 funding (contract no. LSHM-CT-2003-503041), the Swedish Strategic Foundation, and the Swedish Research Council (No K2007-54X-09894-16-3).

PC05/7

#### CLINICAL AND GENETIC ANALYSIS OF POLISH PATIENTS WITH AUTOSOMAL DOMINANT HYPER-IGE SYNDROME

E. Heropolitańska-Pliszka<sup>1</sup>, B. Pietrucha<sup>1</sup>, C. Woellner<sup>2</sup>, B. Grimbacher<sup>2</sup>, E. Bernatowska<sup>1</sup>

<sup>1</sup>Children's Memorial Health Institute, Immunology, Warsaw, Poland, <sup>2</sup>Royal Free Hospital and University College London, London, United Kingdom

Autosomal dominant hyper-IgE syndrome (AD-HIES) is a distinct entity of primary immunodeficiency (PID) characterized by recurrent skin abscesses and pneumonia with pneumatocele formation, elevated levels of serum IgE >2000 IU/ml. Recently heterozygous dominant negative mutations of STAT3 gene have been described in patients with sporadic and AD-HIES.

In PIDs registry of Department of Immunology there are 38 patients with suspicion of HIES. Among them 28 patients fulfil HIES criteria of clinical scoring system (scoring >40 points) presenting with multiple infections, eczema, IgE concentration 3370–55600 IU/ml and persistent eosinophilia >800 cells. Among 15 males and 13 females at the age of 1–32 yr, two deceased at the age of 17 and 15 due to liver abscesses and oral abscess with sepsis respectively. Recurrent pneumonia occurred in 18 children and pneumatoceles in 10 of them, one patient was suffering from lung aspergillosis, one from lung actinomycosis. 17 patients had recurrent skin abscesses, 13 suffered from neonatal staphylococcal sepsis. 16 children presented neonatal rash, 7 patients had pathological bone fractures and 11 presented with abnormal development of permanent teeth, 15 patients have characteristic coarse face. No malignancy was reported. 22 patients underwent genetic analysis, mutations in STAT3 have already been confirmed in 7 patients, including 5 hot-spot sites and 2 novel mutations.

The knowledge of the diverse features of HIES facilitates provisional diagnosis of AD HIES. Delayed diagnosis leads to serious health problems. Opportunity of performing genetic analysis in these cases may lead to definitive recognition of the disease and earlier institution of appropriate treatment.

PC05/8

#### TWO NEW MUTATIONS OF THE SIXTH COMPLEMENT COMPONENT IN TUNISIAN PATIENTS

H. Jlaïli<sup>1</sup>, J. Blouin<sup>2</sup>, M. Kallel-Sellami<sup>1</sup>, R. Abdelmalek<sup>3</sup>, L. Laadhar<sup>1</sup>, Y. Zerzeri<sup>1</sup>, T. Ben Chaabene<sup>3</sup>, V. Fremaux-Bacchi<sup>2</sup>, S. Makni<sup>1</sup>

<sup>1</sup>La Rabta Hospital, Immunology, Tunis, Tunisia, <sup>2</sup>Georges Pompidou European Hospital, Immunology, Paris, France, <sup>3</sup>La Rabta Hospital, Infectious diseases, Tunis, Tunisia

**Introduction:** C6 is one of the five complement terminal pathway components (C5–C9) which interact to form a membrane-attack complex that lead to bacterial lysis. Genetic deficiency of any of these components leads to increased susceptibility to *Neisseria*. Meningitis infections. Hereditary deficiency of C6 (C6D) is transmitted according to an autosomal recessive mode. Three "hot spot" mutation sites have been located at exon 6, 7 and 12.

**Aim:** to investigate C6 mutations in four Tunisian patients.

**Patients and Methods:** The patients were three males who presented *meningococcal* meningitis and one female with a history of chronic lupus. Complement pathways investigation revealed a decrease of the functional haemolytic assays CH50 and AP50. C6D was confirmed by a double ligand ELISA. Genomic DNA was amplified by polymerase chain reaction (PCR) using intronic primers flanking the 17 coding exons of C6. Sequencing of the PCR products was carried out by the dye terminator sequencing method.

**Results:** Three point mutations were characterised:

- One patient has an homozygous single-base deletion 1195DelC in exon 7
- Two patients have a G>A substitution at the splice site in 5' of exon9 (IVS 9-1)
- One patient has a G>A substitution at the splice site in 3' of exon11 (IVS11+1).

**Conclusion:** We report two new mutations: IVS 9-1 G>A and IVS 11+1 G>A.

These results need to be completed by a familial investigation as well as the assessment of these new mutations' prevalence in Tunisian healthy subjects. C6 RNA analysis is also needed to investigate the consequence of splice site mutations on C6 transcription.

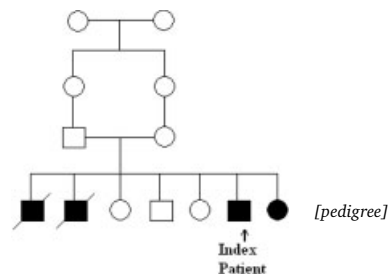
PC05/9

#### AR-HIES PATIENT WHO PRESENTED WITH PML DUE TO JC VIRUS INFECTION

A. Metin<sup>1</sup>, M. Y. Köker<sup>1</sup>, A. Degerliurt<sup>2</sup>, H. Tezer<sup>2</sup>, A. Alp<sup>3</sup>, B. Tunc<sup>2</sup>

<sup>1</sup>Diskapi Children Hospital, Immunology, Ankara, Turkey, <sup>2</sup>Diskapi Children Hospital, Ankara, Turkey, <sup>3</sup>Hacettepe University, Faculty of Medicine, Microbiology, Ankara, Turkey

Hyper-IgE syndrome (HIES) is a rare primary immunodeficiency disease, characterized by the classical triad of chronic pruritic dermatitis of early onset, severe infections of bacterial, viral, mycobacterial and fungal origin and elevated levels of serum IgE. HIES had been shown to be associated with STAT-3 mutations in autosomal dominant form, (Type1) and with TYK-2 mutations in autosomal recessive form (Type2) of the disease. Type 2 HIES constitutes a small fraction of HIES first reported in Turkish consanguineous families. Notably these patients suffer from severe viral infections such as chronic refractory molluscum contagiosum, herpes simplex virus infections and fatal neurological complications. Here we present another six-year old boy with Type2 HIES from a consanguineous Turkish family. Patient history showed the presence of persistent history of chronic eczematous skin lesions since infancy and two episodes of hospitalizations due to pneumonia. Patient had pustular neonatal rash history. There is no pneumatoceles despite recurrent pulmonary infections; no joint hypermobility, no cold abscesses. There is coarse facies, deep set eyes and thickened skin, severe food allergies. Additionally, Immunologic work up revealed markedly raised IgE level and eosinophilia. The patient was diagnosed as hyper IgE syndrome based on his clinical and laboratory findings. The patient presented with severe neurological disease: restlessness, spasticity, hyperactive deep tendon reflexes, Babinsky and clonus. Speech was lost since 15 days. Neurological symptoms and gradual deterioration started six months ago with mild ataxia. Radiological diagnosis is progressive multifocal leucoencephalopathy (PML) with serial MRI. JC virus infection is proved by serum and cerebrospinal fluid JC PCR test. This patient is the 6. child of a family. There was a sibling deaths with chronic dermatitis and infection. 7. child of the family also diagnosed as HIES. We treated the patient with IVIG 500 mg/kg/day for 15 days with no clinical response. As the response to IFN-gamma stimulation is shown in Tyk-2 deficiency we started to give IFN-gamma to this patient with PML using 50µg/m<sup>2</sup>, 3x/week, sc; and to his sister with HIES for viral prophylaxis and also with the aim of modulating the immune response by influencing maturation towards TH1 lymphocytes.



PC05/10

#### DISTURBANCES IN THE HOMEOSTASIS OF TH17 LYMPHOCYTES IN PATIENTS WITH HYPER IGE SYNDROME AND CHRONIC GRANULOMATOUS DISEASE

R. Horvath<sup>1</sup>, A. Polouckova<sup>1</sup>, J. Lastovicka<sup>1</sup>, J. Bartunkova<sup>1</sup>, A. Sediva<sup>1</sup>, R. Spisek<sup>1</sup>

<sup>1</sup>2nd Medical School of Charles University and University Hospital Motol, Immunology, Prague, Czech Republic

Hyper IgE syndrom (HIES) is a primary immunodeficiency recently associated with mutations in STAT-3 gene resulting in an impaired development of Th17 lymphocytes. Hyper IgE patients with reduced frequency of Th17 cells typically present with *Staphylococcus aureus* and/or *Candida* strains infections. Notably, the same spectrum of dominant pathogens is present in patients with chronic granulomatous disease. We therefore analyzed and compared the characteristics of Th17 compartment in both HIES and CGD. Frequency of peripheral blood Th17 lymphocytes and quantity of Th17-derived cytokines (IL-21, and IL-17) were analyzed in 4 patients from 3 families with hyper IgE syndrome and 7 patients with CGD. Two CGD patients underwent allogeneic bone marrow transplantation (BMT) and both pre- and post-transplant samples were analyzed. In all patients primary immunodeficiency was confirmed by genetic analysis. In agreement with previous reports, patients with HIES had very low numbers of Th17 cells (0,178 ± 0.15% of CD4+ T cells). In contrast, Th17 frequency was significantly higher in CGD patients (1,796 ± 0.71% of CD4+ T cells) when compared to both control samples (0,667 ± 0.32%, p=0,004) and HIES patients (p=0,025). Numbers of Th17 cells in CGD patients normalized after successful BMT and declined to the range of healthy controls. Despite the striking overlap in the susceptibility to a narrow spectrum of infections, most commonly *Staphylococcus aureus* and *Candida albicans*, HIES and CGD patients have distinct disturbances in the homeostasis of Th17

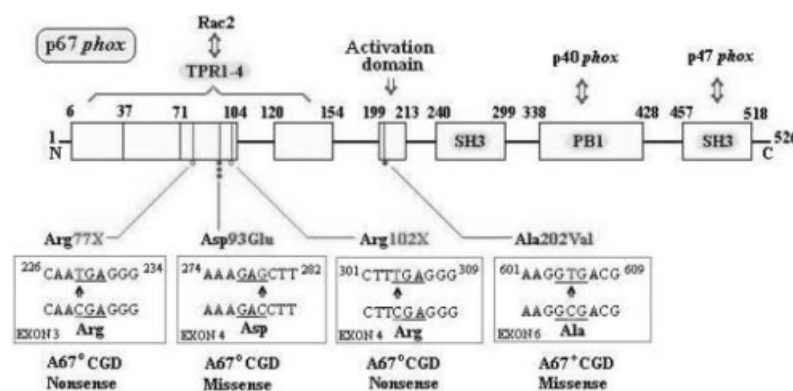
lymphocytes. Development of Th17 lymphocytes in STAT-3 deficient HIES patients is impaired while in CGD the frequency of Th17 cells is higher than in healthy controls. Increase of Th17 cells in CGD is likely to be secondary as a result of defect in neutrophils.

#### PC05/11 FOUR DIFFERENT NCF2 MUTATIONS IN FOUR FAMILIES RESULTING IN AUTOSOMAL RECESSIVE CHRONIC GRANULOMATOUS DISEASE

M. Y. Köker<sup>1,2</sup>, Ö. Sanal<sup>1</sup>, A. Metin<sup>2</sup>, T. Patisroglu<sup>3</sup>, D. Roos<sup>4</sup>

<sup>1</sup>Hacettepe University, Children's Hospital, Immunology, Ankara, Turkey, <sup>2</sup>Diskapi Children Hospital, Immunology, Ankara, Turkey, <sup>3</sup>University of Erciyes, Pediatric Hematology, Ankara, Turkey, <sup>4</sup>Sanquin Research and Landsteiner Laboratory, Academic Medical Center Amsterdam, Amsterdam, Netherlands

One of the rarest forms of autosomal recessive chronic granulomatous disease (AR-CGD) is attributable to mutations in the NCF2 gene, which encodes the polypeptide p67<sup>phox</sup>, a key cytoplasmic protein in the phagocyte NADPH oxidase system. NCF2 is localized on chromosome 1q25, encompasses 40 kb, and contains 16 exons. We report here the clinical and molecular characterization of four AR-CGD patients from four consanguineous Turkish families. The ages of the three female patients were between 6–20 years and a male patient was 6 years old; all patients showed clear clinical symptoms of CGD. Mutation analysis of NCF2 revealed four different homozygous mutations: a novel nonsense mutation in exon 3 c.229C>T, p.Arg77X; a novel missense mutation in exon 4 c.279C>G, p.Asp93Glu; a nonsense mutation in exon 4 c.304C>T, p.Arg102X; and a novel missense mutation in exon 6 c.605C>T, p.Ala202Val. All mutations were present in the homozygous form, and the parents were found to be heterozygotes for these mutations. Only in case of p.Ala202Val was p67<sup>phox</sup> protein expression still partly intact and the NADPH oxidase activity only partially destroyed. We are still investigating the functional capacity of the 202-Val p67<sup>phox</sup> protein.



[Location of point mutations leading to A67 CGD in ]

#### PC05/12 A NEW MUTATION IN CHRONIC GRANULOMATOUS DISEASE

M. J. Bernal<sup>1</sup>, M. T. Martínez de Saavedra<sup>1</sup>, J. A. Brieva<sup>1</sup>, A. Ferreira<sup>2</sup>, A. Sampalo<sup>1</sup>

<sup>1</sup>Hospital Puerta del Mar, Immunology, Cadiz, Spain, <sup>2</sup>Hospital La Paz, Immunology, Madrid, Spain

Chronic Granulomatous Disease (CGD) is a primary immunodeficiency syndrome characterized by impairment of intracellular microbicidal activity of phagocytes with increased susceptibility to severe recurrent bacterial and fungal infections. CGD results from mutations in one of the four NADPH-oxidase components that preclude generation of superoxide and related antimicrobial oxidants. Mutations in CYBB gene encoding gp91-phox are responsible of the most severe and prevalent form of CGD (X-linked). 372 different mutations in CYBB gene causing X-linked CGD have been reported (HGMD).

Here we describe a new mutation in a boy who was diagnosed of severe form of CGD at two years old. The defect consists in a deletion of cytosine in the exon 7 of the CYBB gene, that produces a frameshift from Alanine in 233 position and induces a premature stop codon (g. 19908delC, c695delc, p.Ala233fsX9).

The mutation was detected by sequencing the 13 exons of the CYBB gene and the adjacent intronic regions in the patient and his mother. Mother and grandmother behave as symptomatic carriers with cutaneous and pulmonary disease, respectively. In the DHR-123 flow cytometry functional assay, a low percentage of normal oxidative-positive neutrophils were detected: 22% in the mother and 7% in the grandmother.

This is the first description of this mutation in a family with gp-91 phox deficiency which results in a severe clinical phenotype.

#### PC05/13 TWO NOVEL P67PHOX MUTATIONS IN CHRONIC GRANULOMATOUS DISEASE SPANISH PATIENTS

L. Martínez-Martínez<sup>1</sup>, C. González-Santesteban<sup>1</sup>, M. Piquer<sup>2</sup>, C. Benaiges-Martínez<sup>1</sup>, O. de la Calle-Martín<sup>1</sup>

<sup>1</sup>Hospital Sant Pau, Immunology, Barcelona, Spain, <sup>2</sup>Hospital Sant Joan de Deu, Barcelona, Spain

**Aims:** Chronic granulomatous disease (CGD) is a primary immunodeficiency caused by defects in NADPH oxidase complex. This enzyme catalyzes the respiratory burst in the phagocytes and consists of five subunits: gp91phox, p67phox, p47phox, p40phox and p22phox. Alterations in p67phox, p47phox and p22phox have been associated with recessive autosomal heritance.

**Methods:** We analyzed several CGD-patients who have suffered severe recurrent infections since birth. The respiratory burst was measured by H<sub>2</sub>O<sub>2</sub> production using dihydrorhodamine-1,2,3. mRNA was obtained from peripheral blood cells and RT-PCR assays were performed. Mutations were confirmed by DNA analysis of the probands and relatives.

**Results:** All patients presented with absent respiratory burst activity. The absence of alterations in gp91phox, p22phox and p47phox prompted us to the analysis of p67phox. mRNA analysis of NCF2 gene (coding p67phox) in patients 1 and 2 (male and female siblings) showed an homozygous substitution (c.C229T) that lead to a premature stop (p.Arg77Stop). This alteration had not been previously described. Both parents were carriers of the mutation. p67phox mRNA obtained from patient 3 (6 month-old girl) did not showed the expected size: there were larger and smaller mRNA species. Her parents were studied. Her mother presented the wild-type and larger mRNA species. The father had the wild-type and two shorter mRNAs, lacking exon 5 and exons 5 and 6, respectively. NCF2 gene was further analysed. The maternal allele showed a genomic duplication, that includes exons 9 and 10. This alteration had been previously described by Borgato *et al.* The paternal allele showed a premature stop codon in exon 5 (p.Lys166Stop). Any alteration in intronic splicing motifs justifies the altered spliced mRNAs.

**Conclusions:** We described two novel mutations of NCF2 gene (p67phox) in 3 CGD-Spanish patients. Two siblings presented the p.Arg77Stop mutation. The third patient was showed to be compound-heterozygous for null NCF2 defects: a previously described genomic duplication of exons 9 and 10, that prevent the synthesis of the p67phox protein, and a premature stop codon (p.Lys166Stop) that originates a truncated p67phox protein. The presence of this abnormal protein is allowed by alternative mRNA splicing lacking the stop-containing exon 5.

#### PC05/14 HAEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS- A SINGLE CENTRE EXPERIENCE

M. Klaudel-Dreszler<sup>1</sup>, O. Rutynowska-Pronicka<sup>2</sup>, P. Socha<sup>1</sup>, E. Kowalewska-Majewska<sup>2</sup>, A. Bakula<sup>1</sup>, D. Perek<sup>2</sup>, J. Socha<sup>1</sup>, B. Piątoś<sup>3</sup>, E. Bernatowska<sup>1</sup>

<sup>1</sup>Children's Memorial Health Institute, Department of Gastroenterology, Hepatology and Immunology, Warsaw, Poland, <sup>2</sup>Children's Memorial Health Institute, Department of Oncology, Warsaw, Poland, <sup>3</sup>Children's Memorial Health Institute, Warsaw, Poland

**Introduction:** Haemophagocytic lymphohistiocytosis (HLH) - a life-threatening condition, caused by congenital or acquired defects in cellular cytotoxicity, which symptoms are: fever, hepatosplenomegaly, duo- or pancytopenia, coagulopathy, hyperferritinemia.

**Material:** We present 8 children (4 girls, 4 boys) suspected of primary immunodeficiency or acute liver failure.

**Results:** They manifested prolonged fever, jaundice, hepatosplenomegaly, accompanied by anaemia, thrombocytopenia, hypofibrinogenemia, hypertransaminasemia. Neurological symptoms varied from irritability to brain oedema. Hyperferritinemia and increased erythrophagocytosis in bone marrow were found in 7 patients. Cytotoxic activity of NK cells, evaluated in 6 children, was low in 5. Perforin expression in NK cells was normal in 6 tested patients. We established the diagnosis of Griscelli syndrome type II, macrophage activation syndrome in the course of LED complicating IgA-deficiency, HLH secondary to HCMV-infection in two boys, familial haemophagocytic lymphohistiocytosis type 3 or 4 in 3 girls, XLP in 8-mo-boy. Four children received treatment according to HLH-2004 protocol (dexamethasone, Cyclosporin A, etoposide), 3 only dexamethasone and methotrexate, 2 boys were given Ganciclovir to treat co-existing CMV-infection. A girl cured only with steroids died; 3 girls died because of HLH-reactivation; 8-mo-boy died after MUD-HSCT. Boys with CMV-associated HLH and a teenager with MAS are free of symptoms.

We confirmed FHL due to MUNC-13 mutation in one girl. The genetic evaluation towards FHL type 3 or 4 is ongoing in 2 girls.



**Conclusions:**

1. The diagnosis of HLH should be considered in patients with fever, acute liver insufficiency and pancytopenia of unknown origin.
2. Perforin expression and cytotoxic activity of NK cells are useful tests in diagnostics of HLH.

**PC05/15 CLINICAL PRESENTATION OF SIX HIES PATIENTS FROM TURKEY**A. Metin<sup>1</sup>, M.Y. Köker<sup>1</sup><sup>1</sup>Diskapi Children Hospital, Immunology, Ankara, Turkey

Hyper-IgE syndrome (HIES) is a rare primary immunodeficiency disease, characterized by the classical triad of chronic pruritic dermatitis of early onset, severe infections of bacterial, viral, mycobacterial and fungal origin and elevated levels of serum IgE. HIES had been shown to be associated with STAT-3 mutations in autosomal dominant form, (Type1) and with TYK-2 mutations in autosomal recessive form (Type2) of the disease. Type 2 HIES constitutes a small fraction of HIES first reported in Turkish consanguineous families. Notably these patients suffer from severe viral infections such as chronic refractory molluscum contagiosum, herpes simplex virus infections and fatal neurological complications which were not observed in Type1 HIES.

Here we present six HIES patients, three of them AR-HIES patients, from consanguineous Turkish families, and remaining three AD-HIES from non consanguineous families. The age of 3 male and 3 female patients were between 7-17 years of old. AD-HIES patients (type1) were presented with TBC brain abscesses, pneumonia with cold abscesses and non-Hodgkin (T cell) lymphoma, respectively. AR-HIES (type2) patients were presented with epidermodysplasia verruciformis, severe Herpes simplex infections and progressive multifocal leukoencephalopathy due to JC virus infection, respectively.

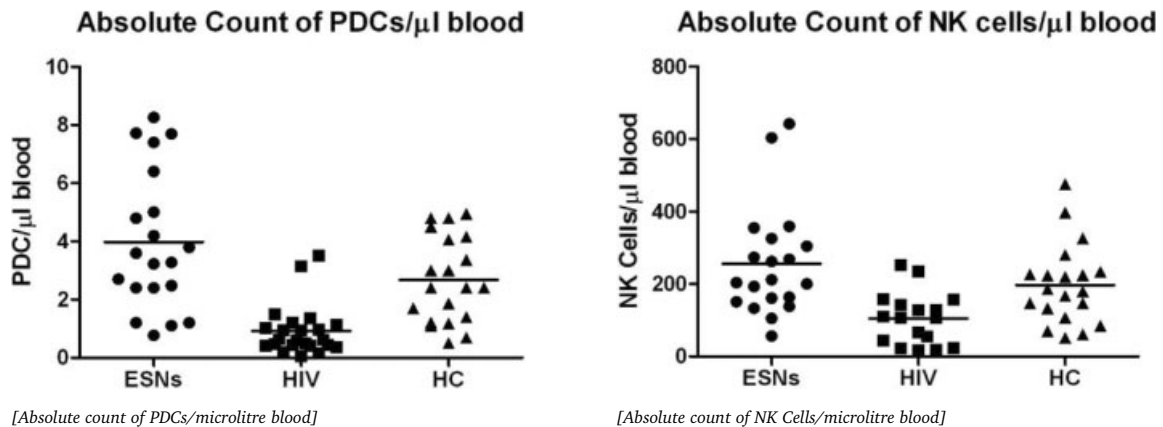
**PC05/16 ENHANCED INNATE IMMUNE RESPONSES HELP RESIST HIV TRANSMISSION IN SEXUALLY EXPOSED INDIVIDUALS**A. Singla<sup>1</sup>, A. Wanchu<sup>1</sup>, S. K. Arora<sup>1</sup><sup>1</sup>Post Graduate Institute of Medical Education and Research, Immunopathology, Chandigarh, India, <sup>2</sup>Post Graduate Institute of Medical Education and Research, Internal Medicine, Chandigarh, India

**Objectives:** The mechanisms involved in controlling the establishment of HIV-1 infection are not fully understood. Besides genetic factors, other host factors like immunological status especially the role of innate immune responses in conferring protection to individuals who are sexually exposed to HIV-1 but remain seronegative (ESN) has not been thoroughly evaluated. The aim of the present study was to investigate quantitative and functional status of NK cells and PDC in ESN compared to HIV-1 infected (HI) adults and healthy controls (HC).

**Methodology:** Twenty ESN were identified from HIV-1 serodiscordant couples with a history of frequent unprotected sexual intercourse. An equal number of age and sex matched HI and HC individuals were also recruited. All ESN were confirmed HIV negative by ELISA and for proviral DNA. Absence of CCR5 D32 mutation was confirmed by PCR. Actual counts and percentages of NK, PDC and CD4+ T cells were evaluated by flow-cytometry. Intracellular expression of IFN- $\gamma$  and TNF- $\alpha$  in NK cells was assessed flow-cytometrically after stimulation with PMA/Ionomycin. IRF7 expression was assessed by real time PCR. Paired t test was applied for statistical analysis of the data using SPSS software. The level of significance was set at  $p < 0.05$ .

**Results:** ESNs demonstrated a higher frequency of NK cells (12.08%) and PDC (0.051%) in comparison to HIV-1 infected subjects. However, the absolute count of PDC (3.98 cells/ $\mu$ L) was significantly higher in ESN compared to HC (2.67 cells/ $\mu$ L) and HI (0.92 cells/ $\mu$ L) individuals. Importantly, the expression of IFN- $\gamma$  and TNF- $\alpha$  by NK cells was significantly higher (52.66% and 52.65%) in ESN when compared with HC (38.79% and 33.46%) and HI (37.68% and 30.57%).

**Conclusion:** Our results suggest that besides other multiple factors, these effector cells may be playing an important role in controlling the establishment of sexually transmitted HIV-1 infection.

**PC05/17 TOLL-LIKE RECEPTOR EXPRESSION AND SIGNAL TRANSDUCTION IN NEWBORN AND NEONATAL CHILDREN**A.M. Tolstopiatova<sup>1</sup>, G.A. Buslaeva<sup>1</sup>, M.V. Degtyareva<sup>1</sup>, N.V. Davidova<sup>2</sup>, S.I. Pavlova<sup>1,2</sup>, I.G. Kozlov<sup>1,2</sup><sup>1</sup>Russian State Medical University, Pediatrics, Moscow, Russian Federation, <sup>2</sup>Federal Research and Clinical Center for Pediatric Hematology, Oncology and Immunology, Moscow, Russian Federation

Infections occupy a leading position in the structure of the morbidity in newborns. An adoptive component of the immune system is characterized by significant immaturity in the neonatal period. Under such circumstances, the system of innate immunity and one of its most important components, namely, pattern recognition receptors, come out on top in anti-infectious protection in newborns.

**Objectives:** To study the role of innate immunity receptors (TLR-2, TLR-4, the mannose receptor – CD206) on peripheral blood mononuclear cells in newborns suffering from fungal, viral, bacterial infections.

**Methods:** The evaluation of the TLR (TLR-2 and -4) surface expression level on peripheral blood granulocytes and monocytes (1) immediately after blood isolation (intact cells) and (2) after 2-hour incubation of blood samples at 37°C in the presence of TLR-specific ligands (lipopolysaccharide and nonopsonized zymosan) is studied. At the same time, the level of surface expression CD62L (L-selectin) is assessed on intact cells and the cells incubated for 15 min with TLR-specific ligands. The shedding of CD62L from the surface of phagocytes presents one of the earliest events evidencing of an adequate signal transduction from TLR inside the cell after their binding with ligands. Thus, suggested protocol allows not only to assess the TLR expression level, but also their functional activity. Small volumes of blood samples (< 0.5 ml), unified technology of flow cytometry and timesaving provide a significant advantage of the protocol.

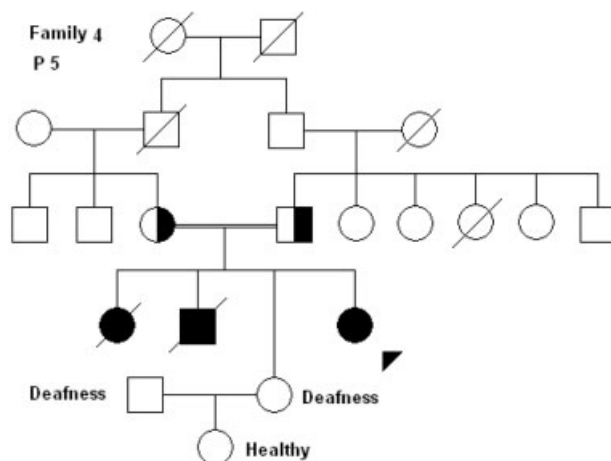
**Results:** The basal level of TLR-2, TLR-4, mannose receptor expression on monocytes and granulocytes in newborns suffering from infectious-inflammatory diseases versus healthy infants is significantly lower.

**Conclusion:** The results obtained will help to establish the criteria to identify the risk groups of newborns suffering from severe fungal, viral, bacterial infections, and to optimize therapy as well.

**PC05/18 A P22 DEFICIENT CGD PATIENT ASSOCIATED WITH HYPOGAMMAGLOBULINEMIA**M.Y. Köker<sup>1,2</sup>, Ç. Tan<sup>3</sup>, Tezcan<sup>3</sup>, T. Turul Özgür<sup>3</sup>, G. Türkkani Asal<sup>3</sup>, Ö. Sanal<sup>3</sup><sup>1</sup>Diskapi Children Hospital, Immunology, Ankara, Turkey, <sup>2</sup>Hacettepe University. Children's Hospital, Immunology, Ankara, Turkey, <sup>3</sup>Hacettepe University. Children's Hospital, Ankara, Turkey

Chronic granulomatous disease (CGD) is a rare inherited disorder characterized by severe susceptibility to bacterial and fungal infections. The disease is caused by a defect in any one of the four components of the NADPH oxidase, i.e. gp91phox, p22phox, p47phox or p67phox, encoded by the X-linked CYBB gene and the autosomal CYBA, NCF1 and NCF2 genes, respectively. We report here the clinical and molecular characterization of a patient with AR-CGD associated hypogammaglobulinemia. Mutation analysis of CYBA showed a missense mutation (previously described in heterozygous form by Rae et al) in exon 2 p.[gly24arg] in the homozygous form and the parents were found to be heterozygote for this mutations. The patient was a 18-year-old girl, whose parents are first cousins, was referred to a local hospital at 2 months of age with cervical lymphadenitis after BCG vaccination, and was treated with Isoniazid (INH) and rifampin for 3 months. Skin abscesses developed during follow-up which responded antibacterial therapy and CGD was diagnosed by NBT and DHR tests at age 2 years. She has been receiving prophylactic itraconazole and TMP-SMX treatment since the diagnosis and doing well. Her elder sister died of pyogenic infection and sepsis, and her elder brother died of *Aspergillus* pneumonia.

- During follow-up she was found to have **hypogammaglobulinemia** with low serum **IgG 115, IgM 19 and IgA 6.67 and IgE 5**. She has lack of anti pneumococcal antibody response and anti-HBs antibody response. In flowcytometric analysis **CD4/CD8 T cell ratio was 0.4 (1.1-1.4)** lower than expected and **CD19 + B cell percentage was with in below the normal range (10%)**. These findings were suggestive for common variable immunodeficiency. However, she did not experience unusual susceptibility to pyogenic infections probably due to prophylactic antibiotics trimethoprim-sulphamethoxazole and itraconazole she has been receiving.



[Family Pedigree]

## PC05/19 X-LINKED CGD IN A CARRIER MOTHER

C.-M. Farber<sup>1</sup>, P. Cochaux<sup>2</sup>

<sup>1</sup>Cliniques Universitaires de Bruxelles Hopital Erasme, Immunodeficiencies, Brussels, Belgium, <sup>2</sup>Cliniques Universitaires de Bruxelles, Genetics, Brussels, Belgium

**Objectives:** to care adequately for a female patient with X-linked CGD.

**Methods:** The mutation in the CGD gene was identified by molecular biology; the X-chromosome inactivation pattern was determined by an in-house assay, auto-antibodies were determined by indirect immunofluorescence (IFA) and radioimmunoprecipitation (RIPA).

**Results:** Mrs T, like her husband, is originary from a small village in Sicily. She developed CGD 2 years after her son was cured by an HLA identical, fraternal stem-cell transplant. Her X-chromosome inactivation pattern was skewed; not anew finding. She also had very high titers of anti DNA antibodies (1:2560) by Crithidia immunofluorescence. RIPA (Farr test) was negative; antibodies to *Leishmania donovani* (1:160), but not to the other *Schistosoma* species

**Conclusions:** We know that CGD can develop in carrier females with skewed X-chromosome inactivation. Mrs T also had clinical and laboratory findings compatible with cutaneous lupus. Other carriers have much lower levels of antinuclear antibodies. We think that this might be explained by a cross-reaction between Leishmania and Crithidia, which is just a stage in Leishmania's life cycle.

## PC05/20 THE STRATEGY OF THE INTRAVENOUS IMMUNOGLOBULIN(IGM) IN THE TREATMENT OF IMMUNODEFICIENCY'S(IMD)

I.K. Munir<sup>1</sup>, M. Ishaq<sup>1</sup>, S. Ishaq<sup>1</sup>

<sup>1</sup>Al-Junaid Hospital, Allergy/Pulmonary, Nowsehra, Pakistan

**Rationale:** Img have been the real hope of the researcher in the field of biotechnology in promoting defensive materials in the fight against the slow decay of the human generations from the curse of insufficient endogenous (Im)production. Thus safeguarding the human generations the reality of today's era.

**Methods:** Immunoglobulins, the endogenous proteins secreted by B lymphocyte cells. Igm are the important components of the humeral mediated immunity with their role in host defense include antigen binding and various effector functions. These include complement activation, complement binding, and binding to various Fc receptors. (IGIV) is made from pooled human plasma, about 90-100 % of which is composed of immunoglobulin G (IgG) for intravenous administration. Igm are indicated for substitutive therapy in patients with antibody deficiencies. Since with the earlier formulation of the Igm in the human history several errors & trials have been furthered to achieve the slow but the concise production of the Igm. While in some conditions, working relatively effectively elsewhere not with that efficacy as had been thought. The production of Igm had been for decades the test of the human knowledge & expertise in this direction. In this efforts the role of the volunteerism is also worth remembering/rewarding as several volunteers have devoted themselves in the trials for promoting a better future & survivals at the cost of their lives, for those human beings who previously use to succumbed to death prematurely. They now have a hope not only of their better survival & to reach a dream never thought decades before.

**Results:** The struggle as is on the move forwards still needs an everlasting momentum behind the idea as the goal still not achieved. And the goal as of effective, ideal & affordable Igm preparation that could be safe enough also.

**Conclusions:** The Igm of the present era are better than placebo in its role model ,but not dependable in the true spirit of ideal life saving biological preparations that could be quoted as a safeguard against several life threatening Imd.

## PC13 – INFLAMMATORY JOINT DISEASES

PC13/1 MICRO-ENVIRONMENTAL CONTROL OF IL-6 R EXPRESSION PREVENTS INFILTRATING T CELLS FROM BECOMING REFRACTORY TO CHRONIC IL-6 EXPOSURE

E. Hidalgo<sup>1</sup>, S.J. Flavell<sup>1</sup>, S.J. Curnow<sup>1</sup>, A.D. Filer<sup>1</sup>, M. Salmon<sup>1</sup>, C.D. Buckley<sup>1</sup>, K. Raza<sup>1</sup>, D. Scheel-Toellner<sup>1</sup>

<sup>1</sup>University of Birmingham, Immunity and Infection, Birmingham, United Kingdom

Chronic exposure to cytokines often leads to downregulation of receptor expression, the target cells become refractory. We are investigating the regulation of the response of T cells in the joint to IL-6. IL-6 is an important regulator of T cell differentiation and survival. IL-6 can exert its biological function in 2 different ways; by directly binding to the IL-6R or CD126 or via transsignalling, which happens when sIL6R-IL6 complexes bind to the IL-6R component CD130. We have been investigating the levels of expression and the regulation of the two chains of the IL-6 R by CD4+ T cells from blood, synovial fluid, and synovial tissue of RA patients. Furthermore, we studied the responsiveness of these cells to IL-6 and soluble sIL6R-IL6 complexes by studying their activation STAT3.

Compared to peripheral blood T cells, the expression of CD126 was very low on T cells in both the synovial tissue and synovial fluid. Intriguingly, the expression of CD130 on T cells from the synovial tissue was comparable to that of peripheral blood T cells, while T cells for the synovial fluid expressed very low levels. The combination of low CD126 and high CD130 expression by T cells in the synovial tissue suggest that these cells may be targets for transsignalling through sIL6R-IL6 complexes. Indeed, when exposed to sIL6R-IL6 complexes, these cells responded with a higher level of STAT3 phosphorylation compared to cells incubated with IL-6. Immunofluorescence studies of the distribution of IL-6 R expression on T cells throughout the inflamed synovial tissue showed that T cells in the perivascular cuff area, expressed higher levels of CD130 than T cells that had migrated further into the tissue. These data suggest that factors present in the perivascular cuff region of the inflamed synovium may maintain CD130 expression on infiltrating CD4+ T cells. Preliminary data suggests that IL10 present in the perivascular cuff may be responsible for the high expression of CD130 on T cells in the rheumatoid synovium. This observation may help to explain why T cells do not become refractory to chronic exposure to IL-6.

# PC13/2 EARLY ACTIVATION OF INKT CELLS IN A RHEUMATOID ARTHRITIS MODEL AND ITS APPLICATION TO DISEASE TREATMENT

J. Biton<sup>1</sup>, A. Miellot-Gafsou<sup>1</sup>, M.-C. Boissier<sup>1</sup>, E. Bourgeois<sup>2</sup>, A. Herbelin<sup>2</sup>, N. Bessis<sup>1</sup>

<sup>1</sup>University Paris 13, Faculty of Medicine, EA 4222, Rheumatology Dpt, Avicenne Hosp APHP, Bobigny Cedex, France, <sup>2</sup>CNRS UMR 8147, University Paris 5, Paris, France

Invariant (i) NKT cells are a unique lymphocyte subtype, CD1d restricted, implicated in the regulation of autoimmunity, and a good source of various Th1, Th2 or Th17 cytokines. Activation of iNKT cells with their exogenous ligand  $\alpha$ -galactosylceramide ( $\alpha$ -GalCer) exerts therapeutic effect in autoimmune diseases such as rheumatoid arthritis (RA). However their physiological role in RA, without any exogenous stimulation, is unclear. We thus aimed at elucidating the pathophysiological role of iNKT cells in collagen-induced arthritis (CIA), a model of RA. We mainly show that iNKT cells were activated in the early phases of the disease (6 days post induction); this activation was characterized, in the liver, but not in spleen or lymph nodes, by secretion of IL-4, IL-17, IFN- $\gamma$ , IL-10, by an overexpression of invariant TCR mRNA, and by a CD69 increased expression. Importantly, functional iNKT cells blockade by an anti-CD1d mAb early treatment induced a clinical improvement of arthritides. This disease improvement by iNKT blockade was accompanied in the early phase of CIA (day 6) by a decreased expression of maturation molecules (CD80, CD86, CD40) on splenic dendritic cells and macrophages and, unexpectedly, by a decreased regulatory T cells suppressive capacities. All together, these findings suggest that, in the beginning of the disease, iNKT cells are activated and may contribute to the pathogenesis of arthritis and can therefore be considered as therapeutic target in rheumatoid arthritis.

# PC13/3 INFLAMMATORY RHEUMATIC DISEASES ARE ASSOCIATED WITH MYOCARDIAL AND CARDIAC SMALL VESSEL INFLAMMATION: A STUDY OF HUMAN HEART AND SKELETAL MUSCLE BIOPSIES

C. Grundtman<sup>1,2</sup>, I. Hollan<sup>3</sup>, Ø. T. Førre<sup>4</sup>, K. Saatvedt<sup>5</sup>, K. Mikkelsen<sup>6</sup>, I. E. Lundberg<sup>2</sup>

<sup>1</sup>Innsbruck Medical University, Innsbruck, Austria, <sup>2</sup>Karolinska Institutet, Karolinska University Hospital, Stockholm, Sweden, <sup>3</sup>Hospital for Rheumatic Diseases, Department of Rheumatology, Lillehammer, Norway, <sup>4</sup>The Feiring Heart Clinic, Feiring, Norway, <sup>5</sup>University of Oslo, Oslo, Norway, <sup>6</sup>Rikshospitalet University Hospital, Oslo, Norway

**Background:** Although the etiology of cardiovascular diseases (CVD) is multifactorial, it is now generally accepted that inflammation plays a major role during all stages of CVD. The burden of chronic systemic inflammation could confer a major risk for development of CVD and atherosclerosis, consequently leading to the known premature morbidity and mortality in patients with inflammatory rheumatic diseases (IRD). The aim of this study was to compare levels of markers of inflammation and endothelial cell activation in cardiac and skeletal muscle of patients with or without IRD undergoing coronary artery bypass grafting.

**Methods:** Paired biopsies of cardiac and skeletal muscles were taken from 22 consecutive IRD patients and 8 non-IRD patients undergoing coronary artery bypass grafting. The biopsies were evaluated randomized and coded by conventional microscopy and digital image analysis for cell markers (CD3, CD4, CD8, CD68, CD163, and CD31), human leukocyte antigens (HLA-ABC, HLA-DR, and HLA-DQ), adhesion molecules (ICAM-1 and VCAM-1), and pro-inflammatory cytokines (IL-1 $\alpha$ , IL-1 $\beta$ , and TNF).

**Results:** IRD patients had a significantly higher expression of adhesion molecules, pro-inflammatory cytokines, and all classes of HLA on cardiomyocytes and endothelial cells, but no increase of mononuclear cells in the myocardium compared to non-IRD patients. Cardiac muscle from IRD patients displayed a significantly higher number of macrophages and increased expression of pro-inflammatory cytokines and HLA molecules compared to skeletal muscle. Moreover, a positive correlation between CRP-levels and HLA-DR, HLA-DQ, and VCAM-1 cardiac expression in combined IRD and non-IRD patients were found.

**Conclusion:** Our data suggest a new and important mechanism for the known early and accelerated development of CVD in patients with chronic systemic inflammation. We show that IRD patients have a concomitant cardiac pathology with inflammation in both cardiac tissue and vessels. Furthermore, cardiac muscle seemed to be more prone to develop these changes than skeletal muscle. The more pronounced expression in patients with IRD compared to patients without IRD could indicate that the systemic inflammation commonly seen in these disorders could contribute to local cardiac inflammation. Conclusively, this might reason why patients with systemic inflammation bear an increased risk for the development and premature mortality of CVD.

# PC13/4 AN IMPROVED MODEL OF CHRONIC RHEUMATOID ARTHRITIS WITH LYMPHOID NEO-ORGANOGENESIS AND PRODUCTION OF AUTOANTIBODIES

U. Baddack<sup>1</sup>, S. Hartmann<sup>1</sup>, A. M. Wengner<sup>1</sup>, C. Loddenkemper<sup>2</sup>, G. Mueller<sup>1</sup>, M. Lipp<sup>1</sup>

<sup>1</sup>Max-Delbrueck Center for Molecular Medicine, Molecular Tumor Genetics and Immunogenetics, Berlin, Germany, <sup>2</sup>Charite – University Hospital Benjamin Franklin, Institute of Pathology, Berlin, Germany

Rheumatoid arthritis (RA) is a common autoimmune disease and associated with the chronic inflammation of the synovium of diarthrodial joints. The disease finally leads to irreversible joint damage resulting in chronic pain and disability. Characteristically for RA is the infiltration of the synovial tissue by granulocytes and large numbers of mononuclear cells. Another hallmark is the development of self-reactive T and B cells, leading to autoantibody production. However, the basic molecular and cellular mechanisms of these events remain poorly understood. We have developed a novel combined mouse model of antigen-induced arthritis showing specific features of the chronic phase in human disease, e.g. frequent formation of ectopic follicular structures and measurable titers of autoantibodies targeting CCP and Collagen type II. In our model formation of ectopic follicles with segregated B and T cell areas can be regularly induced by intra-articular injection of mBSA into the knee joints of pre-immunized BALB/c mice. Remarkably, most follicles comprise active germinal centers (GC) harboring antigen-specific plasma cells. In CXCR5- and CCR7-deficient mice the formation and organization of these ectopic structures is severely impaired proving that both chemokine receptors play an important role in lymphoid neo-organogenesis during chronic inflammation. Our results suggest that continuous inflammatory stimuli with persistent expression of homeostatic chemokines are sufficient to induce lymphoid neo-organogenesis at extra-nodal sites resulting in aberrant chronic immune responses. Additionally it is possible to induce autoreactive antibodies. These findings may help to achieve further insight into chronic autoimmune processes and their underlying pathways.

# PC13/5 TNFRP55 DEFICIENCY AUGMENTS TH1 AND TH17 RESPONSES DURING DEVELOPMENT OF ARTHRITIS

R.J. Elicabe<sup>1,2</sup>, E. Cargnelutti<sup>1,2</sup>, M.I. Serer<sup>1,2</sup>, P. Stege<sup>3,4</sup>, S.R. Valdez<sup>5,6</sup>, G.A. Rabinovich<sup>7,8</sup>, M.S. Di Genaro<sup>1,2</sup>

<sup>1</sup>National University of San Luis, Biochemistry and Biological Sciences, San Luis, Argentina, <sup>2</sup>IMIBIO-SL (CONICET-UNSL), San Luis, Argentina, <sup>3</sup>National University of San Luis, Chemistry, San Luis, Argentina, <sup>4</sup>INQUISAL, CONICET, San Luis, Argentina, <sup>5</sup>National University of Cuyo, Mendoza, Argentina, <sup>6</sup>LARLAC, IMBECU-CONICET, CCT Mendoza, Mendoza, Argentina, <sup>7</sup>National University of Buenos Aires, Buenos Aires, Argentina, <sup>8</sup>IBYME-CONICET, Buenos Aires, Argentina

Reactive arthritis (ReA) is an inflammatory arthritis that arises after certain types of gastrointestinal or genitourinary infectious. The pathogenesis of ReA is incompletely understood. Th1 and Th17 cells are associated with chronic inflammation. The objective was to determine the IL-17 and IFN- $\gamma$  contribution in *Yersinia*-induced ReA in TNFRp55<sup>-/-</sup> mice. We used C57BL/6 TNFRp55<sup>-/-</sup> and wild-type (WT) mice. The animals were orally infected with *Y. enterocolitica* O:3, and arthritis progression was assessed using clinical score and histological analysis. *Yersinia* LPS in the joint was investigated by micellar electrokinetic chromatography (MEKC). Homogenates of joint, mesenteric lymph nodes (MLN) and inguinal lymph nodes (ILN) were obtained at different days after infection (7, 14 and 21), and IFN- $\gamma$ , IL-17, TGF- $\beta$ , IL-6, IL-1 $\beta$  and IL-10 levels were quantified by ELISA. Spleen cells from WT and TNFRp55<sup>-/-</sup> mice were re-stimulated *in vitro* with heat-killed *Yersinia* (HKY) and the supernatants were examined for IFN- $\gamma$  and IL-17 production. Differences between the groups were tested for significance by Mann Whitney U test. A *p* value less than 0.05 was considered statistically significant. We found higher severity of ReA in TNFRp55<sup>-/-</sup> compared with WT mice. LPS was detected in joint of the mice. When we compared local cytokine profile in the joint of TNFRp55<sup>-/-</sup> with WT mice, we found higher articular IFN- $\gamma$  and IL-17 levels in TNFRp55<sup>-/-</sup> mice at day 14 (*p* < 0.02) and 21 (*p* < 0.03) after infection, respectively. We demonstrated early correlation between cytokine production in MLN and ILN. When systemic cytokine production was examined, IL-17 and IFN- $\gamma$  levels were increased in knockout mice (*p* < 0.05). Association between IL-17, IL-6 and TGF- $\beta$  levels was observed. Inflammatory response dominated by articular IL-17, IL-6 and IL-1 $\beta$ , including IFN- $\gamma$  in ILN, was detected in the chronic phase of arthritis in TNFRp55<sup>-/-</sup> mice. Altered IL-10 production and higher IL-12/IL23 p40 level was found TNFRp55<sup>-/-</sup> mice (*p* < 0.05). These data demonstrate redundant pathways, including both IL-17 and IFN- $\gamma$ , may be used to mediate inflammation in absent of TNFRp55. One way by which TNFRp55 signaling regulates Th1 and Th17 responses is down-regulating p40. These results provide further understanding of the cytokine interplay in ReA.

# PC13/6 THE SYMPATHETIC NERVOUS SYSTEM PROMOTES THE DEVELOPMENT OF B CELLS THAT SHOW REGULATORY POTENTIAL IN COLLAGEN-INDUCED ARTHRITIS

G. Pongratz<sup>1</sup>, M. Melzer<sup>1</sup>, R.H. Straub<sup>1</sup>

<sup>1</sup>University Medical Center Regensburg, Internal Medicine I, Rheumatology, Regensburg, Germany

The recent success in the treatment of some autoimmune disease, such as rheumatoid arthritis (RA), with depleting anti-CD20 antibodies, led to a revival of the concept that B cells play a role in the pathogenesis of these disease. However, a complete depletion of CD20<sup>+</sup> B cells, no matter if autoreactive or not, is a rather unspecific method and has the potential to cause complicating side effects such as severe infection. To avoid this problem it is important to understand how B cells might be regulated to influence the course of disease more specifically. A known regulator of B cell function is the sympathetic nervous system (SNS), but recent studies also showed a clear influence of the SNS on the development and severity of experimental arthritis.

**Therefore, it was hypothesized that the SNS acts via regulating B cell function to modulate the development and severity of arthritis.**

We show that the SNS has the potential to support the generation of B cells that possess regulatory potential in collagen induced arthritis (CIA):

1) We activated B cells with anti-CD40 (1mg/ml)/IL-4 (1ng/ml) in the presence or absence of norepinephrine (10<sup>-6</sup>M), and adoptively transferred 3 Mio. of these cells in arthritic DBA1J mice. Compared to arthritic mice that received PBS, mice that received B cells developed a less severe arthritis *per se*, however, this effect was significantly more pronounced when B cells activated in the presence of norepinephrine were used for treatment.



2) We induced arthritis in sympathectomized mice and control mice. At day 28 after induction of arthritis, we exchanged splenic B cells between the groups by adoptive transfer. *Per se*, sympathectomized mice developed more severe arthritis after splenectomy than control mice. However, transferring B cells from the sympathectomized mice into control mice, and *vice versa*, also changed the course of disease, respectively. Both findings point to a role of the sympathetic nervous system in generating B cells that possess regulatory potential in the collagen induced arthritis model.

#### PC13/7 SYSTEMATIC DEVELOPMENT OF NOVEL ANTIBODY BIOMARKERS FOR DIAGNOSTIC PROTEIN BIOCHIPS – THE RHEUMATOID ARTHRITIS CASE STUDY

A. Lueking<sup>1</sup>, A. Kowald<sup>1</sup>, H.E. Meyer<sup>2</sup>, M. Schneider<sup>3</sup>, S. Müllner<sup>1</sup>

<sup>1</sup>Protagen AG, Dortmund, Germany, <sup>2</sup>Ruhr Universität Bochum, Medizinisches Proteom Center, Bochum, Germany, <sup>3</sup>Heinrich-Heine Universität, Düsseldorf, Germany

Rheumatoid arthritis (RA) is a chronic, systemic, inflammatory disease of the joints. Diagnosis and treatment at an early stage of the disease are required to prevent erosive joint destruction, deformity and functional impairment. Diagnosis of RA patients based on autoantibodies against cyclic citrullinated peptides (CCP) is possible with high sensitivity and specificity. However, anti-CCP autoantibodies are preferentially detected in patients with severe RA, but are less frequent in patients with mild RA. Therefore, it is speculated that the currently available panel of diagnostic markers (anti-RF and anti-CCP) does not assess the full heterogeneity of the disease.

Consequently, we are developing a protein biochip with a novel set of diagnostic markers based on the proprietary UNiarray<sup>®</sup> technology. Briefly, stratified patient samples are incubated with the currently largest collection of recombinant human proteins available for screening purposes to detect autoantibodies against specific targets. Using plasma samples from 20 RA patients we identified more than 100 RA-specific candidate autoantigens. The identified human autoantigens were recombinantly expressed, purified and printed together with 2000 additional human recombinant proteins (partially derived from other autoimmune disease screens) on one single protein microarray. For a first verification of the novel markers the protein biochip was used to test 67 plasma samples of RA patients vs. 85 plasma samples of apparently healthy control persons. Statistical ranking and further analysis shows that a smaller subset of markers is sufficient to achieve a high sensitivity and specificity. Within the high-ranked antigens proteins involved in certain signalling pathways are significantly over-represented. Furthermore, several proteins implicated in different immune and neurodegenerative diseases have been identified. A RA-specific protein biochip consisting of this smaller subset of markers was generated and tested with sera from 77 RA patients and 76 apparently healthy control persons. Using different bioinformatical methods such as SVM, neuronal nets or treeBoost classification trees, sensitivity and specificity of approximately 70% is achieved. As an outlook, further possible developments will be discussed.

#### PC13/8 THERAPEUTIC POTENTIAL OF A FILARIAL NEMATODE PRODUCT, ES-62, IN THE TREATMENT OF INFLAMMATORY AUTOIMMUNE DISEASE

M.A. McGrath<sup>1</sup>, I.B. McInnes<sup>1</sup>, W. Harnett<sup>2</sup>, M.M. Harnett<sup>1</sup>

<sup>1</sup>University of Glasgow, Department of Immunology, Infection & Inflammation, Glasgow, United Kingdom, <sup>2</sup>University of Strathclyde, Strathclyde Institute of Pharmacy and Biomedical Sciences, Glasgow, United Kingdom

The phosphorylcholine-containing glycoprotein ES-62, secreted from filarial nematodes, has been shown to suppress aberrant inflammation without immunocompromising the host. For example, ES-62 has demonstrated significant anti-inflammatory effects in the Collagen-Induced Arthritis (CIA) and the MRL/lpr animal models of Rheumatoid Arthritis (RA) and Systemic Lupus Erythematosus (SLE), respectively. The mechanisms underlying such suppression have not been fully elucidated but ES-62 may be acting, at least in part, by targeting the IL-17 inflammatory pathway. Therefore, the primary objective of these studies was to characterise the potential involvement of IL-17 in MRL/lpr disease pathogenesis, and secondly, to examine whether ES-62 modulation of CIA and lupus disease progression results from inhibition of this cytokine. Thus, lymph node (LN) cells from control and ES-62-treated mice from both disease models were stimulated and analysed by flow cytometry for intracellular cytokine staining whilst serum samples were collected for analysis of cytokine levels by ELISA. Furthermore, *in situ* analysis of lymph node, spleen and kidney architecture, as well as cellular infiltration throughout the course of disease, was performed by laser scanning cytometry. Substantial levels of IL-17 were detected in serum samples from MRL/lpr mice prior to onset of inflammatory kidney disease and preceding production of IFN $\gamma$ . By contrast, such IL-17 production was not observed in MRL/MP wild type mice which do not develop disease. Moreover, LN cells from ES-62-treated MRL/lpr mice stimulated *ex vivo* exhibited reduced levels of IL-17, KC chemokine, TNF $\alpha$  and IFN $\gamma$  production relative to control disease mice.

Similarly, reduced secretion of IL-17 from LN cells stimulated *ex vivo* as well as decreased levels of IL-17 detected in serum of ES-62 treated mice was evident in the CIA model. ES-62 was found to modulate IL-17 production from both gd and CD4 T cells, which were the major producers of this cytokine in this model. In conclusion, ES-62 was able to significantly reduce disease severity in CIA and MRL/lpr models of autoimmune inflammatory disease. Such protection was reflected by inhibition of IL-17 production and current studies are now addressing the potential therapeutic role of such IL-17 suppression and how this inhibition of IL-17 is achieved.

#### PC13/9 REGULATORY B CELL NUMBERS ARE REDUCED IN PATIENTS WITH ACTIVE RHEUMATOID ARTHRITIS BUT THEIR SUPPRESSIVE FUNCTION IS PRESERVED

F. Flores-Borja<sup>1</sup>, P.A. Blair<sup>1</sup>, D. Ng<sup>1</sup>, G. Cambridge<sup>1</sup>, M.R. Ehrenstein<sup>1</sup>, C. Mauri<sup>1</sup>

<sup>1</sup>University College London, Medicine, London, United Kingdom

**Objectives:** We have recently demonstrated that Bregs are contained within the transitional B cell subset and exert a strong suppressive effect both in experimental arthritis and lupus-like disease. In the present study we have aimed to translate our results to man and show the existence of an equivalent Breg subset in the peripheral blood of healthy controls.

**Methods:** Peripheral blood mononuclear cells (PBMCs) samples were obtained from healthy donors and patients with rheumatoid arthritis attending the Rheumatology Clinic at University College Hospital. B-lymphocyte subpopulations were sorted or depleted from PBMCs following membrane staining with fluochrome-conjugated antibodies by using a FACSaria cell sorter. CD4<sup>+</sup> T-lymphocytes were negatively selected by removing non-T cells with coated beads and magnetic columns. Production of intracellular cytokines in co-cultures of T:B lymphocytes was evaluated by intracellular staining and flow cytometry after three-day culture and stimulation with PMA/ionomycin. The secretion of immunoglobulins by B-lymphocytes was evaluated by ELISA after 10-14 days of culture.

**Results:** A population of CD24<sup>hi</sup>CD38<sup>hi</sup>CD1d<sup>hi</sup> Bregs produced IL-10 and suppressed the production of the pro-inflammatory cytokines IFN $\gamma$ , IL-2 and TNF $\alpha$  by CD4<sup>+</sup> T-lymphocytes in co-culture assays. Selective depletion of CD24<sup>hi</sup>CD38<sup>hi</sup>CD1d<sup>hi</sup> cells from PBMCs resulted in increased production of pro-inflammatory cytokines by activated CD4<sup>+</sup> T cells. In comparison to healthy, CD24<sup>hi</sup>CD38<sup>hi</sup>CD1d<sup>hi</sup>IL-10<sup>+</sup> Bregs were numerically reduced in patients with active rheumatoid arthritis (RA), yet maintained their suppressive function as shown by their capacity to inhibit Th1 differentiation and the secretion immunoglobulins.

**Conclusion:** These findings demonstrate the existence of a B cell subset with suppressive function in healthy individuals and suggest that, in addition to regulating T cell functions, Bregs might also influence directly/indirectly the function of other B cell subtypes and therefore play a key role in the prevention of autoimmune diseases in humans.

#### PC13/10 SHORT AND LONG TERM EFFECTS OF ANTI-CD20 TREATMENT ON B CELL ONTOGENY IN BONE MARROW OF PATIENTS WITH RHEUMATOID ARTHRITIS

M. Rehnberg<sup>1</sup>, S. Amu<sup>1</sup>, A. Tarkowski<sup>1</sup>, M.I. Bokarewa<sup>1</sup>, M. Brissler<sup>1</sup>

<sup>1</sup>Sahlgrenska Academy at University of Gothenburg, Rheumatology and Inflammation Research, Gothenburg, Sweden

**Objective:** In the present study we evaluated changes in the phenotype of B cells in peripheral blood (PB) and bone marrow (BM) in patients with rheumatoid arthritis following infusion of B cell depleting monoclonal anti-CD20 antibodies (Rituximab).

**Methods:** PB and BM samples were obtained from 37 patients with active rheumatoid arthritis prior to rituximab infusion. Thirteen of these patients have been previously treated with rituximab (mean 22 months) and comprised a long-term follow-up group. BM samples for short-term follow-up were taken 1 month (10 patients) and 3 months after treatment (14 patients). The phenotype of B cells (CD19+CD3-) was characterized by CD27/IgD/CD38/CD24 expression.

**Results:** One and three months following anti-CD20 treatment BM retained up to 30% of B cells while PB were totally depleted from B cells. Analysis of the remaining BM B cells showed prevalence of immature pre-B (CD38++CD24++) and memory (CD27+IgD-) cells, while IgD+ cells were completely depleted. A significant reduction of CD38+ populations defined as early transitional (CD38+CD24+IgD+/-) (p=0.01), and mature B2 cells (CD38+IgD+CD24+/-CD27-) (p=0.0001) was observed shortly after rituximab treatment. These changes in the BM were associated with a dramatic reduction of autoantibody levels in PB (p=0.025). Long-term follow-up showed a complete repopulation of PB with CD19+ B cells. Within the B cell populations we found a significant reduction of memory cells (CD27+IgD-) in BM and in PB (p=0.003), while levels naive B cells (CD27+IgD+) were increased in BM (p=0.02) and in PB (p=0.003).

**Conclusions:** Anti-CD20 treatment achieves a depletion of naive IgD+ B cells short after the treatment. Which resulted in a long time reduction of CD27+ memory B cells. The prolonged inability to up-regulate CD27 results in a discrimination of B cells from germinal centres and a skewed renewal of memory B cells. However, this reduction of switched memory B cells in BM or PB does not prevent repopulation with autoantibody producing clones suggesting that mechanisms regulating the formation of auto-reactive clones are not disrupted by rituximab.

# PC13/11 THE EFFECTS OF ABATACEPT TREATMENT ON CYTOKINE PRODUCTION BY DISTINCT T CELL SUBSETS FROM RA PATIENTS

J. Pieper<sup>1</sup>, O. Snir<sup>1</sup>, S. Johansson<sup>1</sup>, P. Janson<sup>2</sup>, O. Winqvist<sup>2</sup>, R. van Vollenhoven<sup>1</sup>, V. Malmström<sup>1</sup>

<sup>1</sup>Karolinska Institutet, Department of Medicine, Rheumatology Unit, Stockholm, Sweden, <sup>2</sup>Karolinska Institutet, Department of Medicine, Clinical Allergy Research Unit, Stockholm, Sweden

**Objectives:** T cells are believed to play an important role in the pathogenesis of rheumatoid arthritis (RA). They can contribute to chronic inflammation in the joints by several pathways including activating macrophages via IFN- $\gamma$  and TNF- $\alpha$ , and by recruiting neutrophils and activating osteoclasts via IL-17. Thus, modulating T cells responses is beneficial for treating RA.

Abatacept (Orencia) is a newly developed treatment for RA. It competes with the T cell co-stimulatory molecule CD28, and thus modulates T cell responses. However, approximately 1/3 of RA patients display an expanded T cell subset often referred to as CD4<sup>+</sup>CD28<sup>null</sup>. These cells are proinflammatory cells that have down-regulated their surface expression of CD28. Hence, they will not be directly affected by Abatacept treatment. In this study, we investigate which T cell subsets are affected by Abatacept treatment, and whether CD4<sup>+</sup>CD28<sup>null</sup> T cells will expand during treatment.

**Methods:** PBMCs were isolated from RA patients treated with Abatacept, before and 6 months following treatment. The cells were stimulated *in vitro* in a polyclonal ( $\alpha$ -CD3), or antigen-specific (influenza or CMV) fashion. Intracellular cytokine production was investigated in a multiparameter flow cytometry setting. Additionally, the IFN- $\gamma$  producing capacity was studied by epigenetic analysis of the IFN- $\gamma$  enhancer.

**Results:** Following  $\alpha$ -CD3 stimulation we demonstrate robust production of IFN- $\gamma$ , TNF- $\alpha$  and IL-17, whereas the CD4<sup>+</sup>CD28<sup>null</sup> T cell subset consistently fails to make IL-17. Strikingly, this T cell subset demonstrates a highly demethylated IFN- $\gamma$  promoter suggestive of a Th1 driven phenotype.

Of importance, T cells from the circulation of RA patients with clinical response to Abatacept show significantly diminished cytokine production 6 months following therapy as compared with base line (before treatment).

**Conclusion:** T cells can contribute to inflammatory arthritis via several different cytokine pathways, and Abatacept treatment leads to a general modulation of the effector cytokines IFN- $\gamma$ , TNF- $\alpha$  and IL-17. The effect appears to be long lasting since the down regulation is seen upon *in vitro* T cell stimulation in the absence of the drug. To what extent T cell responses are diminished also in clinical non-responders will be investigated in future experiments.

# PC13/12 INCREASED TH17 CELL ACTIVITY IN PATIENTS WITH EARLY RHEUMATOID AND PSORIATIC ARTHRITIS

J. Leipe<sup>1</sup>, K. Thüemmler<sup>1</sup>, H. Schulze-Koops<sup>1</sup>, A. Skapenko<sup>1</sup>

<sup>1</sup>University of Munich, Med. Poliklinik, Division of Rheumatology, Munich, Germany

**Objectives:** Based on recent data from experimental murine models, Th17 cells have been suggested to be a distinct pro-inflammatory subset of CD4 T cells potentially involved in the pathophysiology of autoimmune inflammation. To gain insights into the role of Th17 cells in human autoimmune diseases, we analyzed Th17 cells in patients with prototypic autoimmune diseases, e.g. rheumatoid (RA) and psoriatic arthritis (PsA).

**Methods:** CD4 T cells from patients with early, treatment-naïve RA and PsA and from patients with well-established RA were assessed *ex vivo* and after activation *in vitro* for the frequency of Th17 cells by flow cytometry, and for IL-17 production by ELISA. For control, cells from patients with non-autoimmune arthritis (osteoarthritis, OA) and from age-matched healthy controls were used. Gene expression of RORC was assessed by real-time PCR. The potential of naïve CD4 T cells to differentiate into Th17 cells was evaluated in priming cultures that permitted Th17 development under standardized conditions. Frequencies of Th17 cells and IL-17 production were correlated with disease activity and with response to anti-rheumatic therapy in RA patients.

**Results:** Frequencies of Th17 cells were significantly higher in CD4 T cells from patients with RA and PsA, as compared to controls. Upon activation, CD4 T cells from RA and PsA patients yielded markedly increased Th17 cell frequencies and IL-17 production, and were partially non-responsive to the inhibitory capacity of IL-4 and IFN $\gamma$  on Th17 generation. In contrast, Th1 frequencies and IFN $\gamma$  production were not different between RA and PsA patients and the controls. Consistent with these findings, higher expression of RORC, but not of T-bet, GATA-3 or FOXP3 was found in CD4 T cells from RA patients compared to HC. Differentiation of naïve CD4 T cells from patients with RA and PsA into Th17 cells was also increased compared to controls. Whereas effective immunosuppressive treatment of RA was associated with normalization of Th17 cell frequency and IL-17 production, an increase of both parameters was observed in non-responders to therapy.

**Conclusion:** Our data suggest that Th17 cells and their signature cytokine, IL17 have a prominent role in the pathogenesis of human autoimmune diseases.

# PC13/13 EFFICIENT CONTROL OF AN ESTABLISHED ARTHRITIS BY IMMUNIZATION AGAINST TNFALPHA IN HUMAN TNFALPHA TRANSGENIC MICE

E. Assier<sup>1</sup>, L. Delavallée<sup>1</sup>, G. Grouard-Vogel<sup>2</sup>, G. Vuagniaux<sup>3</sup>, M. Laborie<sup>2</sup>, E. Bernier<sup>2</sup>, D. Zagury<sup>2</sup>, N. Bessis<sup>1</sup>, M.-C. Boissier<sup>1,4</sup>

<sup>1</sup>University Paris 13, Immunology, Bobigny, France, <sup>2</sup>Néovacs, Paris, France, <sup>3</sup>Debiopharm, Lausanne, Switzerland, <sup>4</sup>Avicenne Hospital, Rheumatology, Bobigny, France

**Purpose:** Passive blockade of TNF $\alpha$  shows high efficacy in rheumatoid arthritis, although some concerns remain such as occurrence of resistance and high cost. We have previously demonstrated that active immunization against human (h)TNF $\alpha$  was possible when using a TNF Kinoid (TNF-K), a conjugate of hTNF $\alpha$  and KLH, adjuvanted with ISA-51. TNF-K proved its efficacy in preventive treatments in hTNF $\alpha$  transgenic (Ttg) mice. In the present study, we aimed at demonstrating a therapeutic effect on established arthritis.

**Methods:** Fifteen-week-old Ttg mice were intramuscularly immunized after the onset of arthritis with TNF-K. Control group was treated with PBS. In parallel, another group was treated with infliximab (weekly, for 12 weeks). Arthritides were evaluated clinically and histologically. Animals were euthanized either 12 or 30 weeks after TNF-K priming. TNF $\alpha$  antibodies were evaluated by ELISA and by L929 cells bioassay.

**Results:** TNF-K therapeutic immunization in comparison to controls, showed a dramatic effect on clinical arthritides and histological scores of inflammation or destruction as evaluated 12 weeks after the treatments started. This effect was comparable to those observed with infliximab. The reversion of arthritides was followed by a late flare in TNF-K treated animals; a boost of TNF-K (17 weeks after priming) reactivated a regression of arthritides in comparison to non-boosted animals. Histological score evaluated 30 weeks after priming showed the persistence of the anti-inflammatory effects, although a slight progression was noticed in TNF-K treated animals between the 12th and 30th week following vaccination. Anti-TNF $\alpha$  antibodies decreased along time after the peak around 8 weeks after TNF-K priming.

**Conclusions:** TNF-K therapeutic immunization is efficient in treating an established arthritis in Ttg mice. The blockade of TNF $\alpha$  induced by TNF-K immunization is reversible as demonstrated by clinical, histological and TNF $\alpha$  levels evaluation, and may be re-induced by a late boost of TNF-K. Taken together, these data are consistent with a unique profile of this anti-TNF $\alpha$  immunization strategy, with a clear-cut long lasting effect on chronic inflammation contrasting with a reversible active blockade of TNF $\alpha$ .

# PC13/14 CONSEQUENCES OF BLOCKING IL-6 RESPONSES *IN VIVO*

R. Lissilaa<sup>1</sup>, V. Buatois<sup>1</sup>, G. Elson<sup>1</sup>, L. Chatel<sup>1</sup>, S. Herren<sup>1</sup>, G. Magistrelli<sup>1</sup>, P. Malinge<sup>1</sup>, M. Kosco-Vilbois<sup>1</sup>, W. Ferlin<sup>1</sup>

<sup>1</sup>NovImmune S.A., Plan-les-Ouates, Geneva, Switzerland

The binding of interleukin-6 (IL-6) to the membrane-bound IL-6 receptor alpha-chain (mIL-6R $\alpha$ ) induces the homodimerization and the subsequent phosphorylation of the ubiquitously-expressed signal transducing protein gp130. This signaling event is defined as *cis*-signaling. However, many of the inflammatory properties assigned to IL-6 are believed to be mediated via the naturally occurring soluble receptor (sIL-6R $\alpha$ ). IL-6 forms an agonistic complex (IL-6Rc) with IL-6R $\alpha$  and induces responses in cells which normally would not respond to IL-6 alone. This process mediated by the IL-6Rc, defined as *trans*-signaling, induces activation of a much larger range of cells, i.e. gp130<sup>+</sup>, IL-6R $\alpha$ - cells.

To further investigate the role of this trans-signaling pathway in disease processes, a panel of monoclonal antibodies (mAbs) to IL-6R $\alpha$  were generated. Here we describe two mAbs, 2B10 and 25F10, that bind to IL-6R $\alpha$  and differentially regulate IL-6 signaling. Using BIAcore affinity measurements and ELISA binding assay, these differences were quantitated. Functionally, 2B10 inhibits the formation of the IL-6Rc suggesting that it recognizes the IL-6-binding site on IL-6R $\alpha$ . 25F10, on the other hand, blocks only the *trans*-IL-6 signaling pathway suggesting it recognizes an epitope outside of the IL-6-binding pocket. The consequences of blocking the two IL-6 signaling pathways *in vivo* demonstrate a differential consequence on T cell activation, antigen-dependant humoral responses and disease susceptibility in a mouse model of collagen induced arthritis, CIA.

# PC13/15 INNATE IMMUNITY TRIGGERS IL-32 EXPRESSION BY FIBROBLAST-LIKE SYNOVIOCYTES IN RHEUMATOID ARTHRITIS

L. Sparsa<sup>1</sup>, G. Alsaleh<sup>1</sup>, C. Grussenmeyer<sup>1</sup>, D. Wachsmann<sup>1</sup>, J.-E. Gottenberg<sup>1</sup>, J. Sibilia<sup>1</sup>

<sup>1</sup>Université de Strasbourg, Laboratoire Physiopathologie des Arthrites, Illkirch, France

**Objective:** Interleukin-32 (IL-32) is a recently described cytokine mainly produced by NK cells, T lymphocytes, epithelial cells and blood monocytes stimulated by IFN- $\gamma$ . IL-32 is a strong inducer of proinflammatory cytokines such as TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-8. This cytokine is highly expressed in rheumatoid but not in osteoarthritis synovial tissues. IL-32 expression is strongly correlated with that of TNF- $\alpha$  and IL-1 $\beta$  and with the severity of joint inflammation. We investigated the role of innate immunity in IL-32 expression by synovioocytes, resident and target cells of autoimmunity in rheumatoid arthritis (RA).

**Methods:** Quantitative RT-PCR, confocal analysis and ELISA were performed to evaluate IL-32 mRNA induction and IL-32 release by FLS isolated from patients with RA and subsequently stimulated with TLR2 (BLP), TLR3 (poly I:C), TLR4 (LPS) ligands and with TNF- $\alpha$  and/or IFN- $\gamma$ .

**Results:** BLP, poly I:C and LPS as well as IFN- $\gamma$  and TNF- $\alpha$  induced IL-32 mRNA expression and IL-32 release by RA FLS. IL-32 isoform  $\alpha$  was neither expressed nor secreted. Interestingly, concomitant stimulation of cells with either TNF- $\alpha$  or PAMPs increased IL-32 mRNA expression induced by IFN- $\gamma$  as compared to stimulation with IFN- $\gamma$  alone. The synergistic effect mediated by TNF- $\alpha$  is dependent of IRF-1. Thus, TNF- $\alpha$  and PAMPs work in synergy with IFN- $\gamma$  to induce IL-32 expression by FLS.

**Conclusion:** IL-32 is expressed by FLS and regulated by innate immunity. Thus, rheumatoid synoviocytes might enhance the inflammatory process in RA through the local expression of IL-32, which subsequently stimulates TNF- $\alpha$  and IFN- $\gamma$  production by monocytes, macrophages, and lymphocytes. Since TNF- $\alpha$  and IFN- $\gamma$  upregulate IL-32 expression in a "vicious" pathogenic circle, IL-32 might reveal a promising therapeutic target in RA.

#### PC13/16 CD8+ T-CELL MEDIATED SELF-REACTIVITY IN THE HLA-B\*27 CONTEXT CORRELATES WITH PEPTIDE CONFORMATIONAL DIMORPHISM

E. Nurzia<sup>1</sup>, F. Panimolle<sup>1</sup>, B. Loll<sup>2,3</sup>, W. Saenger<sup>2</sup>, A. Ziegler<sup>4</sup>, B. Uchanska-Ziegler<sup>4</sup>, R. Sorrentino<sup>1</sup>, M. T. Fiorillo<sup>1</sup>

<sup>1</sup>Sapienza – University of Rome, Departement of Cell Biology and Development, Rome, Italy, <sup>2</sup>Freie Universität Berlin, Institut für Chemie und Biochemie/Kristallographie, Berlin, Germany, <sup>3</sup>Max-Planck-Institut für Medizinische Forschung, Abteilung für Biomolekulare Mechanismen, Heidelberg, Germany, <sup>4</sup>Universitätsmedizin Berlin, Campus Benjamin Franklin, Freie Universität Berlin, Institut für Immunogenetik, Charité, Berlin, Germany

**Objective:** The human MHC subtypes HLA-B\*2705 and HLA-B\*2709 are differentially associated with the autoimmune disease Ankylosing Spondylitis (AS), although they differ only in a buried residue (D116H), located in the peptide binding groove. Our aim is to correlate structural and functional differences of the two B27 alleles in presenting Arg-rich viral and self-peptides to CD8+ T cells.

**Methods:** T cell studies are performed in B\*2705 positive AS patients and B\*2705 and B\*2709 healthy donors to assess the presence of specific CTLs responsive to the peptides pLMP2, pVIPR and pGR. Our studies were correlated with the structures of the two HLA-B27 subtypes complexed with the respective peptides as obtained by X-ray crystallography.

**Results:** We show here that the T-cell repertoires of B\*2709-positive individuals lack CD8+ T-cells specific for the self-peptide pVIPR (RRKWRWWHL), derived from the vasoactive intestinal peptide type 1 receptor (VPAC1) which, however, are present in B\*2705-positive individuals. This peptide shares extensive homology with a foreign peptide, pLMP2 (RRRWRLTV), derived from EBV, toward which both B\*2705 and B\*2709 individuals mount a vigorous CTL response. Interestingly, pGR (RRRWRRWRL), a glucagon receptor-derived self-peptide highly similar in sequence to both pVIPR and pLMP2, is able to trigger a specific CTL response in B\*2705 as well as in B\*2709 positive individuals. X-ray crystallography of the two HLA-B27 subtypes complexed with pGR reveals a dual conformation of the bound peptide, whereas pVIPR is presented in a dual conformation only by B\*2705.

**Conclusions:** Conformational dimorphism of HLA-B27-bound peptides appears to be connected with the presence of self-reactive CTL in both HLA-B27 subtypes. This suggests that the presence of these double conformations is likely to impair negative thymic selection, thus allowing the escape of autoreactive T cells that can be activated in the periphery once the self- or a cross-reactive, possibly foreign, peptide is encountered.

#### PC13/17 12/15-LIPOXYGENASE IS INVOLVED IN THE LIMITATION OF INFLAMMATION AND TISSUE DAMAGE IN MURINE INFLAMMATORY ARTHRITIS

G. Krönke<sup>1</sup>, J. Katzenbesser<sup>1</sup>, S. Uderhardt<sup>1</sup>, M. Zaiss<sup>1</sup>, G. Schabbauer<sup>2</sup>, A. Zarbock<sup>3</sup>, M. Koenders<sup>4</sup>, W. van den Berg<sup>4</sup>, L. Joosten<sup>4</sup>, G. Schett<sup>1</sup>

<sup>1</sup>University of Erlangen, Erlangen, Germany, <sup>2</sup>University of Vienna, Vienna, Austria, <sup>3</sup>University of Münster, Münster, Germany, <sup>4</sup>University Nijmegen, Nijmegen, Netherlands

12/15-lipoxygenase (12/15-LO) has been implicated in the generation of both pro- and anti-inflammatory arachidonic acid-derived lipid mediators including 12-HETE, 15-HETE, 13-HODE and lipoxin A4, respectively. Nevertheless the role of this enzyme during the course of the inflammatory reaction remains elusive.

To understand the role of 12/15-LO during chronic inflammation, we determined the effect of 12/15-LO deficiency in two different murine arthritis models (K/BxN-serum transfer model and TNF transgenic mice). Interestingly, arthritis exacerbated in 12/15-LO<sup>-/-</sup> mice and histological analysis of inflamed joints showed an increase in the magnitude of the inflammatory infiltrate and in the area of bone erosions. While the level of anti-inflammatory 12/15-LO-derived lipoxin A4 was dramatically reduced in 12/15-LO-deficient mice, the animals exhibited a decoupled and enhanced expression of pro-inflammatory genes such as IL-1b, IL-6 and KC in their inflamed joints. These findings correlated with an increased activation of the p38MAPK and PI3K pathway.

In consistence, *in vitro* analysis revealed increased phosphorylation of p38MAPK and enhanced expression levels of IL-1b, IL-6 and KC in 12/15-LO<sup>-/-</sup> macrophages after stimulation with TNF, providing additional support for a role of 12/15-LO as a negative regulator of the inflammatory response.

Together these data point toward a pivotal role of 12/15-LO-derived lipid mediators in the resolution of inflammation and indicate a central involvement of this enzyme in the limitation of inflammation-associated tissue damage.

#### PC13/18 HETEROGENEITY OF PERIPHERAL IL-17 PRODUCING CELLS IN RHEUMATOID ARTHRITIS (RA) AND HEALTHY CONTROLS

M. Schlegel<sup>1</sup>, I. Kötter<sup>2</sup>, I. Steiert<sup>1</sup>, C. A. Müller<sup>1</sup>

<sup>1</sup>Sect. Transplantationimmunology and Immunohematology, Tübingen, Germany, <sup>2</sup>Med. Univ. Clinic, Tübingen, Germany

**Objectives:** Interleukin-17 (IL-17 A/F) has been described as signature cytokine of the T-helper subset Th17. Recently it has been reported that CD8+ T cells, NKT cells and possibly also non-T cells are capable to secrete IL-17. Since IL-17 appears to play a critical role in experimental as well as human arthritis, we investigated heterogeneity of the IL-17 secreting cells in PMBC of rheumatoid arthritis patients in comparison to healthy donors.

**Methods:** Ficoll separated PMBC of 20 RA-patients and 20 healthy donors were analysed on a LSRII (Becton Dickinson) after stimulation with PMA/Ionomycin, PHA or medium as control for 20h and BrefeldinA for 2h. Multi-parameter immunofluorescence staining with CD3-FITC, CD4-PacificBlue, CD8-PerCP, CD25APC-Cy7, CD28-APC, CD56-AlexaFluor700 conjugated antibodies as well as EMA for the exclusion of dead cells was performed. IFN- $\gamma$ -PE-Cy7 and IL-17-PE antibodies were used for intracellular cytokine staining. Results were evaluated with FlowJo and GraphPad Prism.

**Results:** The mean frequencies of IL-17<sup>+</sup> lymphocytes were 0,5%/0,6% in PMBC of RA-patients and controls. RA-patients showed less CD3<sup>+</sup> but significantly more CD3<sup>+</sup> IL-17<sup>+</sup> cells than the controls (13% versus 8%). As major subset of the IL-17<sup>+</sup>CD3<sup>+</sup> T-cells CD4<sup>+</sup> T-helper-cells (RA: 61%; C: 55%) were identified. CD3<sup>+</sup>CD8<sup>+</sup> T-cells (22% of the CD3<sup>+</sup> IL17<sup>+</sup> T-cells) as well as CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup> cells represented the second and third major sub-fractions of the CD3<sup>+</sup> IL-17<sup>+</sup> lymphocytes in RA patients and controls. The IL-17<sup>+</sup> CD3<sup>+</sup> cells consisted of CD56<sup>+</sup> NK-cells, CD3<sup>+</sup>CD4<sup>+</sup>, CD3<sup>+</sup>CD8<sup>+</sup> cells. In particular, the CD3<sup>+</sup> CD4<sup>+</sup> population showed a large proportion of IL-17<sup>+</sup> cells, being significantly higher in RA-patients than in the controls (5,35% vs 3,6%). The largest IL-17<sup>+</sup>CD3<sup>+</sup> cell fractions represented CD20<sup>+</sup> B-cells (45%) and CD14<sup>+</sup> monocytes (15%) in two patients and controls analysed. IFN- $\gamma$ <sup>+</sup>IL-17<sup>+</sup> positive cells were seen in the CD3<sup>+</sup>CD4<sup>+</sup> as well as the CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup> populations. PMA/Ionomycin or PHA significantly increased IL-17<sup>+</sup>CD3<sup>+</sup> cells in RA-patients and controls. PMA/Ionomycin also induced a significant rise of IL-17<sup>+</sup>CD3<sup>+</sup> cells in the RA-patients.

**Conclusion:** Beside Th17 additional T cell subsets such as CD3<sup>+</sup>CD8<sup>+</sup> and CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup> T cells as well as non-T-cells such as CD3<sup>+</sup>CD4<sup>+</sup> cells, monocytes and B-cells may contribute to altered production of IL-17 in RA

#### PC13/19 VACCINATION RESPONSE TO PROTEIN AND POLYSACCHARIDE ANTIGENS FOLLOWING ANTI-CD20 TREATMENT IN PATIENTS WITH RHEUMATOID ARTHRITIS

M. Rehnberg<sup>1</sup>, S. Amu<sup>1</sup>, M. Brissert<sup>1</sup>, K. Zendjanchi<sup>2</sup>, G. Håwi<sup>2</sup>, A. Tarkowski<sup>1</sup>, M.I. Bokarewa<sup>1</sup>

<sup>1</sup>University of Göteborg, Rheumatology and Inflammation Research, Göteborg, Sweden, <sup>2</sup>Sahlgrenska University Hospital, Göteborg, Sweden

**Background:** Treatment of rheumatoid arthritis (RA) using immunosuppressive and cell depleting drugs is frequently complicated with infections. The aim of our study was to evaluate vaccination response in patients with RA following B cell depletion.

**Material and methods:** Twenty-eight RA patients were vaccinated with hemagglutinin from Haemophilus influenza (Afluria, Novartis) and with polysaccharides from 23 pneumococcal serotypes (Pneumo23, Aventis MSD). Patients were vaccinated 6 months following treatment with anti-CD20 antibodies (rituximab (Roche AB), post-RTX group, n=10), and 6 days before treatment (pre-RTX group, n=8). The remaining 10 RA patients were not exposed to RTX (control group). Blood samples were collected on day 6 following vaccination to evaluate formation of vaccine-specific B cells, and on day 21 to evaluate vaccine-specific serological responses.

**Results:** On day 6 after vaccination, the majority (80-75%) of patients developed influenza-specific IgG and/or IgA expressing B cells irrespectively of RTX treatment. The number of influenza specific IgM expressing B cells was significantly lower in the post-RTX group as compared to controls (p=0.02). Development of polysaccharide-specific IgG and/or IgA B cells was found in 27-50% of RA patients, which was equally distributed between the RTX-treated groups and the controls. On day 21 after vaccination, the levels of influenza-specific IgM were similar in all three groups, while total IgG were lower in the pre-RTX group as compared to the controls. The production of IgG1-4 subclasses of influenza-specific antibodies were evenly represented in the RTX-treated groups while IgG1 and IgG4 subclasses dominated in the controls. The levels of polysaccharides-specific IgM and IgG were similar in post-RTX group and the controls. The production of IgG2 and IgG3 subclasses dominated among the polysaccharide-specific IgG, both in the RTX-treated groups and in the controls. The production of influenza-specific Ig containing k-light chain was significantly reduced in RTX-treated patients as compared to the controls. The proportion of k/l-light chain containing polysaccharide-specific Ig was similar in the RTX-treated groups and the controls.

**Conclusions:** B cell depletion using anti-CD20 antibodies changes the pattern of vaccine-specific antibody production with respect to IgG subclasses and light-chain composition in patients with RA.



# PC13/20 RITUXIMAB DEPLETES FUNCTIONALLY ACTIVE CD20<sup>+</sup> T CELLS IN RHEUMATOID ARTHRITIS: AN ADDITIONAL MECHANISM OF ACTION?

T. Witte<sup>1</sup>, E. Wilk<sup>1</sup>, N. Marquardt<sup>1</sup>, T. Horvath<sup>1</sup>, K. Kalippke<sup>1</sup>, K. Scholz<sup>1</sup>, R.E. Schmidt<sup>1</sup>, R. Jacobs<sup>1</sup>

<sup>1</sup>Med. Hochschule Hannover, Klinische Immunologie und Rheumatologie, Hannover, Germany

**Objective:** Rituximab is an anti-CD20 antibody used for the depletion of B cells in proliferative and autoimmune diseases. It is still unclear, whether depletion of B cells as antigen-presenting cells and cytokine producers is the only mechanism of action of Rituximab in autoimmune disorders. CD20 is also expressed on a small population of T cells. We now studied the role of these CD3<sup>+</sup>CD20<sup>+</sup> T cells in RA and their correlation with response to Rituximab treatment.

**Methods:** Phenotypes of PBMC from healthy donors and RA patients were examined by four color FACS analysis. Cytokine production was determined by intracellular staining. The number of CD3<sup>+</sup>CD20<sup>+</sup> T cells was measured before and after Rituximab infusion and again at relapse of RA upon follow-up in 9 RA patients.

**Results:** The relative number of CD3<sup>+</sup>CD20<sup>+</sup> T cells was identical in healthy controls (mean 1.6% of PB-T cells, n=142) and in RA patients (mean 1.2% of PB-T cells, n=27). CD3<sup>+</sup>CD20<sup>+</sup> cells are characterized by enhanced production of proinflammatory cytokines (IL-1b, TNF). After Rituximab infusion, CD3<sup>+</sup>CD20<sup>+</sup> T cells were reduced (from 1.1 to 0.4% of PBL). Interestingly, there was no reduction of CD3<sup>+</sup>CD20<sup>+</sup> T cells in the two patients who did not respond to Rituximab.

**Conclusion:** CD3<sup>+</sup>CD20<sup>+</sup> T cells produce cytokines relevant for RA pathogenesis and are depleted by Rituximab. Incomplete depletion appears to correlate with a lack of response. Therefore, the depletion of CD20<sup>+</sup> T cells may be an additional mechanism of anti-CD20 therapy in RA.

# PC13/21 OSTEOCLASTOGENIC PROPERTY OF BLOOD AND BONE MARROW PLASMA FROM RHEUMATOID ARTHRITIS AND OSTEOARTHRITIS PATIENTS

A. Radzikowska<sup>1</sup>, M. Jastrzebska<sup>1</sup>, T. Burakowski<sup>1</sup>, E. Kontny<sup>1</sup>, E. Warnawin<sup>1</sup>, P. Malydyk<sup>2</sup>, W. Maslinski<sup>1</sup>

<sup>1</sup>The Institute of Rheumatology, Dept. of Pathophysiology and Immunology, Warsaw, Poland, <sup>2</sup>The Institute of Rheumatology, Clinic of Orthopaedic Surgery, Warsaw, Poland

**Background:** Recent data indicates that the alterations of the OPG/RANKL ratio are critical in bone loss in RA.

**Objectives:**

1. To compare levels of cell-surface expressed and soluble RANKL and OPG in bone marrow and peripheral blood isolated from patients with RA and OA.

2. To compare osteoclastogenic potential of RA and OA blood and bone marrow plasma.

**Methods:** Bone marrow samples were obtained during routine total hip replacement surgery. Mononuclear cells were isolated from both peripheral blood and bone marrow of patients with RA and OA. The surface expression of RANKL and OPG was determined by flow cytometry using specific antibodies. Levels of soluble RANKL and OPG in bone marrow plasma were evaluated by ELISA. To compare the osteoclastogenic potential of RA and OA blood and bone marrow plasma, adherent PBMCs isolated from healthy blood donors were cultured for 15-21 days in the presence of heat inactivated plasma. Osteoclasts differentiation was examined by TRAP staining and calcified matrix resorption activity.

**Results:** Freshly isolated CD3<sup>+</sup> and CD33<sup>+</sup> cells from peripheral blood expressed higher levels of RANKL than cells isolated from bone marrow. There was a tendency to higher surface expression of those molecules on cells isolated from OA patients. In contrast significantly higher levels of soluble RANKL in bone marrow from RA than from OA were observed. Consequently, the ratio of soluble OPG/RANKL was significantly lower in RA patients in comparison to OA patients. Both RA and OA plasma have osteoclastogenic potential to stimulate differentiation of peripheral blood derived monocytes into TRAP<sup>+</sup> and multinucleated osteoclasts. Interestingly, higher osteoclastogenic potential of RA plasma was reflected by stronger resorptive activity of osteoclasts in comparison to OA.

**Conclusions:** Our results document expression of RANKL and OPG on the surface of monocytes and T cells from peripheral blood and bone marrow. The lower ratio of soluble OPG/RANKL in RA than in OA bone marrow reflects situation in vivo, where microenvironment favors bone degradation. In addition, RA plasma has stronger osteoclastogenic property than OA, reflected by higher resorptive activity of osteoclasts.

**Acknowledgements:** This work was partially sponsored by European Community FP6 Project LSHB-CT-018661 AutoCure.

# PC13/22 RHEUMATOID ARTICULAR ADIPOSE TISSUE IS A POTENT SOURCE OF PROINFLAMMATORY CYTOKINES

M. Jastrzebska<sup>1</sup>, E. Kontny<sup>1</sup>, I. Janicka<sup>1</sup>, W. Maslinski<sup>1</sup>, P. Malydyk<sup>2</sup>

<sup>1</sup>Institute of Rheumatology, Dept Pathophysiology & Immunology, Warsaw, Poland, <sup>2</sup>Institute of Rheumatology, Dept. of Rheumorthopaedy Surgery, Warsaw, Poland

**Objectives:** Numerous cytokines play fundamental role in synovial inflammation and articular destruction, characteristic for rheumatoid arthritis (RA). Cytokines are thought to originate from inflamed synovial tissue. Recently adipose tissue has been suggested to play a role in chronic inflammatory joint diseases, but its ability to synthesize proinflammatory cytokines has not been characterized. The aim of present work was to investigate whether rheumatoid articular adipose tissue secretes cytokines relevant to RA pathogenesis.

**Materials and methods:** Adipose tissue explants, obtained from RA patients who were undergoing knee joint replacement surgery, were cultured (100 mg /ml) for 18 hrs in medium (DMEM) alone or in the presence of LPS (1 µg/ml) or cytokines: TNF-α, IFN-γ, IL-15, IL-17 or IL-23 (10 and 40 ng/ml). Concentrations of TNF-α, IL-6 and IL-8 were measured in culture supernatants by ELISA. The expression mRNA encoding IL-15 and its specific receptor (IL-15Rα) was evaluated in freshly obtained tissue explants by RT-PCR.

**Results:** Secretion of IL-6 (1.1 ng/ml) and IL-8 (1.5 ng/ml) by untreated cells were high and potentially raised upon LPS (25.5 ng/ml and 31.2 ng/ml, respectively) and TNF-α (18.4 ng/ml and 34.5 ng/ml) treatment. IL-15 and IFN-γ were less efficient but also significantly (P< 0.05) elevated IL-6 (5.5 ng/ml and 7.8 ng/ml) and IL-8 (13.8 ng/ml and 8.3 ng/ml) secretion, while IL-17 has no significant effect. Interestingly, IL-23 enhanced only IL-8 secretion (7 ng/ml). Adipose tissue expressed both IL-15 and IL-15Rα mRNA at the level similar to synovial tissue, obtained from the same RA patients. Although spontaneous secretion of TNF-α was negligible, LPS (but not cytokines) triggered marked TNF-α synthesis (438 pg/ml).

**Conclusions:** Rheumatoid articular adipose tissue secretes spontaneously a large amount of IL-6 and IL-8 and the synthesis of these interleukins is further up-regulated by several cytokines (TNF-α, IL-15, IFN-γ). Importantly, both TNF-α and IL-15 may act in autocrine way. Therefore our results give direct evidence that in RA articular adipose tissue is an active participant of proinflammatory cytokine network.

# PC13/23 CITRULLINATION BREAKS T-CELL TOLERANCE IN PATIENTS WITH INFLAMMATORY ARTHRITIDES AND IN HEALTHY PERSONS

K. Van Steendam<sup>1</sup>, M. De Ceuleneer<sup>1</sup>, F. De Keyser<sup>2</sup>, D. Elewaut<sup>2</sup>, K. Tillemans<sup>1</sup>, D. Deforce<sup>1</sup>

<sup>1</sup>Ghent University, Laboratory for Pharmaceutical Biotechnology, Ghent, Belgium, <sup>2</sup>Ghent University Hospital, Department of Rheumatology, Ghent, Belgium

**Objectives:** Rheumatoid Arthritis (RA) and Spondyloarthropathy (SpA) are two inflammatory auto-immune diseases. They can be distinguished by their clinical presentations and the presence of antibodies against citrullinated proteins (ACPA), which are specific for RA. Production of these serological markers indicates a predominant role for B-cells in the pathology of RA. However, little is known about the effect of citrullinated proteins on T-cell reactivity.

**Methods:** PBMC were isolated from healthy volunteers, RA-patients (CCP+ and CCP-) and SpA-patients. The IFNγ-production was evaluated by ELISpot analysis. PBMC (500000) were stimulated with *in vitro* citrullinated (*cit*) and non-citrullinated (*non-cit*) human cell extract, each at a concentration of 20 µg/100 µL. In parallel, 10--5e--6 PBMC were cultured for a week in the presence of *cit* (80 µg/ml) or *non-cit* (80 µg/ml). Supernatants were collected and the secretion of cytokines was evaluated by multi-ELISA detecting IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-17a, IFNγ, TNFα, G-CSF and TGFβ.

**Results:** In healthy controls and SpA-patients the number of spots detected by ELISpot differed significantly between stimulation with 'cit' and 'non-cit'. However, this was not seen in RA-patients. These results were confirmed by ELISA. The ELISpot assay showed no significant difference between the number of spots per 500000 PBMC after stimulation with 'non-cit' or with cDMEM.

Other cytokines, like IL6, IL10, IL17a and G-CSF, did show a distinct increase when PBMC of healthy persons or patients with arthritis were stimulated with 'cit' as opposed to 'non-cit'. On the contrary, a higher production of TGFβ was seen in the 'non-cit' condition.

**Conclusion:** These data indicate that, unexpectedly, citrulline-specific IFNγ producing T-cells are present in the repertoire of healthy people. However, they seem to be underrepresented in the blood of RA-patients compared to healthy controls and SpA-patients. It is possible that the citrulline-specific T-cells in RA may be retained in the joint, the site of inflammation in RA.

The Multi-ELISA shows distinct cytokine profile of PBMC after stimulation with citrullinated proteins.

Our results indicate a break in T-cell tolerance against citrullinated proteins. The presence of citrulline specific T-cells could play an important role in the ACPA production in RA.

# PC13/24 THE SUPPRESSIVE CAPACITY OF JOINT-DERIVED CD25BRIGHTCD4+ REGULATORY T CELLS IS RELATED BOTH TO THE EXPRESSION OF FOXP3 AND TO THE ACTIVATION OF RESPONDER T CELLS

S. Raghavan<sup>1</sup>, J. Herrath<sup>1</sup>, P. Amoudruz<sup>1</sup>, M. Müller<sup>1</sup>, P.T. Larsson<sup>1</sup>, C. Trollmo<sup>1</sup>, V. Malmström<sup>1</sup>

<sup>1</sup>Karolinska University Hospital Solna, Karolinska Institute, Rheumatology Unit, Department of Medicine, Stockholm, Sweden

**Objective:** Higher frequencies of FOXP3 are generally found in CD4<sup>+</sup> T cells from synovial fluid compared to peripheral blood. Here we aimed to dissect if this increase was mainly found in the classical CD25bright compartment and secondly if the FOXP3 frequency was directly linked to suppressive capacity.

**Methods:** Paired peripheral blood and synovial fluid samples were obtained from 9 patients with inflammatory arthritis. From 8 of the 9 patients additional paired blood and synovial fluid samples were obtained from several relapses. Mononuclear cells were stained for CD3, CD4, CD25 and FOXP3. Cytokines (IL-1β, IL-6 and

(TNF) in synovial fluid of the same patients was measured by ELISA. Cells were sorted into CD25bright, CD25int, and CD25-CD4+ T cells. Proliferation was measured in co-culture experiments set up with irradiated autologous CD3- APC, CD25-CD4+ responder T cells and/or CD25brightCD4+ cells. Analysis of cytokines (IFN- $\gamma$ , IL-17, IL-10, IL-13, TNF and IL-6) in supernatants of responder CD25- T cells cultured alone or together with CD25brightT cells was performed.

**Results:** In spite of some variations in FOXP3 frequency at individual relapses the overall frequency of FOXP3 in the CD4+ T cell population was consistently higher in synovial fluid compared to blood. Correlation analysis showed a significant inverse correlation between level of FOXP3 in the CD4+ T cells compartment and IL-6 in the joint fluid. The suppressive function of sorted CD25bright Treg was in some cases related to FOXP3 expression but in others to the activation status of the CD25- responder T cells.

**Conclusion:** Our data suggest that there is no general defect in the synovial FOXP3 Treg population from inflammatory arthritis patients. Consequently, the suppression of inflammation in the joint most likely depends on several parameters such as the FOXP3 frequency in CD4+ T cells, the cytokine milieu, but also on the activation status of cells targeted by the Treg cells.

#### PC13/25 RAPAMYCIN-TREATED DENDRITIC CELLS AS TOLEROGENTIC CELL-BASED THERAPY IN ARTHRITIS

J. Quentin<sup>1</sup>, L.-M. Charbonnier<sup>2</sup>, C. Jorgensen<sup>3</sup>, P. Louis-Plence<sup>1</sup>

<sup>1</sup>inserm 844, Université Montpellier 1, Montpellier, France, <sup>2</sup>Institute for Medical Immunology/Université Libre de Bruxelles, Bruxelles, Belgium, <sup>3</sup>inserm 844, Université Montpellier 1, CHU Lapeyronie, Montpellier, France

**Background:** Dendritic cells (DC) are professional antigen-presenting cells which have unique properties to steer the outcome of immune responses. We previously demonstrated that repetitive injections of immature DCs (iDC) was able to protect mice from developing collagen-induced arthritis (CIA). Rapamycin is a macro-lide antibiotic with potent immunosuppressive properties introduced in recent years as therapy in organ transplantation. The immunosuppressive activity of rapamycin has been ascribed primarily to its inhibitory effects on T cells. More recently the effect of rapamycin on differentiation, antigen uptake, and immunostimulatory capacity of human DC was examined. In the present study we investigated the effect of rapamycin on the phenotype and the immunogenicity of iDC in mixed-lymphocyte reaction, and evaluated the tolerogenic potential of such rapamycin-treated DC in the CIA mouse model of arthritis.

**Methods:** Bone marrow-derived DCs were generated in the presence of 10ng/ml of rapamycin during 6 days. Maturation of rapamycin-treated DC was induced or not by addition of LPS. DC cytokine secretion profiles were quantified by ELISA and their phenotype monitored by facs analysis. In *in vivo* experiments, repetitive injections of 5x10<sup>5</sup> rapamycin-treated DC was performed in naive mice and regulatory populations monitored in various lymphoid organs. In the CIA mouse model, repetitive injections of iDC or rapamycin-treated DC were performed 3 weeks after their immunization.

**Results:** The phenotype of rapamycin-treated DC was slightly different from conventional iDC, with a decreased expression of CD40 and co-stimulatory molecules such as CD86. The decrease of the immunogenicity of rapamycin-treated DC was confirmed in MLR. In *in vivo*, we observed an increase of the IL10-secreting CD4<sup>+</sup> CD49b<sup>+</sup> T lymphocytes in the liver and spleen of the mice injected with the rapamycin-treated DC. After their repetitive injections in arthritic mice, a decrease of the IFN- $\gamma$  secretion was observed in the liver of DC-treated mice, leading to a decrease of the severity of established arthritis more importantly than following injection of conventional iDC.

**Conclusion:** Our results indicate that the vaccination with rapamycin-treated DC has an impact on the clinical outcome of autoimmune arthritis, suggesting that the tolerogenic potential of iDC is reinforced when they are generated in presence of rapamycin.

#### PC13/26 HIGHER LEVELS OF FAS-EXPRESSING B-CELLS AND BETTER CLINICAL RESPONSE TO B-CELL DEPLETION THERAPY IN EBV-INFECTED RA PATIENTS

M. Magnusson<sup>1</sup>, M. Brissert<sup>1</sup>, M. Rhenberg<sup>1</sup>, S. Amu<sup>2</sup>, K. Zendjanchi<sup>1</sup>, M. Lindh<sup>3</sup>, M.I. Bokarewa<sup>1</sup>

<sup>1</sup>Göteborgs Universitet, Rheumatology & Inflammation Research, Göteborg, Sweden, <sup>2</sup>Institute of Molecular Medicine, Trinity Centre, Dublin, Ireland, <sup>3</sup>Sahlgrenska University Hospital, Göteborg, Sweden, Department of Virology, Göteborg, Sweden

Viruses may be part of the pathogenesis in RA. Also, susceptibility to viral infection may be increased in RA patients as a result of immunosuppressive therapy. This prompted us to relate viral status (EBV, parvovirus B19, CMV, polyoma, HSV1 and HSV2) in RA patients to clinical response during the course of immunosuppressive therapy with rituximab (anti-CD20, B-cell depleting antibodies).

Replication of EBV was identified using RT-PCR in bone marrow from 15 of 35 patients with RA (EBV+ group) of which 4 also expressed parvovirus. Parvovirus was further detected in 8 more patients (parvo+ group). The remaining 12 patients expressed none of the analysed viruses (virus-negative group). The clinical activity of RA did not vary between the groups – the mean of DAS 28 levels were 5.9 in the EBV+ group, 6.1 in the virus-negative group and 5.5 in the Parvo+ group. All the patients received anti-CD20 treatment and 6 months post treatment, the frequency of responders (Change in DAS 28  $\geq 1.3$ ) was significantly higher in the EBV+ group (87%) as compared to virus-negative group (45%), which was also reflected in significantly lower DAS28-values at six months post treatment. In the parvo+ group 62.5% were responders, which was not significantly different from other groups. The levels B-cells, rheumatoid factor, and total Ig (A, G and M) did not differ between patient groups. However, EBV-infected patients had significantly higher levels of CD95+ (FAS/APO-1) B-cells at baseline as compared to EBV-negative groups. Also, the shared epitope (HLA DRB1, genotype 0101) was more common in the EBV+ group (46%) as compared to the virus-negative group (8%). Following anti-CD20 treatment, all EBV-infected patients cleared their EBV-infection from the bone marrow and the circulation, while parvovirus was still detectable in all parvovirus-infected patients.

EBV and parvovirus genomes are found in bone marrow of a significant number of therapy resistant RA patients. Presence of EBV was clearly associated with a better clinical response to B-cell depletion using anti-CD20 antibodies, possibly due to increased susceptibility to apoptosis in B-cells due to higher expression of the apoptosis-inducing receptor FAS/APO-1 found in this patient group (EBV+ group).

#### PC13/27 MODULATION THE FC GAMMA RECEPTORS EXPRESSION IN RHEUMATOID ARTHRITIS BY NEUTROPHIL DEGRANULATION PROCESS

M. Bostan<sup>1</sup>, G.G. Matei<sup>1</sup>, M. Hirt<sup>1</sup>, S. Marineata<sup>2</sup>, L.I. Brasoveanu<sup>1</sup>

<sup>1</sup>Institute of Virology Stefan S. Nicolau, Center of Immunology, Bucharest, Romania, <sup>2</sup>Medical Center Vademecum, Rheumatology, Bucharest, Romania

Activation of neutrophils (PMNs) through Fc gamma Receptors initiates functional responses such as oxidative activity, degranulation and phagocytosis. We investigated the effect of FMLP (N-formyl-methionyl-leucyl-phenylalanine) on FcgammaR expression associated to neutrophils isolated from patients with rheumatoid arthritis (RA-PMNs). Roles of the microfilament and cytoskeletal apparatus in this process were evaluated using cytochalasin B (CB), an inhibitor of microfilament functions. Therefore, PMNs were treated with FMLP and/or CB, and expressions of Fc gammaRIIb and Fc gammaRIIa were analysed by flow cytometry. FMLP induces significant changes in the expression of Fc gammaRs in RA and show that CB enhanced FMLP-induced FcgammaRs down-regulation. In addition we investigated the role of secretory products from FMLP and CB stimulated RA-PMNs on Fc gammaRs expression and observed that they significantly reduce the Fc gamma receptors expression on the RA-PMNs. The down-regulation of both FcgammaRIIb and FcgammaRIIa is completely abolished by PMSE, supporting the role of serine proteases in this process. All these data confirmed the role of PMN-released products on FcgammaRs down-regulation in RA. In addition, we studied the degranulation of RA-PMNs induced by combination of CB and FMLP treatment, evaluated by b-glucuronidase release. RA-PMNs degranulate in response to FMLP and this effect was clearly enhanced by CB. Our experiments show that combination of FMLP and CB, or the secretory products of activated PMNs can also inhibit the phagocytosis process of PMNs in RA. FMLP may not only exert an efficient inflammatory response, but also neutralize this phenomenon through inhibition of different FcgammaRs functions.

Key words: rheumatoid arthritis, neutrophils, Fc gamma Receptors, degranulation, flow cytometry

#### PC13/28 HO-1 END PRODUCTS BILIVERDIN AND CARBON MONOXIDE REVEAL BENEFICIAL EFFECTS IN MURINE COLLAGEN INDUCED ARTHRITIS

M. Bonelli<sup>1</sup>, A. Savitskaya<sup>1</sup>, E. Rath<sup>1</sup>, F. Bach<sup>2</sup>, J.S. Smolen<sup>1</sup>, C. Scheinecker<sup>1</sup>

<sup>1</sup>Medical University of Vienna, Vienna, Austria, <sup>2</sup>Harvard Medical School, Boston, United States

**Introduction:** Heme oxygenase 1 (HO-1) which degrades Heme to free iron, biliverdin and carbon monoxide (CO) plays an important role in inflammation. It has already been shown that HO-1 also regulates osteoclastogenesis which is driven by inflammatory cytokines. There are, however, conflicting data regarding the role of HO-1 in rheumatoid arthritis since treatment with tin protoporphyrin IX, an inhibitor of HO-1, significantly reduced joint inflammation and cartilage destruction in the murine collagen-induced arthritis model (CIA). We therefore investigated the role of CO and biliverdin in the CIA mouse model.

**Methods:** CIA was induced in DBA/1 mice at week 12 of age. On day 0 mice were immunized by chicken collagen II (CII), emulsified in complete Freund's adjuvant. After fourteen days mice received a booster injection of CII. Mice were scored for paw swelling and grip strength. Anti-CII antibody levels were determined by ELISA. In addition serum samples were analyzed by Flow Cytomix Multiplex Kits for IFN-gamma, IL-6 and IL-17 on day 30. After the first clinical signs of arthritis one group of animals was treated with Biliverdin (35mg/kg) twice daily for 14 days. Bilirubin serum levels were measured 15 minutes post intraperitoneal injection of Biliverdin. The second group was treated for 14 days with CO (250 ppm) for one hour. After 50 days all animals were sacrificed and paraffin sections of the affected joints were analyzed for histomorphologic signs of inflammation, cartilage and bone destruction.

**Results:** All animals immunized with CII developed serum anti-CII antibodies. Antibody levels were slightly decreased in Biliverdin and, even more pronounced, in the CO-treated group. Animals in both the Biliverdin and the CO-treated group showed an improvement in signs of clinical disease activity as compared to control animals. In addition decreased serum levels of IL-6, IL-17 and IFN-gamma were observed on day 30 in CO and Biliverdin treated animals. In line with the clinical data, histological analysis revealed less inflammation, erosion and reduced numbers of osteoclasts in both treatment groups.

**Conclusion:** Our data demonstrate a beneficial effect of HO-1 on inflammation and bone destruction in a murine model of RA.

**PC13/29 A NOVEL RECOMBINANT VACCINE EFFECTIVELY INDUCES C5A-SPECIFIC NEUTRALIZING ANTIBODIES AND PREVENTS ARTHRITIS**K. S. Nandakumar<sup>1,2</sup>, Å. Jansson<sup>3</sup>, N. Rydell<sup>3</sup>, A. Blom<sup>4</sup>, C. Lundberg<sup>3</sup>, R. Holmdahl<sup>1,2</sup><sup>1</sup>Karolinska Institutet, Medical Inflammation Research, Department of Medical Biophysics and Biochemistry, Stockholm, Sweden, <sup>2</sup>Lund University, Medical Inflammation Research, Department of Experimental Medicine, Lund, Sweden, <sup>3</sup>Resistentia Pharmaceuticals AB, Uppsala, Sweden, <sup>4</sup>Lund University, Department of Laboratory Medicine, Malmö, Sweden

Complement factor 5 (C5) is crucial for anti-bacterial immunity, in connecting innate and adaptive immune responses and in the removal of immune complexes and inflammatory products. The anaphylatoxin C5a, an activation product of C5, is an important mediator of inflammation by attracting effector cells to the inflammatory foci. Several attempts have been made to neutralize the effect of C5 and C5a during inflammation for amelioration of the clinical disease. Most importantly, neutralization of C5a without affecting the essential function of C5 becomes attractive to attenuate inflammation without compromising the host defense against infections. Sustained neutralization of C5a by exploitation of host immunity will be more optimal and cost effective for therapeutics. Hence, we have constructed and expressed fusion proteins of C5a and maltose binding protein (MBP) and used them for vaccination against arthritis. C5a-specific antibodies were induced in mice vaccinated with C5a-MBP recombinant proteins and sera from these mice neutralized the activation of C5a but not C5. We tested the efficacy of specific C5a neutralization in various arthritis mouse models [collagen induced arthritis (classical and relapsing models) and collagen antibody induced arthritis]. Vaccination of mice with C5a-MBP led to significant reduction of arthritis incidence and severity during both the acute and chronic phases of the disease. Furthermore, C5a-MBP vaccination was also significantly reduced a relapsing chronic arthritis in mice. Exploitation of host immune response to generate C5a neutralizing antibodies without compromising C5 activity is a new strategy for developing highly effective vaccine for pathogenic antibody dependent inflammatory diseases.

**PC13/30 FREQUENCY OF RS3761847 SNP (TRAF1/C5 REGION) IN NORTHERN GREEK PATIENTS WITH RHEUMATOID ARTHRITIS. ASSOCIATION WITH DISEASE ACTIVITY AND CLINICAL RESPONSE**A. Sarantopoulos<sup>1</sup>, P. Boura<sup>1</sup>, C. Nakas<sup>2</sup>, K. Tselios<sup>1</sup>, G. Papaioannou<sup>1</sup>, I. Theodorou<sup>3</sup><sup>1</sup>Aristotle University of Thessaloniki, Hippokraton Hospital, Clinical Immunology Unit, 2nd Department of Internal Medicine, Thessaloniki, Greece, <sup>2</sup>Laboratory of Biometry, University of Thessaly, School of Agricultural Sciences, Volos, Greece, <sup>3</sup>Laboratoire Central d'Immunologie Cellulaire et Tissulaire, CHU Pitié Salpêtrière (AP-HP), INSERM U543, Université Pierre et Marie Curie, Paris, France

Genome Wide Association studies have permitted identification of new polymorphic genetic loci linked to autoimmune diseases. A SNP (rs3761847) on chromosome 9 at the TRAF1/C5 region is linked to susceptibility to Rheumatoid Arthritis (RA) in large cohorts mainly including Caucasians recruited both in Europe and Northern America.

**Objectives:** Aim of the present study is to identify possible correlations of rs3761847 SNP not only with the development of RA in Greek patients, but also with disease activity and clinical response to anti-TNF treatment.

**Methods:** 180 RA patients (age 59.5±13.5) and 100 healthy individuals have been studied for allelic discrimination (adenine/guanine) on the rs3761847 position.  $\chi^2$  test was performed to identify differences between the two populations. RA patients were further sub-grouped and analyzed regarding disease activity or response to administered anti-TNF treatment.

**Results:** A/G prevalence on RA patients and controls did not vary significantly. (A/G=58,85/41,15 in RA patients and 59,02/40,98 in controls). 38/180 patients with severe RA were receiving anti-TNF treatment. On this subgroup, prevalence of A was 50,88% and G 49,12%, whereas on the rest of RA patients the rates were 61,3% and 38,7% respectively (table 1).

	A (%)	G (%)
RA patients receiving anti-TNF therapy (38/180)	50,88	49,12
RA patients receiving DMARDs (142/180)	61,3	38,7

[Table 1. Allelic discrimination on RA patients]

When patients were further sub-grouped regarding responsiveness to anti-TNF treatment, the A/G ratio between responders and non responders did not differ.

**Conclusion:** On this study, alleles A or G were not found to be associated with the development of RA. However allele G carriers showed a more severe pattern of disease. This indicates that in northern Greek population, allele G is not associated with the development of RA but once RA is established, its presence is associated with a more severe form of disease. Genetic profiles between different ethnical populations may vary, accompanying different patterns of disease. Study of TRAF1/C5 area is of immediate interest.

**PC13/31 NAPA OF BORRELIA BURGDORFERI DRIVES TH17 INFLAMMATION IN LYME ARTHRITIS**G. Codolo<sup>1,2</sup>, A. Amedi<sup>3</sup>, A.C. Steere<sup>4</sup>, E. Papinutto<sup>1,5</sup>, A. Polenghi<sup>1</sup>, G. Del Prete<sup>6</sup>, C.T. Baldari<sup>7</sup>, G. Zanotti<sup>5</sup>, C. Montecucco<sup>2</sup>, M.M. D'Elia<sup>3,6</sup>, M. de Bernardi<sup>1,8</sup><sup>1</sup>Venetian Institute of Molecular Medicine, Padova, Italy, <sup>2</sup>University of Padua, Department of Experimental Biomedical Sciences, Padova, Italy, <sup>3</sup>University of Florence, Department of Internal Medicine, Florence, Italy, <sup>4</sup>Center for Immunology and Inflammatory Diseases, Division of Rheumatology, Allergy and Immunology, Massachusetts General Hospital, Harvard Medical School, Boston, United States, <sup>5</sup>University of Padua, Department of Chemistry, Padova, Italy, <sup>6</sup>Azienda Ospedaliera Universitaria Careggi, Department of Biomedicine, Florence, Italy, <sup>7</sup>University of Siena, Department of Evolutionary Biology, Siena, Italy, <sup>8</sup>University of Padua, Department of Biology, Padova, Italy

**Objective:** This study was undertaken to evaluate the role of the innate and acquired immune responses elicited by the protein NapA of *Borrelia burgdorferi* in Lyme arthritis, which is characterized by an inflammatory infiltrate, consisting mainly of neutrophils and T cells. In the past years Th1 cells were proposed to play a central role in Lyme arthritis. More recently Th17 cells were shown not to be essential in inducing Lyme arthritis in mice suggesting that other mediators and T cells are involved. In particular the attention was focused on Th17 cells.

**Methods:** The cytokine profile of synovial fluid T cells specific for NapA was investigated in 5 patients with Lyme arthritis. It was also evaluated the cytokine and chemokine profile induced by NapA in neutrophils, monocytes/macrophages and endothelial cells. Finally, the effect of protein administration in vivo, in rat synovia, was considered.

**Results:** T cells from synovial fluid of patients with Lyme arthritis produced interleukin (IL)-17 in response to NapA. In agreement with these data, NapA was found to induce the expression of IL-23 in neutrophils and monocytes, and IL-6, IL-1 $\beta$  and transforming growth factor (TGF)- $\beta$  in monocytes, all cytokines crucial for the differentiation of the Th17 subset. Finally, we demonstrated in vivo that NapA is able to recruit lymphocytes in rat synovia. Accordingly, it was found to promote the release of monocytes- and lymphocytes-attractive chemokines from endothelial cells and macrophages.

**Conclusion:** Collectively our results suggest that NapA might be one of the major bacterial products of *Borrelia burgdorferi* responsible for triggering and sustaining inflammation within synovia, with a strong ability to recruit monocytes and lymphocytes from the blood. Moreover, we found that NapA is able to drive the expression of IL-6, IL-1 $\beta$ , IL-23, and TGF- $\beta$  by innate immune cells and, in virtue of such an activity, to elicit a synovial T helper 17 response which, in turn, is expected to play a crucial role in the pathogenesis of Lyme arthritis.

**PC13/32 THE ROLE OF IL-1BETA, IL-6, TGF-BETA1 AT THE PATHOGENESIS OF RHEUMATOID ARTHRITIS**R. Yatsyshyn<sup>1</sup>, Y. Neyko<sup>1</sup>, N. Yatsyshyn<sup>1</sup><sup>1</sup>Ivano-Frankivsk National Medical University, Internal Medicine, Ivano-Frankivsk, Ukraine

**Background:** The role of TGF-beta1 in Rheumatoid Arthritis is considered protective, whereas that of IL-6 both protective and pro-inflammatory. Among a variety of actions, TGF-beta1 antagonizes the action of IL-1beta, inhibits the expression of collagenase-1 and stimulates the production of the metalloprotease inhibitor-1 (TIMP-1). IL-6, alone or in combination with its soluble receptor induces the TIMP-1 expression in human synoviocytes and chondrocytes and blocks IL-1-induced collagenolytic activity, but also it stimulates bone resorption and the production of MMP-13 by fibroblasts. It's well documented that TGF-beta1 stimulates the production of IL-6 by lung fibroblasts and epithelial cells.

**Objectives:** The purpose of our study was to investigate the role of TGF-beta1 and IL-6 in the human synovial fibroblasts and chondrocytes at the RA, since definitive data about these cells are not available.

**Methods:** Fibroblasts were isolated from synovial membrane and articular cartilage obtained from 9 patients with RA undergoing joint arthroplasty. All experiments were performed with cells of 3rd to 5th passages. The IL-6 levels in cell culture conditioned media were determined by ELISA. Total RNA was extracted from cells by RNeasy mini kit and RT-PCR was performed by one-step kit.

**Results:** Fibroblasts were cultured in serum-free DMEM for 48h in the presence or absence of TGF-beta1 (1ng/ml). In the presence of TGF-beta1, the IL-6 levels in conditioned medium significantly increased, (2.2-4.6times) and (3.4-5.2 times) for fibroblasts, respectively. The observed increase was dose (0.01-1ng/ml) and time (12-48h) dependent. RT-PCR indicated that fibroblasts up-regulated IL-6 mRNA in response to TGF-beta1 (0.01-1.0ng/ml) in a concentration-dependent manner. Using specific protein kinases inhibitors, it was found that only calphostin C significantly suppressed the TGF-beta1-induced IL-6 expression. When fibroblasts were incubated with 1ng/ml TGF-beta1 for 24 or 48h in the presence of indomethacin (1ug/ml), a decrease of IL-6 concentration (24%) was observed.

**Conclusion:** It was demonstrated that TGF-beta1 significantly enhances the production of IL-6 by human synovial fibroblasts. The IL-6 induction by TGF-beta1 is carried out by protein kinase C-dependent pathway. Therefore, the role of TGF-beta1 should not be considered purely protective since, under different circumstances, it may promote inflammatory or anti-inflammatory activities, via induction of IL-6.



**PC13/33 ANTI-TYPE II COLLAGEN-IC-INDUCED PRODUCTION OF IL-1 $\beta$  AND TNF- $\alpha$ , STIMULATE PRODUCTION OF MATRIX METALLOPROTEINASES FROM MONOCYTES/RHEUMATOID ARTHRITIS SYNOVIAL FIBROBLAST CO-CULTURES**M. Mullazehi<sup>1</sup>, L. Mathsson<sup>1</sup>, O. Feldt<sup>1</sup>, K. Schubert<sup>1</sup>, M. Korotkova<sup>2</sup>, V. Malmström<sup>2</sup>, J. Rönnelid<sup>1</sup><sup>1</sup>Uppsala University, Unit of Clinical Immunology, Uppsala, Sweden, <sup>2</sup>Department of Medicine, Unit of Rheumatology, Karolinska Institutet, Stockholm, Sweden

**Objective:** To establish an in vitro model that might explain the association between early joint destruction and the appearance of collagen type II (CII) antibodies in early Rheumatoid Arthritis (RA) patients. This model utilizes immune complexes (IC) containing CII-antibodies as stimulus and macrophages and synovial fibroblasts, two principal cell types in RA pannus tissue, as responder cells.

**Methods:** Peripheral blood mononuclear cells (PBMC) and RA synovial fibroblasts (RASf) were stimulated with IC individually as well in co-cultures. Monocytes were depleted to define the responder cells, and TNF- $\alpha$  and IL-1 $\beta$  were neutralized to study the effect on MMP production. TNF- $\alpha$ , IL-1 $\beta$ , MMP-1, MMP-8 and MMP-13 were measured in cell culture supernatants using ELISA.

**Results:** IC induced production of TNF- $\alpha$ , IL-1 $\beta$  and MMP-1 in PBMC cultures, and TNF- $\alpha$ , IL-1 $\beta$ , MMP-1 and MMP-8 in PBMC/fibroblast co-cultures, in a dose-dependent manner. IC-induced MMP-1 responses were stronger and more associated with induced production of IL-1 $\beta$  as compared to MMP-8 responses. Baseline production of IL-1 $\beta$  and MMP-1 increased significantly in co-cultures as compared to individual cultures, whereas this was not the effect for TNF- $\alpha$  and MMP-8. Monocyte depletion decreased TNF- $\alpha$ , IL-1 $\beta$  and MMP-1 production, while the effect on MMP-8 production was variable. Cytokine neutralization revealed that IL-1 $\beta$  was a stronger inducer of MMP-1 than was TNF- $\alpha$ .

**Conclusion:** Synergistic actions between (RASf) and PBMC result in enhanced anti-CII IC-induced production of IL-1 $\beta$  and MMP-1. IL-1 $\beta$  and MMP-1 are regulated in parallel as anti-CII IC-induced IL-1 $\beta$  supports the production of MMP-1. MMP-8 seems to be regulated by other means. Anti-CII IC-induced TNF- $\alpha$  seems to be inferior to IL-1 $\beta$  concerning MMP-1 induction. The fact that IC stimulated synovial macrophages and fibroblasts to produce MMPs which are the first enzymes to cleave the interstitial collagens may explain the anti-CII-associated joint destruction apparent in early RA.

**PC13/34 REGULATORY T-CELLS FROM RHEUMATOID ARTHRITIS PATIENTS ARE DEFINED BY SPECIFIC MICRORNA SIGNATURES**K. Smigielska<sup>1</sup>, P. G. Jellema<sup>1</sup>, M. van West<sup>1</sup>, J. Westra<sup>2</sup>, M. Boots<sup>3</sup>, C. G. M. Kallenberg<sup>2</sup>, L. F. M. H. de Leij<sup>1</sup>, A. van den Berg<sup>1</sup>, E. Brouwer<sup>2</sup>, B. J. Kroesen<sup>1</sup><sup>1</sup>University Medical Center Groningen, Pathology & Medical Biology, Groningen, Netherlands, <sup>2</sup>University Medical Center Groningen, Rheumatology & Clinical Immunology, Groningen, Netherlands, <sup>3</sup>Schering Plough Research Institute, Immunology, Oss, Netherlands

**Objectives:** miRNAs have emerged as driving factors in disorders that arise from deranged developmental processes. Current knowledge provides a firm conceptual foundation to presume pathophysiological involvement of specific miRNAs in the development, progression and activity of autoimmune diseases. In this study we set out to establish miRNA expression signatures of effector T-cells (Teff) and regulatory T-cells (Tregs) derived from healthy controls (HC) and Rheumatoid Arthritis (RA) patients.

**Methods:** CD4+/CD25- (Teff) and CD4+/CD25++ (Treg) T-cell subsets from untreated RA patients as well as sex and age matched HC were FACS sorted. We used a quantitative RT-PCR based miRNA profiling assay allowing the analysis of 180 different miRNAs.

**Results and conclusions:** Approximately 10 miRNAs showed very high expression levels with Ct-values between 20 and 25 in the CD4+ cells. From this top-10 list of most abundantly expressed miRNAs, 9 were present both in the RA patients and HC and thus comprise a general (CD4+) T-cell signature.

Differential expression between Tregs and Teff cells in general was determined. Both in RA patients and HC we found a number of miRNAs to be differentially expressed between Tregs and Teff cells irrespective of disease. In addition, a number of miRNAs were shown to be differentially expressed in Tregs from RA-patients compared to HC, as well as in Teff cells from RA patients compared to HC. This indicates that Tregs and Teff are characterized by specific miRNA signatures and that the miRNA signature of Tregs and Teff in RA-patients is different from that of HC.

In silico analysis of putative targetgenes was performed for the miRNAs that were most differently expressed between Treg and Teff cells of the RA patients and HC. This resulted in a number of candidate targetgenes involved in T-cell receptor signaling, regulators of cytoskeletal function, regulators of MAPK and calcium signaling, and regulators of cell migration. A number of putative targetgenes is recognized by more than one of the identified miRNAs. This is consistent with the concept that multiple different miRNAs regulate the same targetgene and co-evolution of specific signaling pathways with miRNAs acting as master switches in these pathways.

**PC13/35 IMMUNOPHENOTYPE AND FUNCTIONAL STUDY OF B AND T CELL EMERGING SUBCLASSES AFTER ANTI-CD20 TREATMENT IN PATIENTS WITH RHEUMATOID ARTHRITIS. CORRELATIONS TO CLINICAL RELAPSE AND PATHOPHYSIOLOGY OF RA**A. Sarantopoulos<sup>1</sup>, K. Tselios<sup>1</sup>, P. Skendros<sup>1</sup>, S. Kamali<sup>1</sup>, I. Gougourelas<sup>1</sup>, P. Boura<sup>1</sup><sup>1</sup>Hippokraton Hospital, Aristotle University of Thessaloniki, Clinical Immunology Unit, 2nd Department of Internal Medicine, Thessaloniki, Greece

B and T cells share an important role on maintaining inflammation in rheumatoid pannus. Nevertheless, T-B cell interactions after B cell depletion therapy have not been thoroughly studied.

**Aim of this study** is to analyze emerging B cell subpopulations and T cell immunophenotype after rituximab treatment and to identify possible markers of RA flair.

**Materials and methods:** 22 RA patients (58.5 $\pm$ 7.7 age, 164 $\pm$ 107 duration of disease) have been studied. Patients received rituximab for at least 1.5 year and were assessed every 2 months for clinical response to treatment (DAS28, HAQ-DI) and changes of the serological profile (ESR, CRP, RFs and anti-CCPs). On the same evaluation periods, patients were assessed for T cell and B cell subpopulations (T cells for CD3, CD8, CD25, CD28, CD40L and B cells for CD40, CD45RO/RA, HLADR). Immunoglobulin production was also estimated by measuring Ig classes and IgG subclasses.

**Results:** Rituximab therapy resulted in decrease of DAS28 score, functional impairment improvement (HAQ-DI < 0.8 in mean) and lowering of CRP. ESR and auto antibodies (RFs and anti-CCPs) did not further increase. Analysis of T cell populations revealed that CD4+/CD25+ cells diminished. CD40L expression on CD4+ cells did not statistically change. Emerging B-cell populations strongly expressed CD40, CD45RA but not HLADR. Immunoglobulin production and IgG subclasses were not strongly altered.

**Conclusions:** T cell populations after rituximab therapy rearranged their activation phenotype, indicating alternative pathways of T activation (CD40L) following B cell depletion. CD40L must be further studied, as it seems that it may be used to predict RA relapse after B cell depletion therapy. Emerging B cells highly express activation and memory phenotype. Germinal centers in rheumatoid pannus may contribute to the development of such phenotype. Immunoglobulin production did not significantly alter, indicating that rituximab therapy does not seem to affect antibody mediated immunological responses. Correlations of these findings regarding T and B cell homeostasis with immunohistochemical pannus pathology may further illuminate RA pathogenesis.

**PC13/36 EFFECTS OF AN ANTAGONIST OF THE BOMBESIN/GASTRIN-RELEASING PEPTIDE RECEPTOR ON COLLAGEN-INDUCED ARTHRITIS**P. G. Oliveira<sup>1</sup>, L. G. Pinto<sup>2</sup>, R. Grespan<sup>2</sup>, R. Roesler<sup>3</sup>, G. Schwartzmann<sup>4</sup>, F. Q. Cunha<sup>2</sup>, R. M. Xavier<sup>1</sup><sup>1</sup>HCPA/UFRGS, Rheumatology, Porto Alegre, Brazil, <sup>2</sup>USP-RP, Pharmacology, Ribeirão Preto, Brazil, <sup>3</sup>UFRGS, Pharmacology, Porto Alegre, Brazil, <sup>4</sup>HCPA/UFRGS, Internal Medicine, Porto Alegre, Brazil

**Objective:** To evaluate the effects of RC-3095, a specific antagonist of the gastrin-releasing peptide receptor, as anti-inflammatory therapy in collagen induced arthritis.

**Methods:** 20 male DBA/1J mice were randomly divided into control (not manipulated), placebo (saline administered subcutaneously 50 ml/kg, twice a day for 10 days after onset the disease) and treatment (0.3 mg/kg of RC-3095 administered subcutaneously twice a day for 10 days after onset the disease) groups. The arthritis was induced on the day 0 with an intradermal injection of 50  $\mu$ l of emulsion with 200 mg of bovine type-II collagen in complete Freund's adjuvant 1.5 cm distal from the base of the tail. The animals were monitored daily for signs of arthritis, and the treatment began 1 day after CIA became clinically detectable. On the 11th day the hind paws were collected for joint histology and immunohistochemistry for gastrin-releasing peptide receptor. Clinical evaluation was accomplished daily, through standardized scoring of the edema, articular index and hypernociception. Differences between experimental groups were compared by Mann-Whitney test.

**Results:** Treatment with RC-3095 (0.3mg/Kg, s.c., twice a day) significantly ameliorated signs of CIA, as assessed by a decrease in all clinical features and inhibitory effect on neutrophil influx. There were significant differences in the articular index, oedema and mechanical hypernociception compared to placebo group ( $p=0.001$ ,  $p=0.002$  and  $p=0.003$ , respectively). RC-3095 treated animals had a remarkable inhibition of all histological findings of arthritis, presenting only mild inflammatory infiltration and nearly normal joint architecture of the posteriors paws. The immunohistochemistry studies demonstrated a markedly increased expression of GRP receptor in the inflammatory infiltrate, synovial tissue and cartilage in the animals of the placebo group with the severe articular inflammation. RC-3095 significantly decreased this GRPR expression ( $p=0.041$ ).

**Conclusions:** RC-3095 was able to improve experimental arthritis and attenuate joint damage in all clinical, histological and immunohistochemistry parameters studied. These data indicate that interference with the neuropeptide GRP pathway is a potential new strategy for the treatment of arthritis.

HCPA/FIPE, CAPES, CNPq

# PC13/37 A GENETIC PREDISPOSITION UNDERLIES INCREASED MATRIX METALLOPROTEINASE-7 (MMP-7) EXPRESSION IN RHEUMATOID INFLAMMATION

M. Kazantseva<sup>1</sup>, J. Highton<sup>2</sup>, P. Hessian<sup>1</sup><sup>1</sup>University of Otago, Physiology, Dunedin, New Zealand, <sup>2</sup>University of Otago, Medical and Surgical Sciences, Dunedin, New Zealand

**Objective:** There is considerable tissue destruction associated with inflammation in rheumatoid arthritis (RA). Microarray data suggested increased levels of matrix metalloproteinase-7 (MMP-7) transcript in rheumatoid subcutaneous nodule tissue. This study investigated the links between two functional single nucleotide polymorphisms (SNPs), -181A/G and -153C/T, in the promoter of MMP-7 gene and increased MMP-7 expression in nodule tissue.

**Methods:** MMP-7 gene expression was compared in 24 rheumatoid nodules and 11 synovial membranes from 32 patients with RA using real-time PCR (RT-PCR) analysis. Paired samples were available from 3 patients. Genomic DNA was extracted from slices of frozen nodule tissue. Genotypes for the MMP-7 -181(A/G) and -153(C/T) polymorphisms were analysed using restriction fragment length polymorphism (RFLP) analysis. The Kruskal-Wallis test was used to compare MMP-7 expression in different genotype groups. Inflammatory cells producing MMP-7 protein were identified using double immunofluorescence with antibodies specific for MMP-7 (pro- and active enzyme), and CD14 (a macrophage marker).

**Results:** Seven of 24 nodules (29%) showed MMP-7 expression above the threshold value (1.20 ng RNA; nodule group mean  $\pm$  SE). This group demonstrated significantly higher levels of MMP-7 transcript ( $2.73 \pm 0.65$  ng RNA; mean  $\pm$  SE) when compared to remaining nodules ( $0.14 \pm 0.05$  ng RNA;  $P < 0.001$ ) or to synovial tissues ( $0.11 \pm 0.02$  ng RNA;  $P < 0.001$ ). MMP-7 expression was significantly higher in rheumatoid nodule tissue from RA patients homozygous for G allele at position -181 within the MMP-7 promoter as compared to the wild-type, homozygous for the A allele ( $2.35 \pm 1.03$  vs.  $0.06 \pm 0.02$  ng RNA;  $P < 0.01$ ). Immunofluorescent staining showed MMP-7 is produced by a subset of CD14<sup>+</sup> monocyte/macrophages infiltrating the nodule.

**Conclusion:** Based on MMP-7 gene expression rheumatoid subcutaneous nodules can be divided into two distinct groups, with high and low MMP-7 expression. Monocyte/macrophages infiltrating the nodule are responsible for the MMP-7 production. We demonstrated for the first time in an inflammatory situation, an *in vivo* association between the -181A/G polymorphism and increased MMP-7 expression in rheumatoid nodule lesions. Our findings provide a first step towards understanding the molecular mechanisms for increased human MMP-7 gene expression in rheumatoid inflammation.

# PC13/38 LACK OF RESPONSE TO CLASSIC TREATMENT IS ASSOCIATED WITH ANTI-CYCLIC CITRULINATED PEPTIDE ANTIBODIES BUT NOT WITH DRB1-SE STATUS IN PATIENTS WITH RECENT-ONSET RHEUMATOID ARTHRITIS

B. Manzanares<sup>1</sup>, F. Martinez<sup>2</sup>, R. Gonzalez<sup>1</sup>, M. Frias<sup>1</sup>, L. Castro<sup>1</sup>, E. Collantes<sup>2</sup>, J. Peña<sup>1</sup><sup>1</sup>Reina Sofia Hospital, Immunology, Cordoba, Spain, <sup>2</sup>Reina Sofia Hospital, Rheumatology, Cordoba, Spain

**Objective:** To determine the presence of HLA DRB1 alleles, anti-cyclic citrullinated peptide antibodies (antiCCP antibodies) and rheumatoid factor (RF) in patients with recent-onset who do not respond to follow-up treatment with the classical DMARDs hydroxychloroquine (HCQ), salazopyrine (SLZ) or methotrexate (MTX).

**Patients and methods:** We studied 153 patients with recent-onset rheumatoid arthritis (less than one year) with diagnostic criteria of the American College of Rheumatology (ACR) and residing in Cordoba (southern Spain). Forty-two of these patients met the criteria of non-responsiveness to classical DMARDs (partial response: DAS28  $3.2 < 5.1$  or zero  $> 5.1$ ) on treatment with MTX and leflunomide (LF), LF alone, or anti-TNF- $\alpha$  biological therapies (BT), either alone or in association with MTX. For all the patients we studied DRB1 alleles, including the sub-types with SE, levels of anti-cyclic citrullinated peptide antibodies, rheumatoid factor, ages of the patients at the onset of the disease, the time of development of the disease until the suspension of MTX therapy and the start of treatment with MTX with LF, LF alone or BT with or without MTX, the number of DMARDs administered, values of DAS 28 (at each visit), HAQ (initial and every six months) and radiological progress according to the radiological method of Sharp, as modified by Van der Heijde (initial and every year). Statistical method: logistic regression analysis.

**Results:** In the non-responsive patients we found a higher frequency of antiCCP antibodies ( $p=0.03$ ; OR=3.79) and of the DRB1\*0405 allele ( $p=0.012$ ; odds ratio (OR) 6.31, 78% of the female patients ( $p=0.006$ ), a larger number of DMARDs and a higher prevalence of rheumatoid factor (RF) ( $p=0.002$ ). The logistic regression analysis associated non-responsiveness to classical DMARDs solely with the presence of antiCCP antibodies ( $p=0.025$ ; OR 3.17).

**Conclusions:** The presence of antiCCP antibodies and not DRB1-SE status associated non-responsiveness to the initial treatment with classical DMARDs.

# PC13/39 PSYCHOSOCIAL CONFRONTATION STRESS REDUCES THE SUSCEPTIBILITY TO COLLAGEN TYPE II INDUCED ARTHRITIS IN MALE WISTAR RATS

C. Wolff<sup>1</sup>, K. Schunke<sup>2</sup>, V. Stefanski<sup>3</sup>, R. H. Straub<sup>1</sup><sup>1</sup>University Medical Center, Regensburg, Germany, <sup>2</sup>University of Bayreuth, Bayreuth, Germany, <sup>3</sup>Leibniz Institute of Zoo and Wildlife Research, Berlin, Germany

**Purpose:** Psychosocial stress has been shown to influence the neuroendocrine and immune system and, thus, it is thought to influence autoimmune diseases such as rheumatoid arthritis (RA). The purpose of this study was to investigate the effects of social stress on susceptibility and severity of collagen type II – induced arthritis in rats.

**Methods:** In male Wistar rats, psychosocial stress was induced by continuous confrontation with a stronger resident male opponent for seven days. After 3 days of continuous confrontation, arthritis was induced by intradermal injection of collagen type II in incomplete Freund's adjuvant at the base of the tail (day 0). Animals were killed and samples were taken on different days after induction of arthritis. The composition of blood cells was evaluated by flow cytometry. Skin samples around the collagen injection area at the base of the tail were taken and immunocompetent cells were studied by immunohistochemistry. Plasma catecholamines and corticosterone were measured by RIA and cytokines in supernatants of spleen and lymph node cells were detected by Luminex.

**Results:** Control animals developed more often collagen type II – induced arthritis than stressed rats. However, when stressed rats developed arthritis, we observed less severe arthritis than in controls. Lymph node cells from stressed animals secreted more IL-10 but less TNF compared to controls. Importantly, the number of circulating granulocytes and monocytes was significantly higher in stressed rats than in controls (between day 0 and 7 after immunization). This was opposite for lymphocytes, and the fractions of T helper lymphocytes and cytotoxic T-cells. Furthermore, the number of macrophages in the skin of the tail was higher in controls than in stressed rats (day 4 and 14 after immunization).

**Conclusions:** Psychosocial stress leads to reduced susceptibility to collagen type II induced arthritis.

# PC13/40 THE ROLE OF INTERFERON- $\gamma$ ON THE IMMUNOMODULATORY PROPERTIES OF MURINE MESENCHYMAL STEM CELLS IN T CELL PROLIFERATION AND IN COLLAGEN-INDUCED ARTHRITIS

E. Schurgers<sup>1</sup>, H. Kelchtermans<sup>1</sup>, T. Mitera<sup>1</sup>, L. Geboes<sup>1</sup>, P. Matthys<sup>1</sup><sup>1</sup>Catholic University of Leuven, Leuven, Belgium

**Objectives:** To analyze the potential immunosuppressive properties of mesenchymal stem cells (MSC) on T cell proliferation *in vitro* and *in vivo*, and in collagen-induced arthritis (CIA), an animal model for rheumatoid arthritis. An additional aim is to analyze the role of interferon- $\gamma$  (IFN- $\gamma$ ) in these processes.

**Methods:** MSC were isolated from bone marrow of DBA/1 wild-type and IFN- $\gamma$  receptor knock-out (IFN- $\gamma$  KO) mice and expanded *in vitro*. T cell proliferation was evaluated *in vitro* by thymidine incorporation of anti-CD3 stimulated CD4<sup>+</sup> T cells and accessory cells in the presence or absence of MSC. Suppressive mediators in these co-cultures were analyzed by quantitative PCR. *In vivo* T cell proliferation was analyzed by measuring the number of proliferating CD4<sup>+</sup> and CD8<sup>+</sup> T cells in spleens and lymph nodes of mice challenged with anti-CD3 and treated or not with wild-type or IFN- $\gamma$  KO MSC. CIA was induced in DBA/1 mice by an intradermal injection of collagen type II emulsified in Complete Freund's adjuvant and mice were treated with MSC by two intravenous injections of  $1 \times 10^6$  wild-type or IFN- $\gamma$  KO MSC.

**Results:** The enriched MSC displayed an appropriate phenotype by their flow cytometric profile and their ability to differentiate *in vitro* into osteoblasts and adipocytes. *In vitro*, wild-type MSCs dose-dependently suppressed anti-CD3-induced T cell proliferation up to 90% whereas IFN- $\gamma$  KO MSCs had a maximum inhibitory potential of 59%. The increased suppression of wild-type MSC coincided with up regulation of inducible nitric oxide synthase (iNOS) and was inhibited by the addition of an iNOS inhibitor during co-culture. *In vivo*, neither wild-type nor IFN- $\gamma$  KO MSC were able to counteract the anti-CD3-induced expansion of CD4<sup>+</sup> and CD8<sup>+</sup> T cells nor the severity of CIA or the humoral or cellular immune response towards collagen type II.

**Conclusion:** Whereas MSC inhibit anti-CD3-induced proliferation of T cells *in vitro*, an effect that is partially mediated by IFN- $\gamma$ -induced nitric oxide, MSC do not affect T cell expansion *in vivo*, nor do they influence the disease course of CIA. Thus there is a clear discrepancy between the *in vitro* and *in vivo* effects of MSC on T cell proliferation.

# PC13/41 CASPASE-8 PROCESSING IN JUVENILE IDIOPATHIC ARTHRITIS: OCCURANCE OF A P22 CLEAVAGE PRODUCT

T. Telieps<sup>1,2</sup>, O. Feyen<sup>2</sup>, T. Niehues<sup>3</sup>, I. Schmitz<sup>1</sup><sup>1</sup>Heinrich-Heine-University, Medical Microbiology and Hospital Hygiene, Düsseldorf, Germany, <sup>2</sup>University Children's Hospital Düsseldorf, Dept. of Pediatric Oncology, Hematology and Clinical Immunology, Düsseldorf, Germany, <sup>3</sup>HELIOS Klinikum Krefeld, Centre for Child and Adolescent Health, Krefeld, Germany

Juvenile Idiopathic Arthritis (JIA) is the most common rheumatic disease during childhood. The exact pathophysiological mechanisms are unknown. Nevertheless, JIA is considered an autoimmune disease. Inflammation of the synovial space with infiltration of lymphocytes, especially T cells, is a hallmark feature of JIA. In healthy humans apoptosis may be involved in the prevention of influx of lymphocytes into the joint and the number of T cells in the joints is normally low. Since altered apoptosis can cause autoimmunity, as can be seen e.g. by CD95 mutations causing Autoimmune Lymphoproliferative Syndrome (ALPS), we wanted to analyze the role of CD95-induced apoptosis in the survival of T cells in the synovia leading to arthritic inflammation.

Cell surface markers (e.g. CD45RO, CD45RA, CD95) in synovial fluid and peripheral blood were investigated by flow cytometry. We analyzed apoptosis in mononuclear cells and highly activated T cells of JIA patients, patients with other autoimmune diseases and healthy controls in response to CD95 and dexamethasone stimulation via AnnexinV/PI staining. Cleavage and/or expression of caspases and apoptosis regulators (FLIP, Bcl-2, Bcl-x<sub>L</sub>) were analyzed in mononuclear cells using immunoblot technique.

Although T cells in the synovial space were highly activated, apoptosis upon CD95 stimulation, but not dexamethasone treatment, was impaired in JIA and other autoimmune diseases compared to healthy controls. We observed a non-canonical cleavage pattern of Caspase-8 resulting in a p22 fragment and high expression of FLIP in synovial fluid mononuclear cells of JIA patients.

Alterations in expression and cleavage of proteins involved in CD95-induced apoptosis may result in impaired apoptosis. This may lead to prolonged survival of highly activated cells in inflamed joints. Glucocorticoid-induced apoptosis was intact in patients arguing against a general apoptosis defect. Since strong expression of FLIP/Short in synovial fluid mononuclear cells may simply reflect the highly activated status of the cells we believe that non-canonical processing of caspase-8 contributes to the resistant phenotype of JIA T cells. The implications of the p22 fragment regarding apoptosis and cell survival have to be further analyzed and may help to identify the exact defect in intracellular signaling of synovial lymphocytes from children with JIA.

#### PC13/42 TYROSIN KINASES ARE ESSENTIAL MEDIATORS IN THE DEVELOPMENT OF ARTHRITIS REGULATING ANTIGENE PRESENTATION AND FORMATION OF DENDRITIC CELLS

M. Dehlin<sup>1</sup>, M. Erlandsson<sup>1</sup>, S. Andersson<sup>1</sup>, I.-M. Jonsson<sup>1</sup>, M.I. Bokarewa<sup>2</sup>

<sup>1</sup>University of Göteborg, Rheumatology and Inflammation Research, Göteborg, Sweden, <sup>2</sup>University of Gothenburg, Rheumatology and Inflammation Research, Göteborg, Sweden

**Background:** Tyrosine kinases are a family of intracellular signaling substances participating in cell proliferation, development and apoptosis. Inhibition of tyrosine kinases has been shown efficient in treatment of hematological malignancies and solid cancers as well as diabetes mellitus. We have recently shown that Flt3-ligand (Flt3L) is overexpressed in joints of patients with RA. Moreover, activation of tyrosine kinase Flt3 by an excessive production of Flt3L inside joints induces erosive arthritis in mice.

**Objectives:** In the present study we evaluated the role of tyrosine kinases for the development of experimental arthritis.

**Materials and Methods:** Arthritis was induced using mBSA as an antigen. Mice were treated with low molecular inhibitor of tyrosine kinases, sunitinib (SU11248). Treatment was initiated at the time of mBSA vaccination (long-term treatment) and at the time of intra-articular instillation of mBSA (short-term treatment). Joints were evaluated morphologically 7 days after intra-articular instillation of mBSA. Levels of Flt3L as well as levels of antibodies against mBSA and against citrullinated peptides were measured by ELISA. Effects of sunitinib on the expression of Flt3 and on the development of dendritic cells were evaluated in bone marrow and splenocytes by flow cytometry.

**Results:** Treatment with sunitinib alleviated mBSA induced arthritis reducing intensity of synovitis and frequency of bone erosions in the long-term treated mice. Similar tendencies were observed in short-term treatment groups. Long-term treatment with sunitinib prevented formation of mBSA specific antibodies as well as it reduced production of aCCP antibodies. Circulating levels of Flt3L were not affected by long-term treatment of mice with sunitinib while expression of Flt3 in bone marrow was reduced. Additionally, sunitinib diminished development of dendritic cells in vivo. Here populations of monocyte-derived (B220-CD11c+CD3-CD11b+) and lymphocyte-derived (B220-CD11c+CD8+CD11b-) conventional dendritic cells were most affected.

**Conclusions:** We show that tyrosine kinase activity is important part in the pathogenesis of arthritis. Two mechanisms in the development of arthritis are potentially regulated by tyrosine kinases – antigen presentation followed by antibody production and development of dendritic cells. Intervention by down-regulating tyrosine kinase activity may be a useful tool in treatment of human rheumatoid arthritis.

#### PC13/43 THE ACTIVATION STATUS OF PERIPHERAL BLOOD CD4<sup>+</sup> T CELLS FROM RHEUMATOID ARTHRITIS PATIENTS DEPENDS BOTH ON PATIENTS' AGE AND ON THE AGE AT DISEASE ONSET

J. Pawłowska<sup>1</sup>, A. Dąca<sup>1</sup>, Z. Smoleńska<sup>1</sup>, M. Soroczyńska-Cybula<sup>1</sup>, J.M. Witkowski<sup>1</sup>, E. Bryl<sup>1</sup>

<sup>1</sup>Medical University of Gdansk, Department of Pathophysiology, Gdansk, Poland, <sup>2</sup>Medical University of Gdansk, Department of Family Medicine and Outpatients Connective Tissue Clinic, Gdansk, Poland

**Objectives:** Rheumatoid arthritis (RA) is characterized by profound changes in immune system, not limited to local synovitis, but also including changed activation and function of peripheral blood T lymphocytes. It has been recently suggested that age at disease onset and/or patients' age have influence on disease activity and clinical outcome. Our aim was to find out if the activation status of peripheral blood T cells obtained from RA patients is different depending on patients' age and/or age of RA onset.

**Methods:** Seventy five adult RA patients (aged 44 ± 16 years) were included in our study. Analysis was performed based on the following age criteria: 1. >40 compared with <= 40, 2. >50 compared with <= 50, 3. >60 compared with <= 60. The surface phenotype of peripheral blood lymphocytes including the detection of activation markers: anti-CD69, anti-CD25, anti-CD28, anti-CD95 and anti-HLA-DR on both CD4 and CD8 cells was measured by multicolor flow cytometry.

**Results:** Our results showed that the duration of disease positively correlated with patients' age, but not with onset age. There was a positive correlation between disease activity (DAS28) and onset age, but lack of such a correlation with patients' age. Patients with severe RA were significantly (on average by 7 years) older at disease onset than patients with low/moderate disease activity; no such differences existed for patients' age and duration of disease. Percentages of activated CD4<sup>+</sup>25<sup>+</sup>, regulatory CD4<sup>low</sup>25<sup>high</sup> and CD4<sup>+</sup>95<sup>+</sup> T cells correlated both with patients' age and disease onset age, but percentage of CD4<sup>+</sup>HLA-DR<sup>+</sup> cells was affected only by RA onset age. Significant differences in the proportions of CD4<sup>+</sup>69<sup>+</sup> cells were observed only among the oldest (60+) compared groups, while CD4<sup>low</sup>25<sup>high</sup> among the youngest. The differences in CD4<sup>+</sup>HLA-DR<sup>+</sup> subpopulation was observed in the first youngest compared groups divided based on onset age.

**Conclusions:** Our results showed that activation status of peripheral blood CD4<sup>+</sup> T cells in RA depends both on onset age and patients' age. Dissection of the patients the 'below 40' and above 40' age groups regarding the age at disease onset seems to be the most informative concerning the immune cells' activity status.

#### PC13/44 PROTEIN LJM 111, COMPONENT FROM SALIVARY GLAND EXTRACT (SGE) OF LUTZOMYIA LONGIPALPIS, INHIBITED INFLAMMATORY PARAMETERS ON EXPERIMENTAL ARTHRITIS MODEL

R. Grespan<sup>1</sup>, H.P. Lemos<sup>1</sup>, C.J.F. Oliveira<sup>2</sup>, V. Carregaro<sup>2</sup>, C.R. Teixeira<sup>3</sup>, J.G. Valenzuela<sup>3</sup>, F.Q. Cunha<sup>1</sup>

<sup>1</sup>University of São Paulo, Department of Pharmacology, Ribeirão Preto, Brazil, <sup>2</sup>University of São Paulo, Department of Biochemistry and Immunology, Ribeirão Preto, Brazil, <sup>3</sup>Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, United States

**Objectives:** Aiming to investigate the proteins responsible by the anti-inflammatory effects from SGE and the mechanism involved.

**Methods:** Migration assays was used to evaluate neutrophil recruitment to peritoneal or to articular cavity from OVA- or mBSA-challenged immunized mice, respectively, treated or not with plasmids containing genes that codify for proteins from SGE or with the protein LJM 111. The mechanical hypernociception from mBSA-challenged immunized mice treated or not with LJM 111 was evaluated in knee joint using Von Frey. Cytokines levels (IL-17, TNF-α, IFN-γ and IL-10) of supernatant from cells obtained from lymph nodes of immunized mice and stimulated with mBSA treated or not with LJM 111 were measured by enzyme-linked immunosorbent assay (ELISA). The co-stimulatory molecules expression (MHC-II and CD-86) from bone marrow- derived dendritic cells (BMDc) stimulated with LPS and treated or not with LJM 111 were analysed by flow cytometer. The means of different treatments were compared by analysis of variance (ANOVA), followed by Bonferroni's t test.

**Results:** Plasmid containing gene that codify for protein LJM 111, but not for others proteins, inhibited neutrophil migration to peritoneal cavity in immunized mice. The protein LJM 111 inhibited dose-dependently the neutrophil migration to articular cavity and reduced the hypernociception induced by mBSA-challenge in immunized mice. In addition, LJM 111 reduced IL-17, TNF-α and IFN-γ levels when compared with cells obtained from lymph nodes of immunized mice and stimulated with mBSA without treatment. Thereafter, we sought to investigate how this protein was reducing the cytokine release. Thus, we observed that LJM 111 reduced the co-stimulatory molecules expression (MHC-II and CD-86) from BMDc stimulated with LPS when compared without treatment. Besides that, LJM 111 reduced TNF-α and increased IL-10 levels in supernatant of cultured BMDc stimulated with LPS.

**Conclusion:** These findings showed that the protein LJM 111, component from SGE, reduced inflammatory parameters in experimental arthritis model. These results together suggest that peptides from this protein are viewed as potential therapeutic immunointervention in the rheumatoid arthritis.

Supported by: FAPESP.

#### PC13/45 LIPOPOLYSACCHARIDE INDUCES INCREASED BONE RESORPTION BY STIMULATING HOMING OF OSTEOCLAST PROGENITORS TO THE PERIOSTEAL BONE SURFACE

H. Cvijic<sup>1</sup>, M. Ikić<sup>1</sup>, E. Lazic<sup>2</sup>, S. Kuzmac<sup>2</sup>, N. Kovacic<sup>2</sup>, V. Katavic<sup>2</sup>, A. Marusic<sup>2</sup>, D. Grcevic<sup>1</sup>

<sup>1</sup>Zagreb University School of Medicine, Department of Physiology and Immunology, Zagreb, Croatia, <sup>2</sup>Zagreb University School of Medicine, Department of Anatomy, Zagreb, Croatia

**Objectives:** Lipopolysaccharide (LPS) from gram-negative bacteria causes chronic inflammation and subsequent bone loss, and is involved in the pathogenesis of several bacterially induced bone diseases. We investigated the effects of LPS on bone metabolism and osteoclast differentiation from hematopoietic cells.

**Methods:** C57BL/6 mice were injected during 4 weeks (10 µg LPS/g body weight) and sacrificed at different time-points. Cells from several tissue sources (bone-marrow, homogenized bone shafts, spleen and peripheral blood) were cultured with RANKL (40 ng/mL) and M-CSF (15 ng/mL) to stimulate osteoclast (OCL) dif-



ferentiation. OCL were identified as TRAP-positive multinucleated cells  $\geq$  three nuclei/cell. Femoral sections (5  $\mu$ m) were stained with Goldner-trichrome and TRAP. Microtomography ( $\mu$ CT) was performed using high resolution SkyScan1172. OCL progenitors were characterized by flow-cytometry as a population negative for lymphoid markers (B220, CD3, NK1.1) and positive for CD115 and CD117, within both CD11b negative/low and CD11b positive populations. Gene expression analysis of OCL differentiation genes was performed by qPCR.

**Results:** Three weeks after LPS stimulation, the number of OCL differentiated from cells extracted from bone shafts ( $504.8 \pm 74.28$ ) was higher compared with controls ( $383.3 \pm 30.48$ ;  $p < 0.005$ ). This was in correlation to the decrease in bone volume and trabecular thickness detected by  $\mu$ CT. Femoral sections showed that LPS altered bone metabolism by inducing increased osteoresorption in bone cortex starting from the periosteal surface. This was confirmed by gene expression analysis of bone shafts, showing increased expression of OCL differentiation genes RANK and cFms. Flow-cytometry indicated that enhanced bone resorption starting at the periosteal surface may be caused by homing of peripheral OCL progenitors, since we found 2-3-fold increase of OCL progenitor cell populations in peripheral blood and spleen 10 days after LPS treatment. The presence of increased number of OCL progenitors among peripheral hematopoietic cells was supported by higher number of differentiated OCL from both blood and spleen ( $181 \pm 32.16$  and  $299.25 \pm 61.37$  respectively) compared with control ( $43.8 \pm 25.7$  and  $189 \pm 54.88$  respectively,  $p < 0.005$ ).

**Conclusion:** LPS administration stimulates homing of OCL progenitors to periosteal bone surface and supports osteoclast differentiation. Our further aim is to identify factors induced by LPS that mediate this osteoclastogenic effect and cause enhanced bone resorption.

#### PC13/46 IS DONOR DNA FROM ALLOGRAFTS INCORPORATED INTO RECIPIENT LYMPHOID CELLS?

M. Zagazda<sup>1</sup>, J. Tyska<sup>1</sup>, J. Rutkowska<sup>1</sup>, W.L. Olszewski<sup>1,2</sup>

<sup>1</sup>Medical Research Center, Polish Academy of Sciences, Department of Surgical Research & Transplantation, Warsaw, Poland, <sup>2</sup>Central Clinical Hospital, Ministry of Internal Affairs, Department of Transplantation Surgery, Warsaw, Poland

**Introduction:** Processing and incorporation of fragments of DNA and oligonucleotides by mammalian and bacterial cells is a continuing physiological process. It is strongly intensified in inflammation, cancer and after tissue and organ transplantation. The outcome of DNA transfer between mammalian cells remains not well understood. It has been suggested that donor DNA may play a role in rejection or creating partial tolerance.

**Aim:** To study whether donor DNA may be identified in recipient immune cells and if so, whether it locates in cytoplasm or penetrates into nucleus.

**Methods:** In sex-mismatched combination male rat DNA was injected i.v. into 10 female rats. Recipient blood (PBM), lymph node (LN) and spleen (SPL) mononuclear cells were examined 24 hr later for the presence of SRY gene characteristic for Y-chromosome. SRY was detected using polymerase chain reaction (PCR) method and real-time PCR. The PCR products was analyzed by electrophoresis in 12.5% polyacrylamide gel (PAGE; Phast System, Amersham Pharmacia Biotech) and silver stained (Silver Staining Kit; Amersham Pharmacia Biotech).

**Results:** SRY gene was detected in female PBM, LN and SPL cell cytoplasm in 2 out of 10 rats. Moreover, it was detected in PBM nuclei in 4 out of 10 rats and in LN cell nuclei also in 4 out of 10 rats.

**Conclusion:** Detection of donor male DNA in nuclei isolated from female cells suggests its spontaneous transport into recipients cells and their nuclei. The question remains open whether this finding may have any relevance to the rejection or tolerance process.

#### PC13/47 SURVIVAL OF PMN IN RHEUMATOID ARTHRITIS (RA) CORRELATES WITH INDUCTION OF P21<sup>CIP1/WAF1</sup> (CDKN1A) EXPRESSION

D.A. Schmitt<sup>1</sup>, J. Auer<sup>1</sup>, R.E. Voll<sup>2</sup>, H.U. Beuscher<sup>1</sup>

<sup>1</sup>University Hospital Erlangen, Clinical Microbiology, Immunology and Hygiene, Erlangen, Germany, <sup>2</sup>University Hospital Erlangen, IZKF Research Group N2, Nikolaus-Fiebiger-Center of Molecular Medicine, Erlangen, Germany

Polymorphonuclear neutrophil granulocytes (PMN) are the major cell population in synovial fluid (SF) of patients with RA, and animal models suggest that PMN play a crucial role in initiation and progression of joint inflammation. Disease promoting activities of PMN in joints are thought to be associated with an extended live span, although the underlying molecular mechanisms of which are largely unknown. This study therefore aimed to characterize the effects of SF on the spontaneous apoptosis of PMN. Annexin V staining and subsequent FACS analysis revealed that SF of RA patients suppressed apoptosis of blood PMN in a dose dependent manner. Similar results were obtained when PMN were subjected to oligonucleosomal DNA fragmentation assays. No apoptosis delay was observed in the presence of SF from patients with osteoarthritis and psoriatic arthritis, indicating that suppression of apoptosis in PMN is specific for SF of RA patients. RT-PCR analysis of the expression of anti-apoptotic proteins in PMN revealed a dose dependent induction of p21<sup>CIP1/WAF1</sup> (CDKN1A) in response to SF, while no effects were observed on the expression levels of anti-apoptotic proteins such as myeloid cell leukemia sequence 1 (mcl-1), forkhead box O3 (FOXO-3a), survivin and serum-glucocorticoid kinase (sgk). Kinetic experiments showed that optimal levels of p21<sup>CIP1/WAF1</sup> mRNA accumulated between 4h and 8h after challenging the cells with SF, and thus preceded the occurrence of detectable apoptosis by 8h. In an attempt to investigate the biological significance of the cell culture experiments, mRNAs derived from SF-PMN of 9 independent RA patients were subjected to microarray analysis. The data revealed increased expression levels for p21<sup>CIP1/WAF1</sup> mRNA, while those of other anti-apoptotic proteins, including members of the Bcl2 family remained unchanged. These results suggest, that in RA extension of the live span of PMN is mediated through induction of p21<sup>CIP1/WAF1</sup>.

#### PC13/48 HYPOXIA AND INFLAMMATION

M.C. Bosco<sup>1</sup>, F. Blengio<sup>1</sup>, L. Varesio<sup>1</sup>

<sup>1</sup>Giannina Gaslini Institute, Laboratory of Molecular Biology, Genova, Italy

A common denominator of several pathological conditions, including and inflammation, is represented by hypoxia, a condition of low oxygen tension in the tissue. Hypoxia exerts a tight and complex level of control on mononuclear phagocyte trafficking and immunoregulatory/effector functions at pathological sites, which may have implications for the pathogenesis of chronic inflammation. Mononuclear phagocytes are very sensitive to hypoxia and they are recruited in large numbers as primary monocytes from the circulation to diseased tissues. One clear example of a tissue in which hypoxia plays a pathogenetic role is represented by the rheumatic joints where the PO<sub>2</sub> ranges between 0.8-7%. Physiologic O<sub>2</sub> concentrations in healthy joints range between 7-10%. We characterized the global changes in gene expression occurring in primary monocytes and DCs under hypoxic conditions and we will present our current understanding of the molecular pathways regulating hypoxic gene expression in these cells. These studies lead to new perspectives on the impact of hypoxia on mononuclear phagocyte functional behavior within pathological tissues and to the definition of the mechanisms linking low PO<sub>2</sub> to the control of inflammatory responses. Hypoxia is not the only stimulus present in the inflammatory lesion. To understand the in vivo situation, we must take into consideration the interaction of mononuclear phagocytes with other cells present in ischemic/diseased tissues and the local balance between hypoxia and other microenvironmental alterations, such as glucose depletion, accumulation of lactate and other metabolic byproducts, and low pH, or additional inflammatory co-stimuli, that include cytokines (TNF, IL-1), small metabolites (picolinic acid) and microbial products (LPS), which modulate transcription of hypoxia-responsive genes. We demonstrated the induction of CCL20 in vivo in the synovial fluid by intraarticular hypoxia. This chemokine is important because it is involved in the recruitment of immature dendritic cells, effector/memory T lymphocytes, and naive B cells. Induction of CCL20 by hypoxia represents a mechanism controlling the kinetics and composition of cellular infiltration in the synovial microenvironment, which may contribute to the chronicity of inflammation in juvenile idiopathic arthritis. This pathogenetic mechanism for chronic synovial inflammation associated with JIA can now be a target of new therapeutic strategies.

#### PC13/49 LACTOBACILLUS RHAMNOSUS KL-37 EXOPOLYSACCHARIDE INHIBITS LPS-DEPENDENT ADJUVANT EFFECT ON THE PRODUCTION OF ANTI-OVALBUMIN AND ANTI-COLLAGEN ANTIBODIES AND AMELIORATES THE DEVELOPMENT OF COLLAGEN INDUCED ARTHRITIS IN DBA/1 MICE

B. Nowak<sup>1</sup>, M. Ciszek<sup>1</sup>, M. Śrótek<sup>1</sup>, A. Gamian<sup>2</sup>, J. Marcinkiewicz<sup>1</sup>

<sup>1</sup>Jagiellonian University Medical College, Immunology, Krakow, Poland, <sup>2</sup>Medical School Wrocław, Medical Biochemistry, Wrocław, Poland

**Objective:** To examine:

- (1) whether exopolysaccharide isolated from *Lactobacillus rhamnosus* KL-37 (EPS-37) has an effect on antibody production in mice immunized with protein antigen (ovalbumin or collagen) in the presence of bacterial adjuvant (LPS) and
- (2) the role of EPS-37 in the development of collagen induced arthritis (CIA) in mice.

**Methods:** The effect of EPS-37 on humoral immune response (antibody production) was tested in two different antigen systems, immunizing CBA or DBA/1 mice with ovalbumin (OVA) or collagen type II (CII) respectively using LPS as an adjuvant. Additionally immunization of DBA/1 mice with CII+LPS was repeated 5 times to induce CIA. Serum anti-OVA and anti-CII antibodies (IgG, IgG1, IgG2a) were measured by ELISA. In addition the development of CIA was evaluated by visual observation of joint inflammation (incidence, severity), measurement of paw thickness and the evaluation of myeloperoxidase (MPO) activity in the hind paws joints.

**Results:** Immunization of mice with either OVA or CII in the presence of LPS enhanced greatly the level of antigen-specific antibodies in serum. EPS attenuated the effect of LPS and significantly inhibited the production of anti-OVA and anti-CII antibodies alike. The suppression of anti-CII antibody production and the reduced titer of anti-CII IgG, and especially IgG2a, in serum correlated with amelioration of CIA in DBA/1 mice.

**Conclusion:** EPS-37 inhibited both anti-OVA and anti-CII antibody formation. Reduced, by EPS-37 treatment, serum anti-CII antibody titer (especially IgG2a) resulted in the inhibition of the development of arthritis in mice. However, once the antibodies against CII have formed and reached high levels, EPS-37 was no longer effective to improve the symptoms of arthritis. Therefore EPS-37 may be of value to prevent exacerbation of CIA symptoms during bacterial infections.

**PC13/50 THE SELECTIVE INFILTRATION OF CD56<sup>BRIGHT</sup> NK CELLS INTO SYNOVIAL FLUID OF RHEUMATOID ARTHRITIS AND OSTEOARTHRITIS PATIENTS AND THE DIFFERENT CYTOKINE/CHEMOKINE PATTERNS IN THE SYNOVIAL FLUIDS OF RA AND OA PATIENTS**

B. Mueller<sup>1</sup>, B. Simm<sup>1</sup>, M. Braun<sup>1</sup>, B. Mosetter<sup>2</sup>, M. Witt<sup>3</sup>, B. Moradi<sup>4</sup>, H. M. Lorenz<sup>5</sup>, N. Blank<sup>5</sup>, R. Gruber<sup>3</sup>, J. Marticke<sup>6</sup>, G. O. Hofmann<sup>6</sup>, D. J. Schendel<sup>7</sup>, C. S. Falk<sup>1</sup>  
<sup>1</sup>DKFZ, Heidelberg, Germany, <sup>2</sup>Helmholtz Zentrum, Munich, Germany, <sup>3</sup>Synlab, Weiden, Germany, <sup>4</sup>University Clinic of Heidelberg, Orthopaedic Surgery, Heidelberg, Germany, <sup>5</sup>University Clinic of Heidelberg, Heidelberg, Germany, <sup>6</sup>University Clinic of Jena, Neurology, Jena, Germany, <sup>7</sup>Helmholtz Zentrum, München, Germany

NK cells play an important role in anti-tumor responses as well as in autoimmunity. They can be divided into two major subsets defined as CD16<sup>+</sup>CD56<sup>dim</sup> and CD16<sup>+</sup>CD56<sup>bright</sup>. Whereas CD16<sup>+</sup>CD56<sup>dim</sup> NK-cells are more naturally cytotoxic the CD16<sup>+</sup>CD56<sup>bright</sup> NK-cell subset has the capacity to produce abundant cytokines. To define the phenotypical and functional characteristics of infiltrating NK cells, we analyzed exemplarily synovial fluid of Rheumatoid arthritis (RA) patients (n=33) and osteoarthritis (OA) patients (n=36) in comparison to autologous peripheral blood lymphocytes and healthy donors. The phenotype of the synovial and peripheral lymphocytes, NK cells, in particular, was analyzed by analyzing >20 lineage, activation and NK markers by multicolour-FACS. The patterns of 50 cytokines, chemokines and growth factors in synovial fluids and sera were determined by the Luminex-based multiplex method. Our results confirmed the known predominance of the CD56<sup>bright</sup>CD16<sup>+</sup> NK subset in the synovial fluid while peripheral NK subsets were not altered. Some of the 20 markers showed even a stronger correlation with NK cell infiltration than CD16. The synovial fluids of RA but not OA patients were characterized by a statistically significant increase in proinflammatory cytokines and chemokines and NK cells were shown to contribute to this difference. The selective infiltration of CD16<sup>+</sup> NK cells into the synovial fluid together with the proinflammatory cytokine milieu plays an important role in the discrimination between RA and OA and may provide novel aspects regarding therapeutic intervention.

**PC13/51 INHIBITION OF IL-1 $\beta$ -INDUCED NF- $\kappa$ B ACTIVATION BY  $\alpha$ -PINENE OBTAINED FROM A NATURAL SOURCE**

S. Rosa<sup>1</sup>, J. Gonçalves<sup>2</sup>, A. Rufino<sup>1</sup>, L. Salgueiro<sup>3</sup>, A.F. Mendes<sup>1</sup>, C. Cavaleiro<sup>3</sup>, C. Lopes<sup>1</sup>

<sup>1</sup>University of Coimbra, Center for Neurosciences and Cell Biology and Faculty of Pharmacy, Coimbra, Portugal, <sup>2</sup>University of Coimbra, Center for Neurosciences and Cell Biology, Coimbra, Portugal, <sup>3</sup>University of Coimbra, Laboratory of Pharmacognosy, Faculty of Pharmacy/CEF, Coimbra, Portugal

Nuclear Factor- $\kappa$ B plays a critical role in driving cartilage degradation and inflammation in arthritic diseases. Essential oils are complex mixtures of hydrophobic low molecular weight plant secondary metabolites, composed of a huge diversity of terpenoids and/or phenylpropanoids that are promising samples to screen for biological activities or modulation of molecular targets. The aim of this work was to search for small molecules that can counteract NF- $\kappa$ B activation in chondrocytes. For this, the essential oil obtained from leaves of *Juniperus oxycedrus* spp. *oxycedrus* was screened for inhibitory activity, fractionated and analyzed for composition elucidation.

The essential oil from *J. oxycedrus* was fractionated by liquid chromatography on a silica gel column, eluting with *n*-pentane and diethyl oxide. Each fraction was concentrated and analyzed by gas chromatography and gas chromatography-mass spectroscopy for composition elucidation. The human chondrocytic cell line, C-28/I2, was used to evaluate NF- $\kappa$ B activation. The cells were treated for 30 min with the essential oil or two of its fractions, followed by treatment with Interleukin-1 $\beta$  (IL-1) (30 ng/ml) for 5 or 30 min to evaluate, respectively, the cytoplasmic levels of phosphorylated and total I $\kappa$ B- $\alpha$  by western blot and NF- $\kappa$ B-DNA binding activity by an ELISA-based assay. The MTT reduction assay was used to rule out cytotoxic effects. Statistical significance was assessed by one-way ANOVA with Dunnett's post test.

The essential oil from *J. oxycedrus* decreased IL-1-induced I $\kappa$ B- $\alpha$  degradation in a dose-dependent manner. The major fraction, containing more than 90%  $\alpha$ -pinene, decreased IL-1-induced I $\kappa$ B- $\alpha$  phosphorylation by approximately 80%. Moreover, total I $\kappa$ B- $\alpha$  increased from 10% of the control in IL-1-treated cells to nearly 52% in cells pretreated with the  $\alpha$ -pinene-containing fraction. This fraction also reduced IL-1-induced NF- $\kappa$ B-DNA binding activity by 33% relatively to cells treated with IL-1 alone.

$\alpha$ -pinene, the major component of the essential oil from the leaves of *J. oxycedrus*, probably accounts for most of its ability to inhibit IL-1-induced NF- $\kappa$ B activation. The results obtained show that  $\alpha$ -pinene is effective in preventing NF- $\kappa$ B activation in chondrocyte-like cells, thus suggesting that it may be promising as an anti-arthritis drug.

This work was supported by FCT, project PTDC/SAU-OSM/67936/2006 and PhD fellowships SFRH/BD/19763/2004 and SFRH/BD/47470/2008.

**PC13/52 CYTOMETRIC PROFILING IN ANKYLOSING SPONDYLITIS**

M. Steinbrich-Zöllner<sup>1</sup>, A. Grützkau<sup>2</sup>, J. Grün<sup>2</sup>, P. Wu<sup>1</sup>, M. Rudwaleit<sup>1</sup>, H. Appel<sup>1</sup>, A. Radbruch<sup>2</sup>, J. Sieper<sup>1</sup>

<sup>1</sup>Charité, Campus Benjamin Franklin, Department of Gastroenterology, Infectiology and Rheumatology, Berlin, Germany, <sup>2</sup>German Rheumatology Research Center, Berlin, Germany

**Objectives:** Multi-parametric flow cytometry pilot study revealed a group of differentially regulated leukocyte surface markers in ankylosing spondylitis (AS) patients' peripheral blood. The most prominent two molecules, chemokine receptor CXCR4 and glycolipid antigen presenting molecule CD1c, has been selected for further investigation by the implementation of the newest advances of multicolour cytometry technology.

**Methods:** Two staining cocktails (5 and 7 colour) combining lineage, migration and lipid antigen presenting markers were applied to total leukocytes obtained by erythrocyte lysis of the whole heparinised blood. Relative cell numbers and mean fluorescence intensities (MFI) of generated populations were analysed with unpaired t-test with Welch's correction. Samples of 10 AS patients with active disease, treated exclusively with non-steroidal anti-inflammatory drugs, were compared to 6 normal donors (ND).

**Results:** Patients' blood was characterised by upregulation of CD1c molecule on B lymphocytes (MFI of CD1c on CD19+ lymphocytes, p=0,006). The number of CD19+CD1c+ lymphocytes as a percentage of CD19+ was increased (p=0,006). CXCR4 was upregulated on the entire CD8 lymphocyte population (MFI of CXCR4 on CD3+CD8+ lymphocytes, p=0,0008) and especially on the particular CD8 subset (MFI of CXCR4 on CD3+CD8+CD45RA+CCR7- lymphocytes, p=0,0003). Relative event numbers in daughter gates were respectively changed. Moreover, monocytes showed elevated expression of CXCR4 (MFI of CXCR4 in monocyte-gate p=0,009) and reduced expression of CD62L (MFI of CD62L in monocyte-gate, p=0,01). Relative event numbers were changed respectively in daughter gates (i.e. reduced CD62L+CXCR4- monocyte subset as a percentage of the monocyte-gate, p=0,001).

**Conclusions:** CD1c molecule upregulation on B lymphocytes might suggest the role of glycolipid antigen presentation in AS immunopathology. Overexpression of CXCR4 possibly indicates leukocyte activation and enhanced trafficking. Downregulation of CD62L by monocytes might refer to their involvement in extravasation. The relevance of these molecules for AS immunopathology requires further investigation. Inflammatory processes localised in the joints can be sensed and characterised on the level of immune cells in peripheral blood by multicolour flow cytometry.

**PC13/53 USING A PROTEOMIC STRATEGY TO INVESTIGATE THE AUTOIMMUNE RESPONSE IN RHEUMATOID ARTHRITIS**

K. Lau<sup>1</sup>, S. Chatfield<sup>2</sup>, R. Thomas<sup>3</sup>, R. Shankar<sup>1</sup>, N.A. Williamson<sup>1</sup>, A. W. Purcell<sup>1</sup>

<sup>1</sup>University of Melbourne, Biochemistry and Molecular Biology, Parkville, Australia, <sup>2</sup>Royal Melbourne Hospital, Parkville, Australia, <sup>3</sup>University of Queensland, Princess Alexandra Hospital, Centre for Immunology and Cancer Research, Brisbane, Australia

Rheumatoid arthritis (RA) is an autoimmune disease characterised by chronic inflammation in the joints. A model for the pathogenesis of RA featuring citrullinated proteins as an autoantigen has been proposed (Klareskog, 2009). However, the proteins involved in the autoimmune response is not well characterised. One method to do this is to use a proteomic approach using 2D gel electrophoresis. Previous studies where synovial fluid samples obtained from RA patients were analysed identified both citrullinated and non citrullinated proteins that may have a role in disease (Fritz, 1990; Drynda, 2004; Kim, 2006; Kinloch 2008). However these proteins have not been reliably reproduced in subsequent studies due to inconsistencies with the 2D gel electrophoresis technique when comparing two or more samples. One technology that can address this issue is two dimensional difference gel electrophoresis (2D-DIGE) (Mustafa, 1997).

**Objectives:** To use 2D-DIGE to compare synovial fluid from patients with and without rheumatoid arthritis and analyse protein expression between the two groups. These synovial fluids were also analysed for citrullinated proteins.

**Method:** 6 samples of synovial fluid from patients with rheumatoid arthritis (RA) and 6 samples of synovial fluid from non rheumatoid arthritis (non RA) patients were used in 2D-DIGE. Proteins of interest were determined using DeCyder version 6.5 (GE Healthcare). Citrullinated proteins were identified by western blotting and mass spectrometry.

**Results:** From 2D-DIGE, 5 proteins were found to be differentially expressed between the two groups. 4 of these were identified using mass spectrometry: Complement component 4A, immunoglobulin constant  $\mu$  heavy chain, immunoglobulin  $\kappa$  variable chain and immunoglobulin  $\kappa$  constant chain. One of the citrullinated proteins identified so far in RA synovial fluid samples was fibronectin.

**Conclusion:** An increased level of autoantibodies (rheumatoid factor, anti-citrulline antibodies) in rheumatoid arthritis patients has been documented in the literature. Complement component 4A has been associated with rheumatoid arthritis (Falus, 1989; Mimori, 1990) but the biological basis for this is not understood. Citrullinated fibronectin has been identified in previous studies (Chang 2005, Kim 2006) but its role in disease is not known. Further work is being done to identify the other citrullinated proteins found in synovial fluid.

PC13/54 OCCURRENCE OF RHEUMATOID ARTHRITIS-ASSOCIATED AUTOANTIBODIES IN *LEISHMANIA DONOVANI* INFECTIONE. Ahlin<sup>1</sup>, A. Elshafie<sup>1,2</sup>, J. Rönnelid<sup>1</sup><sup>1</sup>Uppsala University, Clinical Immunology, Uppsala, Sweden, <sup>2</sup>Alribat University Hospital, Department of Laboratories and Blood Banking, Khartoum, Sudan

**Background:** Visceral Leishmaniasis (VL) or Kala-azar, is the most severe form of leishmaniasis and is caused by parasites of the *Leishmania* genus. Post-Kala-azar dermal leishmaniasis (PKDL) is a complication of VL characterised by severe rashes and skin lesions in mostly young patients who have recovered from VL. Circulating immune complexes (CIC) are increased in chronic leishmaniasis. Rheumatoid factor (RF) is a common finding in IC-associated chronic infectious diseases. In one small study the occurrence of anti-Cyclic Citrullinated Peptide (anti-CCP) antibodies has been described in Brazilian patients infected with *Leishmania major*. We have investigated the occurrence of different RA-associated autoantibodies in a larger cohort of Sudanese patients infected with *Leishmania donovani*.

**Methods:** Serum samples were collected from 83 VL patients, 47 PKDL patients, 55 healthy Sudanese controls and 34 Sudanese patients seeing a rheumatologist due to joint complaints. Levels of CIC, anti-CCP and anti-human collagen type II antibodies (anti-CII) were investigated with ELISA and RF by nephelometry.

**Results:** The results are summarised in table 1 and presented as number of autoantibody positive samples out of total and with the mean value among the positive samples (positive/total, mean).

	VL	PKDL	Joint complaints	Control
RF, >20 IU/mL	73/83, 225	30/42, 47	18/31, 123	26/55, 28
Anti-CCP, >25 E/mL	9/80, 46	2/47, 64)	7/34, 620	1/52
Anti-CII, >29 AU/mL	74/82, 85	37/45, 58)	12/34, 91	29/52, 78

[Table 1]

**Conclusions:** The anti-CCP positivity among VL patients might imply shared pathogenic characteristics with anti-CCP positive arthritis. However, the anti-CCP positive samples in the VL group are at a moderate level as compared to the levels found in anti-CCP positive Sudanese rheumatology patients. This together with the observed general increase in other RA-associated autoantibodies in *Leishmania*-infected patients might instead argue that this is an effect of extensive inflammation and immune activation. Our findings of increased RF and anti-CII levels among young Sudanese controls stress the importance of defining local cut-off levels when studying immune activation in non-Caucasian populations.

## PC13/55 ANTIBODIES AGAINST CITRULLINATED ANTIGENS AND HLA HAPLOTYPES IN RHEUMATOID ARTHRITIS

R. Engelmann<sup>1</sup>, B. Müller-Hilke<sup>1</sup><sup>1</sup>University of Rostock, Institute of Immunology, Rostock, Germany

Rheumatoid arthritis (RA) is a chronic autoimmune disease primarily affecting the joints. Pathological processes leading to RA involve both, the innate and the adaptive immune system. The present study focuses on the adaptive immune system, and in particular on autoantibodies against citrullinated peptide antigens (ACPAs). These are highly sensitive and specific for RA, yet no pathogenic role has been assigned as of today. In detail, we compared two ACPA populations directed against cyclic citrullinated peptides (CCP) and mutated citrullinated vimentin (MCV) respectively, and found a significant correlation between their titers. As this correlation may be due to either cross specificities or mutual immune regulation, we analyzed both. To test for cross reactivity, we developed a sequential binding and detection assay and found considerable overlapping reactivity in a subgroup of RA patients. To test for mutual immune regulation in the form of T cell help we analyzed class switching and in detail measured the IgG subclass distribution of ACPAs. We found a prevalence of IgG1 and IgG4 with IgG4 being highly conspicuous because of its shortage among total serum levels in patients' sera (IgG1>IgG2>IgG3>IgG4) and among anti-Varicella zoster antibodies. Investigating an immune regulatory impact from the HLA we correlated ACPA subclass titers and DRB1 haplotypes. Interestingly, double doses of HLA-DRB1\*04 led to elevated IgG1- and IgG3-ACPA titers. There was no such effect observed for the shared epitope suggesting a tendency towards Th1 responses in HLA-DRB1\*04 controlled immune systems.

## PC13/56 KIR3DL1 GENE POLYMORPHISM AND SUSCEPTIBILITY TO ANKYLOSING SPONDYLITIS IN THE SPANISH POPULATION

R. Díaz-Peña<sup>1</sup>, J.R. Vidal-Castieira<sup>1</sup>, R. Alonso-Arias<sup>1</sup>, J. Martínez-Borra<sup>1</sup>, A. López-Vázquez<sup>1</sup>, C. López-Larrea<sup>1</sup><sup>1</sup>Hospital Universitario Central de Asturias, Oviedo, Spain

**Objective:** Kill cell immunoglobulin-like receptors (KIR) form a group of regulatory molecules that specifically recognize human leukocyte antigen (HLA) class I molecules. In the present study we have analysed the possible contribution of KIR3DL1 and KIR3DS1 alleles in the susceptibility to AS in addition to HLA-B27 in the Spanish population.

**Methods:** One matched HLA-B27-positive population from Spain was selected for this study (101 patients with AS and 100 healthy matched controls). The patients with AS were diagnosed at the Rheumatology Units of the Hospital Universitario Central and Hospital Monte Naranco, Oviedo. All B27 and KIR3DL1/3DS1 individuals were typed by SSOP and SSP.

**Results:** We observed that the increase of KIR3DS1\*013 allele frequency in AS patients is clearly independent of the presence or absence of Bw4I80 ( $p < 0.001$ , OR=7.86 and  $p < 0.0005$ , OR=3.98, respectively). Moreover, the "null" allele KIR3DL1\*004 was the unique KIR3DL1 inhibitory allele, that showed a protective effect in AS susceptibility ( $p < 0.05$ ). The protective effect was dependent of Bw4I80.

**Conclusion:** We proposed that the increased activation potential of NK and/or T cells associated with KIR3DL1/3DS1-B27-peptide may predispose to AS. Moreover, presence of inhibitory allotypes as KIR3DL1\*004 showed a protective effect in AS susceptibility in presence of the associated allele KIR3DS1\*013.

## PC13/57 THE ISOLATED COMPOUNDS FROM THE ETHYL ACETATE OF ALFALFA SPROUTS INHIBIT THE SECRETION OF PRO-INFLAMMATORY CYTOKINES BY MURINE PRIMARY IMMUNE CELLS

Y.-H. Hong<sup>1</sup>, Y.-H. Kuo<sup>2</sup>, B.-F. Lin<sup>1</sup><sup>1</sup>Institute of Microbiology and Biochemistry, National Taiwan University, Taipei, Taiwan, Republic of China, <sup>2</sup>National Taiwan University, Department of Chemistry, Taipei, Taiwan, Republic of China

**Objectives:** Alfalfa (*Medicago Sativa* L.) sprouts are a common material in ready-to-eat vegetables for making a salad. Its sprouts or leaves also have been used to alleviate inflammatory status in traditional herbal medicine remedy. In our previous studies, the ethyl acetate extract of alfalfa sprouts (ASEA) has been indicated to have the inhibitory effects on the secretion of pro-inflammatory cytokines and the severity of lupus nephritis. However, the effective components in this extract are not clearly determined. This study tried to find out these components.

**Methods:** BALB/c murine splenocytes and peritoneal macrophages were used to screen out the most potential fractions isolated from ASEA by the inhibiting ability of cytokines production, and elicit the candidate components via further purification.

**Results:** This bioassay-guided purification of ASEA yielded four components: L1, L2, L3, and coumestrol. The anti-inflammatory ability of these isolated pure compounds was tested. The results showed that concanavalin A-stimulated splenocytic IFN- $\gamma$  secretion was significantly decreased by L1, L2, or L3 at the treated concentration of 1, 0.2, or 5  $\mu$ g/ml respectively. In peritoneal macrophages, lipopolysaccharide-stimulated IL-6 secretion was significantly inhibited by L1, L2, or L3 at the treated concentration of 1  $\mu$ g/ml. The ranking of anti-inflammatory ability is L2 > L1 > L3. Furthermore, our data presented that coumestrol has no effect on the secretion of these pro-inflammatory cytokines.

**Conclusion:** This study suggested that the isolated compounds, L1, L2, and L3, are contributions to the anti-inflammatory effect of ASEA. These compounds further could be used for therapeutic application in the inflammatory disease.

## PC13/58 DIAGNOSTIC VALUE OF ANTI-RA33 AUTOANTIBODIES IN PATIENTS WITH AUTOIMMUNE DISEASES

Z. Szabo<sup>1</sup>, J. Nemeth<sup>1</sup>, K. Miklos<sup>1</sup>, J. Simon<sup>1</sup>, J. Furesz<sup>1</sup><sup>1</sup>Ministry of Defence State Health Centre, Department of Central Laboratory, Budapest, Hungary

**Objectives:** Anti-RA33 autoantibodies target the heterogenous nuclear ribonucleoproteins (hnRNP), mainly protein A2. hnRNP among others play a role in transcription, pre-mRNA processing in the nucleus, cytoplasmic mRNA translation and turnover. Anti-RA33 antibodies are found in patients with rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and mixed connective tissue disease (MCTD). Differential diagnosis of autoimmune diseases is often beset with difficulties. Serological markers help setting up the diagnosis. Our aim was to investigate the diagnostic value of anti-RA33 antibodies in RA and compare it with the characteristics of anti-citrullinated peptide antibodies (anti-CCP) and rheumatoid factor (RF), furthermore, to analyze the incidence of anti-RA33 in patient with other arthropathy and other autoimmune diseases.

**Methods:** 200 patients (60 with RA, 42 with SLE and 47 with other autoimmune diseases and 51 with other arthralgia) were tested for anti-RA33, anti-CCP and RF. Anti-RA33 and anti-CCP antibodies were analyzed by ELISA test, RF was measured by turbidimetry.

**Results:** In the group of patients with RA the rate of positive results was: 82% tested with anti-CCP, 20% tested with anti-RA33 and 85% tested with RF. As regards patients affected by SLE, 10% of them was positive for anti-CCP, 28% for anti-RA33 and 40% for RF.

As for patients with other autoimmune disease 6% was anti-CCP positive, 10% anti-RA33 positive and 14% RF positive.

In the group of patients with other arthropathies there were no positive results with anti-CCP test meanwhile 4% was found to be positive with anti-RA33 test and 6% with RF test.



**Conclusions:** Our results suggest that anti-CCP and RF tests are useful in the routine diagnosis of RA while anti-RA33 test does not give further information. On the other hand anti-RA33 test may give help in the diagnosis in case of seronegative RA. In our presentation, cases for which only anti-RA33 test was positive are analyzed.

# PC13/59 ASSESSMENT OF AUTOANTIGEN-SPECIFIC T CELL RESPONSES IN RHEUMATOID ARTHRITIS

J. Locke<sup>1</sup>, J.D. Isaacs<sup>1</sup>, W.-F. Ng<sup>1</sup>

<sup>1</sup>University of Newcastle upon Tyne, Musculoskeletal Research Group, Newcastle upon Tyne, United Kingdom

The strong link between rheumatoid arthritis (RA) and HLA-DR shared epitopes has implicated CD4<sup>+</sup> T-cells in the disease pathogenesis. It has been postulated that CD4<sup>+</sup> T-cells activated by autoantigens presented by RA-associated HLA-DR molecules may be responsible for driving joint inflammation and cartilage damage. However, CD4<sup>+</sup> T-cell responses to candidate autoantigens have proved difficult to be consistently identified in RA patients, which may reflect the use of insensitive or inappropriate detection techniques.

**Methods:** In this study, we measured proliferative responses of peripheral blood mononuclear cells (PBMC) from healthy volunteers and RA patients against phytohaemagglutinin (PHA), purified tuberculin protein derivative (PPD) and the putative RA autoantigen, human cartilage glycoprotein 39 (HCgp39). Proliferation of bulk PBMC was assessed by <sup>3</sup>H-thymidine incorporation. CD4<sup>+</sup> T-cell proliferation was measured via carboxyfluorescein succinimidyl ester (CFSE) dilution. Cytokine production in the supernatant was measured using custom-made multiplex chemical electroluminescence technology and enzyme-linked immunosorbent assays (ELISA).

**Results:** We found that while there was good correlation between <sup>3</sup>H-thymidine incorporation and CFSE dilution assays in assessing responses to PHA, the correlation was poor when assessing responses to PPD. Furthermore, the magnitude of <sup>3</sup>H-thymidine incorporation correlated poorly to the percentage of CD4<sup>+</sup> T-cells that had proliferated in response to the test antigen, as defined by CFSE dilution in the CD4<sup>+</sup> T-cell population. When proliferation of CD4<sup>+</sup> T-cells to HCgp-39 was measured in 15 RA patients using CFSE dilution, a weakly positive response was detected in only 2 patients. We also showed that the use of foetal bovine serum (FBS) in culture medium led to significant non-specific proliferation of PBMC and “positive” responses to HCgp-39 in CFSE dilution assays. Non-specific proliferation can be minimised by replacing FBS with autologous serum.

**Conclusions:** In summary, our data did not support the findings of previous studies demonstrating increased proliferation in response to HCgp-39 in RA patients. Our data also highlighted the pitfalls of using <sup>3</sup>H-thymidine incorporation assays in assessing T-cell proliferation and the need for more specific and reliable methods to assess autoantigen-specific T-cell responses in clinical studies. Furthermore, the use of FBS should be avoided in functional assays of human cells.

# PC13/60 DICHLOROACETATE ALLEVIATES DEVELOPMENT OF COLLAGEN TYPE II INDUCED ARTHRITIS IN FEMALE DBA/1 MICE

L. Bian<sup>1</sup>, I.-M. Jonsson<sup>2</sup>, M. Verdrangh<sup>2</sup>, C. Ohlsson<sup>2</sup>, M. Bokarewa<sup>2</sup>, A. Tarkowski<sup>2</sup>, M. Magnusson<sup>2</sup>

<sup>1</sup>University of Gothenburg, Department of Rheumatology and Inflammation Research, Gothenburg, Sweden, <sup>2</sup>University of Gothenburg, Gothenburg, Sweden

**Background:** Rheumatoid arthritis (RA) is characterized by persistent synovial cell proliferation and inflammatory cell infiltration in joints. Dichloroacetate (DCA) is traditionally used for the treatment of lactic acidosis and mitochondrial disorders and was recently found to have potent anti-tumor effects by its pro-apoptotic and anti-proliferative properties. The aim of our study was to investigate if DCA can ameliorate arthritis in the collagen type II – induced arthritis model (CIA).

**Materials and methods:** DBA/1 mice were immunized with collagen type II to induce arthritis and were treated with DCA or water. Signs of arthritis were evaluated during the course of experiment and histologic sections of joints were examined for inflammation and erosion. The levels of estradiol were indirectly estimated by uterus weight. Serum circulating pro-inflammatory IL-6 and IgG anti-CII antibody were analyzed. Ovariectomized mice were used to determine the role of estrogen on arthritis modulated by DCA.

**Results:** Female, but not male DBA/1 mice treated with DCA displayed much slower onset of CIA, less severe arthritis and significantly decreased cartilage and joint destruction than mice treated with water. These findings were also reflected by lower serum levels of IL-6 and anti-CII specific IgG in the treated group. Ovariectomized mice, in contrast to sham-operated controls, were not protected from CIA by DCA.

**Conclusion:** DCA delays the onset and alleviates the progression of collagen II-induced arthritis, most likely in an estrogen-dependent manner. DCA can be a potential tool for treatment of rheumatoid arthritis.

# PC13/61 EFFICACY OF ACTIVE IMMUNIZATION WITH TNFALPHA-KINOID IN BALB/C MICE WITH IMMUNOSUPPRESSANT CO-ADMINISTRATION

E. Assier<sup>1</sup>, L. Semerano<sup>2</sup>, L. Delavallée<sup>1</sup>, A. Denys<sup>1</sup>, G. Grouard-Vogel<sup>3</sup>, E. Bernier<sup>3</sup>, M. Laborie<sup>3</sup>, N. Bessis<sup>1</sup>, M.-C. Boissier<sup>2</sup>

<sup>1</sup>University Paris 13, Immunology, Bobigny, France, <sup>2</sup>Avicenne Hospital, Rheumatology, Bobigny, France, <sup>3</sup>Néovacs, Paris, France

**Background:** Passive blockade of TNFalpha through monoclonal antibodies shows high efficacy in rheumatoid arthritis (RA), although some concerns remain such as occurrence of resistance. The kinoid immunization strategy targets cytokines by inducing natural polyclonal antibody response and circumvents the induction of the anti-monoclonal antibody response. Kinoids are heterocomplexes made by conjugating the targeted cytokine to a carrier protein such as the keyhole limpet hemocyanin (KLH). Immunization with an anti-TNFalpha-Kinoid (TNFalpha-K) proved its efficacy in a hTNFalpha transgenic (TTg) mice model of arthritis.

**Objectives:** Immunosuppressant drugs are widely prescribed in association with anti-TNFalpha strategies and may interfere with the efficacy of kinoid immunization. In the present study, we aimed at studying interactions between active immunization with TNFalpha-K and immunosuppressant drugs.

**Methods:** Anti-TNFalpha immunization was performed by injecting intra-muscularly TNFalpha-K formulated in ISA51 adjuvant (SEPPIC), three times (D0, 7, 28) in Balb/C mice. Mice were also injected intraperitoneally with either PBS (control group) or Cyclophosphamide (CYC), Methyl-Prednisolone (MP) or Methotrexate (MTX). Anti-hTNFalpha and anti-KLH antibody (Ab) levels were assessed by ELISA, and the anti-hTNFalpha neutralizing capacity (NC) of sera by L-929 bioassay.

**Results:** All mice developed high titers of anti-hTNFalpha Ab. Chronic administration of MTX (1mg/kg, 3 times/week) or MP (0.2 mg/kg, 3 times/week) from D0 until D67 did not significantly modify the levels of anti-TNF Ab (ELISA and NC). Furthermore, high doses of MP (5 mg/kg) or MTX (2.5 mg/kg) on D35, 38, 41, 44 did not alter the levels of anti-hTNFalpha Ab (ELISA and NC) either. In contrast, a significant decrease in anti-hTNFalpha Ab was observed after administration of CYC (200 mg/kg D35 and D44): as assessed both by ELISA at D60 and D70 (p< 0.0005), and by the neutralizing assay (p< 0.01). Anti-KLH Ab titers were not modified in any group.

**Conclusion:** These data show that TNFalpha-K efficacy, as evaluated by the targeted biological effect, is not significantly altered by current treatments used in RA, such as MP and MTX. However, a late administration of CYC has an inhibitory effect on the anti-TNFalpha Ab production.

# PC13/62 ASSOCIATION OF BAT1 WITH RHEUMATOID ARTHRITIS SUSCEPTIBILITY

S. Gonzalez<sup>1</sup>, L. López-Huergo<sup>1</sup>, J. Ballina-García<sup>2</sup>, L.-S. Alejandro<sup>1</sup>

<sup>1</sup>University of Oviedo, Functional Biology, Oviedo, Spain, <sup>2</sup>Hospital Universitario Central de Asturias, Rheumatology, Oviedo, Spain

**Objectives:** BAT1 gene is located in the MHC region telomeric to HLA-DRB1. BAT1 is involved in the production of inflammatory cytokines such as TNF-α and, consequently, it regulates the inflammatory response. Two functional polymorphisms located at -22 and -348 in the promoter of the BAT1 gene which regulate the expression of BAT1 have been described. The objective of our study is to analyze whether the polymorphisms -22 (G/C) and -348 (C/T) of the BAT1 gene are associated with susceptibility to rheumatoid arthritis (RA).

**Methods:** One hundred fifty-six patients with RA and 154 controls were genotyped for HLA-DRB1 and the polymorphisms -22 and -348 of the BAT1 gene.

**Results:** Our experiments: HLA-DRB1\*040 alleles were associated with RA susceptibility (33.9% vs 20.1%; p = 0.04). Among these, HLA-DRB1\*0401 (13.4% vs 5.1%; p = 0.04) and HLA-DRB1\*0404 (5.7% vs 1.2%; p = 0.2) were increased in patients with RA. Additionally, carriage of BAT1 -348T polymorphism was strongly associated with RA (23.7% vs 12.1%; p = 0.0002). Significantly, BAT1 -348T was in linkage disequilibrium with HLA-DRB1\*0404 and HLA-DRB1\*0405. However, BAT1 -348 T was associated independently with HLA-DRB1 shared-epitope alleles (42.6% vs 18.9%; p = 0.001).

**Conclusion:** The BAT1 -348T polymorphism is associated with RA susceptibility independently of HLA-DRB1. The role of BAT1 in the regulation of tumour necrosis factor-α suggests that BAT1 may regulate the inflammatory response observed in patients with RA.

# PC13/63 ANTI-INFLAMMATORY EFFECT OF 3-(4-HYDROXYPHENYL)-4-(4-(METHYLTHIO)PHENYL)-1H-PYRROLE-2,5-DIONE (HMP), SELECTIVE INHIBITOR OF COX-2 ACTIVITY, IN VITRO AND IN VIVO

Y.-S. Noh<sup>1,2</sup>, Y.-W. Cho<sup>2</sup>, J.Y. Lee<sup>2</sup>, K.-T. Lee<sup>1</sup>

<sup>1</sup>Kyung-Hee University, Department of Pharmaceutical Biochemistry, College of Pharmacy, Seoul, Korea, Republic of, <sup>2</sup>Kyung-Hee University, Department of Biomedical Science, College of Medical Science, Seoul, Korea, Republic of, <sup>3</sup>Kyung Hee University, Research Institute for Basic Sciences and Department of Chemistry, College of Sciences, Seoul, Korea, Republic of

**Objectives:** Our study was designed to assess the anti-inflammatory activity of the cyclooxygenase (COX)-2 selective inhibitor, 3-(4-hydroxyphenyl)-4-(4-(methylthio)phenyl)-1H-pyrrole-2,5-dione (HMP) derived from celecoxib, *in vitro* and *in vivo* inflammatory model.

**Methods:** We estimated the anti-inflammatory effects of HMP on carrageenan-induced acute joint inflammation model. And the neutrophil migration was indirectly determined by MPO activity assay and level of PGE<sub>2</sub> was measured by EIAs in carrageenan-stimulated rat paws. It was also investigated the analgesic effect on acetic-induced writhing test. To investigate the mechanism of the anti-inflammatory action, we examined the effects of HMP on the lipopolysaccharide (LPS)-induced PGE<sub>2</sub> production and COX-2 protein expression in RAW 264.7 cells. The effect of HMP was also evaluated on COX-1 and COX-2 enzymatic activity.

**Results:** Pretreatment of HMP (25 and 50 mg/kg, p.o.) inhibited carrageenan-induced acute joint inflammation through inhibiting the formation of paw edema, neutrophil migration and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) production in rat. Moreover, it exhibited anti-peripheral nociceptive effect in mice. HMP significantly inhibited

the lipopolysaccharide (LPS)-induced prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) production in RAW 264.7 cells. Consistent with these findings, HMP selectively inhibited COX-2 enzyme activity. On the other hand, HMP had no effect on the LPS-induced expression of COX-2 at the protein and mRNA. In addition, the release of pro-inflammatory mediators such as nitric oxide (NO), interleukin-1 $\beta$  (IL-1 $\beta$ ) and interleukin-6 (IL-6) were reduced by HMP via down-regulation of their gene expression. Moreover, the PGE<sub>2</sub> production from mouse peritoneal macrophages in response to LPS was diminished by HMP.

**Conclusion:** These results suggest that HMP, selective COX-2 inhibitor, had the anti-inflammatory effect on carrageenan-induced acute joint inflammation and peripheral nociceptive response via inhibition of COX-2 enzyme activity and PGE<sub>2</sub> production. Therefore, HMP has the potential to be used as anti-joint inflammatory disease agent.

PC13/64 **CHANGES OF COSTIMULATORY MOLECULES EXPRESSION IN RHEUMATOID ARTHRITIS TREATED WITH CTLA-IG (ABATACEPT)**

R. Paganelli<sup>1,2</sup>, M. D'Urbano<sup>2</sup>, E. Celletti<sup>1</sup>, M. C. Turi<sup>1,2</sup>, F. Paolini<sup>1</sup>  
<sup>1</sup>University G. d'Annunzio, Medicine & Sciences of Aging, Chieti, Italy, <sup>2</sup>CESI- Foundation Ud'A, Unit of Biogerontology and Cytokines, Chieti, Italy

We determined the changes of lymphocyte expression of co-stimulatory and activation molecules in 3 patients affected by Rheumatoid Arthritis (RA) undergoing treatment with the costimulation inhibitor CTLA-Ig (Abatacept). The patients (2 F, 1 M) aged 67-71 yrs, had a history of long standing RA unresponsive to multiple anti-rheumatic drugs, including DMARDs and TNF blockers. They were started on Abatacept 750 mg i.v. on day 0, then day 15 and every 4 weeks thereafter. Concomitant therapy with other anti-inflammatory drugs, except steroids, was allowed. Blood samples were collected prior to infusion. Disease activity and health quality were assessed by DAS28 and HAQ. At time 0 median DAS28 was 3.96, and all patients had CRP levels above normal and high values of IgM-RF and anti-CCP antibodies. The percentage of T and B lymphocyte subsets remained stable throughout a median of six months'treatment (range 3 to 9). A non significant decrease of CD28 expression occurred on CD4+ cells (40 to 30% ), mirrored by slight increase of CD152, with a return to baseline values within six months. We observed a significant continuous decrease of PD1 expression on CD3+ T lymphocytes (3.4 to 1.1 %). At the same time, expression of both CD80 and CD86 on B lymphocytes increased slightly. These changes reflect the new regulatory balance of stimulatory/anergic signals on the cell surface due to costimulatory blockade by CTLA-Ig. However they were found on fresh unstimulated blood lymphocytes and may not reflect the overall function of costimulatory activation.

PC13/65 **DEFINING TNF $\alpha$ - AND LPS-INDUCED GENE EXPRESSION PROFILES IN MONOCYTES TO UNRAVEL COMPLEXITY OF MONOCYTES TRANSCRIPTOMES IN HEALTH AND DISEASE**

B. Smiljanovic<sup>1</sup>, J.R. Gruen<sup>1</sup>, T. Haeupl<sup>2</sup>, R. Baumgras<sup>1</sup>, A. Radbruch<sup>1</sup>, A. Gruetzka<sup>1</sup>  
<sup>1</sup>German Rheumatism Research Center-Berlin, Berlin, Germany, <sup>2</sup>Charité – Humboldt University Berlin, Rheumatology, Berlin, Germany

**Objectives:** Cytokines contribute to the host defense by an overall tuning of the immune system. Nevertheless, an excessive and uncontrolled production of pro-inflammatory mediators can be responsible for the onset and maintenance of chronic inflammatory diseases like rheumatoid arthritis (RA). The cytokine production that accompanied pathophysiological processes of chronic inflammation is reflected within the monocytes transcriptome. The present study was designed to define stimulus-specific expression patterns in tumor necrosis factor-alpha (TNF $\alpha$ ) and LPS-stimulated monocytes *in vitro*.

**Methods:** Subsequent to stimulation of whole blood with TNF $\alpha$  and LPS for 1.5 h, monocytes were isolated with a purity of > 99%. Using microarray technology as a comprehensive approach for identifying thousands of differentially expressed genes at a given time, we identified *in vitro* TNF $\alpha$ - and LPS-induced gene expression profiles. These global expression profiles were compared to that of monocytes isolated from RA patients.

**Results:** *In vitro* stimulation of whole blood samples with TNF $\alpha$  and LPS resulted in 4529 and 5036 differentially expressed genes, respectively. Both stimuli induced very similar profiles in monocytes, but also TNF $\alpha$ - or LPS- specific inflammatory patterns were identified.

Gene expression analysis of *in vivo* activated monocytes isolated from RA patients revealed 963 differentially expressed genes in comparison to healthy donors. Our results revealed significant numbers of differentially expressed cytokines and their receptors, and apoptosis-associated genes. To provide more specific insight into their expression we described a cytokine-cytokine receptor profile consisting of 121 genes and an apoptosis-survival profile including 240 genes.

**Conclusion:** Our findings indicate that an approach of defining *in vitro* induced TNF $\alpha$  and LPS gene expression profiles is able to decipher disease specific profiles of RA. 35.9% of differentially expressed genes in RA overlapped with the *in vitro* TNF $\alpha$  signature, while 34.5% of RA genes were contributed to the LPS signature. TNF $\alpha$  and LPS imprints in RA monocytes were detected within the cytokine-cytokine receptor and apoptosis-survival profiles. These imprints could provide an overview into stimulus-specific genes that are associated with chronic inflammation. In addition they could be used as biomarkers for understanding pathomechanisms underlining diseases and for monitoring and predicting drug responsiveness.

PC13/66 **MOLECULAR AND FUNCTIONAL CHARACTERIZATION OF P2X<sub>7</sub> GENE CODING POLYMORPHISMS IN RHEUMATOID ARTHRITIS**

A.K. Al-Shukaili<sup>1</sup>, A. Al-Gailani<sup>2</sup>, A. Al-Ansari<sup>1</sup>, J. Al-Kaabi<sup>3</sup>  
<sup>1</sup>Sultan Qaboos University, Microbiology & Immunology, Muscat, Oman, <sup>2</sup>Sultan Qaboos University, Biology, College of Science, Muscat, Oman, <sup>3</sup>Sultan Qaboos University Hospital, Medicine, Rheumatology unit, Muscat, Oman

**The P2X<sub>7</sub> receptor**, a member of the P2X family of nucleotide-gated channels, is predominantly expressed by monocytic cells such as monocytes, macrophages and dendritic cells. Moreover, activation of P2X<sub>7</sub> by ATP does not only lead to an ionic current, but also to alterations in cell morphology to cell permeabilization and to cytolysis after prolonged exposure to the agonist. P2X<sub>7</sub> receptor ability to induce posttranslation processing of IL-1 $\beta$ , release of IL-1 $\alpha$  and IL-18 implicated in the regulation of inflammation response.

The P2X<sub>7</sub> receptor is a 595 amino acids long protein encoded by a gene found on chromosome 12q24 and contains 13 exons. Genetic factors play a major role in this functional variability and 6 polymorphisms within the coding region of the P2X<sub>7</sub> gene have been shown to abolish receptor function, and 1 found to increase the affinity of ATP to the receptor as shown below and in the Table.

Polymorphism	Function
1. Glu496 to Ala (1513 A to C) 2. Ile 568 to Asn (1729 T to C) 3.Pro451 to Leu ( 4. Thr357 to Ser (1096 C to G) 5. Ala 348 to Thr (1068 G to A) 6.Arg 307 to Gln (946 G to A) 7. His 155 to Tyr (489 C to T)	1. Loss Function-Impair release of IL-1 $\beta$ 2. Prevent Normal trafficking 3. Impair pore formation and 4. Reduce ATP -induced apoptosis and killing of mycobacterium 5. Slightly reduce the activation of the receptor 6. Cause failure of ATP binding to the extracellular domain of P2X <sub>7</sub> 7.Gain function-Increase affinity of ATP to the receptor

[polymorphism within the P2X<sub>7</sub> gene]

The objective of this study is to analyze the role of the P2X<sub>7</sub> receptor and the functional polymorphisms (mentioned above) in RA patients. No significant differences between RA patients and controls in allele frequencies of three polymorphism [His 155 to Tyr (489 C to T), Glu496 to Ala (1513 A to C, ) Ala 348 to Thr (1068 G to A) ] were found. The gain function and loss function of SNP of P2X<sub>7</sub> gene does not appear to be a susceptibility gene locus for the development of RA.

PC13/67 **SCHNITZLER AND SAPHO SYNDROMES: A MOLECULAR LINK BETWEEN P2X<sub>7</sub> RECEPTOR AND INFLAMMASOME COMPLEX**

S. Falzoni<sup>1</sup>, A.L. Giuliani<sup>1</sup>, C. Pizzirani<sup>1</sup>, A. Lo Monaco<sup>2</sup>, M. Colina<sup>2</sup>, F. Trotta<sup>2</sup>, F. Di Virgilio<sup>1</sup>  
<sup>1</sup>University of Ferrara, Department of Experimental and Diagnostic Medicine, Section of General Pathology, Ferrara, Italy, <sup>2</sup>University of Ferrara, Department of Experimental and Clinical Medicine, Section of Rheumatology, Ferrara, Italy

Autoinflammatory diseases are a group of disorders characterized by recurrent unprovoked inflammatory events in absence of autoantibodies or autoreactive T cells. At least eight forms are well characterized :1) Familial Mediterranean Fever (FMF) 2) hypergammaglobulinemia D with periodic fever (HIDS), 3) tumor necrosis factor receptor-associated periodic syndrome (TRAPS), 4) Muckle-Wells syndrome (MWS) 5) familial cold autoinflammatory syndrome (FCAS), 6) chronic infantile neurologic cutaneous articular (CINCA) syndrome, 7) Pyogenic sterile Arthritis, Pyoderma gangrenosum and Acne syndrome (PAPA) and 8) Blau syndrome. Recently also Schnitzler and SAPHO (Synovitis Acne Pustulosis Hyperostosis Osteitis) syndromes have been added to the list. With the exception of FMF, these disorders are rare. In many of these diseases there is a fundamental alteration in the IL-1 (FCAS, MWS, CINCA, HIDS, FMF, PAPA) or TNF pathways (TRAPS). Recent advances in molecular genetics of FCAS, MWS and CINCA have led to the identification of the likely cause of deregulated IL-1 secretion. Processing and release of IL-1 $\beta$  occur in a macromolecular complex termed inflammasome which includes the proteins ASC, NALP3, Caspase-1 and Cardinal. The mechanism of inflammasome activation is obscure. However it is well known that extracellular ATP is the most potent physiological activator so far identified. Extracellular ATP-mediated activation of caspase-1 occurs via a receptor named P2X<sub>7</sub> (P2X<sub>7</sub>R). Mice deficient of constituents of the inflammasome (eg ASC) are also unresponsive to P2X<sub>7</sub>R-mediated stimulation but molecular link between the P2X<sub>7</sub>R and the inflammasome has not been elucidated. Here we have investigated the role of the P2X<sub>7</sub>R and of the inflammasome components ASC and NALP3 in the pathogenesis of Schnitzler and SAPHO syndromes. A better understanding of the mechanism by which the P2X<sub>7</sub>R drives inflammasome activation, pro-caspase-1 cleavage and IL-1 maturation may help to design novel treatments for autoinflammatory diseases.

**PC13/68 THE ROLE OF POLYMORPHNUCLEAR NEUTROPHILS (PMN) IN THE INITIATION OF COLLAGEN INDUCED ARTHRITIS (CIA)**E. Rath<sup>1</sup>, R. Byrne<sup>1</sup>, M. Bonelli<sup>1</sup>, A. Savitskaya<sup>1</sup>, B. Niederreiter<sup>1</sup>, J.S. Smolen<sup>1</sup>, C. Scheinecker<sup>1</sup><sup>1</sup>Medical University of Vienna, Vienna, Austria

**Background:** PMN seem to participate in the initiation of rheumatoid arthritis (RA) but their precise role in the development of synovial inflammation is only partially understood. We therefore analyzed the role of PMN in the initial steps of inflammation in the mouse model of collagen induced arthritis (CIA).

**Materials and methods:** CIA was induced in C57/BL6 mice by immunization with chicken type II collagen (CII) in Freund's complete adjuvant (CFA) at the base of the tail. On day 21, a second injection of CII in CFA was administered.

Mice were monitored for clinical signs of arthritis 3 times a week and tested for anti-CII serum antibodies by ELISA. Mice were sacrificed before clinical signs of arthritis occurred and at various timepoints after the onset of arthritis.

Hind and front paws were collected and cryostat sections as well as paraffin sections were prepared. Joint sections were analyzed after immunohistochemistry (hematoxylin/eosin, tartrate-resistant acid phosphatase – TRAP, toluidine blue, Neu7/4) and immunofluorescence staining (Neu7/4, C3a, MHC class II, CD11c) by confocal laser scanning microscopy. In addition real time in vivo imaging of PMN migration was performed.

**Results:** Mice developed clinical signs of arthritis 38±7 days after the first immunization with collagen. Severe periarticular and intraarticular neutrophilic infiltrates and cartilage destruction were detectable already on the first day of clinical arthritis. Strong staining of complement factor C3a was detectable in areas of inflammation and in the bone marrow of diseased animals. No histological signs of inflammation were detectable before the development of clinical signs of arthritis. In addition no C3a deposits were detectable before disease onset, suggesting that complement activation concurs with the influx of PMN. MHC class II was upregulated on Neu7/4 positive neutrophils in areas of inflammation.

**Conclusions:** Periarticular neutrophilic infiltrates are very prominent in CIA and are detectable already in very early stages of clinical disease. No histological signs of inflammation preceded clinical signs of arthritis. Neutrophils in inflamed areas displayed MHC class II expression which might contribute to their functional capacities. Ongoing experiments have been designed to investigate the migratory behaviour of PMN as well as therapeutic settings.

**PC13/69 CD137 AND CD137 LIGAND AND ITS SOLUBLE FORMS IN PLASMA AND SYNOVIAL FLUID FROM PATIENTS WITH JUVENILE IDIOPATHIC ARTHRITIS**M. Stiefel<sup>1</sup>, T.G. Müller<sup>1</sup>, R. Hühn<sup>1</sup>, M.S. Staeger<sup>1</sup>, J. Föll<sup>2</sup><sup>1</sup>Children's Cancer Research Center, Martin Luther-University, Department of Paediatrics, Halle, Germany, <sup>2</sup>Children's Cancer Research Center, Martin Luther-University; Department of Pediatric Hematology and Oncology, Childrens Hospital, University, Department of Paediatrics, Halle, Regensburg, Germany

**Objectives:** Juvenile idiopathic arthritis (JIA) is one of the most common inflammatory diseases in childhood and the pathogenesis is still unknown. Beside of genetic factors, e.g. single nucleotide polymorphism in cytokines or factors of the innate immune system, there is evidence which indicates a central role of the adaptive immune system in the pathogenesis of JIA. In the last years the role of CD137 and its ligand were investigated and found to play a role in sustaining inflammatory activity of T-cells. Elevated levels of soluble forms of CD137 and its ligand CD137L were detected in sera of adult patients with autoimmune disorders and haematological malignancies ADDIN EN.CITE ADDIN EN.CITE.DATA .

**Methods:** We collected plasma samples and synovial fluid from children and adolescents with juvenile idiopathic arthritis, adults with degenerative joint disease and plasma samples from children without autoimmune diseases. The soluble forms of CD137 (sCD137) and CD137L (sCD137L) were measured with an enzyme-linked immunoadsorbent assay (ELISA) and the membrane bound forms of CD137 and CD137L were analyzed with flow cytometry.

**Results:** In contrast to the control group (children without JIA and adults with degenerative joint disease) some children with JIA have elevated levels of soluble and cell surface linked forms of CD137 and CD137L. However, we observed also differences in the CD137 and CD137L level among the group of patients with JIA depending on disease activity and application of immune-modulating agents. Nevertheless, we found no correlations between conventional clinical parameters of disease activity (e.g. number of joints involved, erythrocyte sedimentation rate, CrP levels) and the levels of sCD137, sCD137L or cell surface linked CD137 and CD137L.

**Conclusion:** CD137 and its ligand might be involved in pathogenesis of JIA. However, these parameters did not correlate directly with several clinical parameters.

**PC13/70 CORRELATION BETWEEN CONTENTS OF ANTIBODIES TO CARDIOLIPIN AND AUTOANTIBODIES TO PURINE METABOLISM ENZYMES IN RHEUMATOID ARTHRITIS PATIENTS**A.V. Alexandrov<sup>1</sup><sup>1</sup>Research Institute for Clinical and Experimental Rheumatology, Volgograd, Russian Federation

**Objectives:** It is known that the process of T-cell immunity development in organism and cooperation of immune cells are associated with activity of Purine Nucleoside Phosphorylase (PNP) and Adenosine Deaminase (ADA). The development of antiphospholipid syndrome (APS), atherosclerosis, rheumatoid arthritis (RA) associates with Th1 type of immune reaction.

The aim of the study was to research the correlation between the process of autoantibodies (AB) to ADA and PNP production and level of AB to cardiolipin in RA patients.

**Methods:** 30 healthy persons and 46 RA patients without clinical manifestations of APS and negative on lupus anticoagulant were included in study. The AB of IgG class to ADA and PNP were determined by technique of indirect ELISA test using immobilized forms of investigated enzymes designed by us.  $\beta_2$ -Glycoprotein-I dependent AB to cardiolipin (aCL) of IgA, IgG/IgM classes were determined using commercial test-kits "Anti-Cardiolipin IgA" and "Anti-Cardiolipin IgG/IgM" (Orgentec Diagnostica GmbH).

**Results:** The insulated increase of AB to ADA was found in 30,4% (1<sup>st</sup> group), AB to PNP – in 17,4% (2<sup>nd</sup> group) of RA patients. Simultaneous increase of AB to ADA and AB to PNP levels (3<sup>rd</sup> group) was watched in 21,7% of cases. aCL were found in 26,1% of RA patients. aCL IgA+IgG (4 of 14) were found most often in 1<sup>st</sup> group of RA patients. In 2<sup>nd</sup> group maximum number of RA patients was found positive on aCL IgG+IgM (3 of 8). In 3<sup>rd</sup> group RA patients positive on aCL IgA+IgG+IgM (4 of 10) were prevailed. It should be noted that increased levels of aCL IgA+IgG were associated with pulmonary hypertension (p=0,018), and aCL IgA+IgG+IgM – with venous thromboses (p=0,041). The increase of IgA concentration was not revealed (p > 0,05) in serum of RA patients that eliminates false positive results of aCL determination of IgA.

**Conclusion:** Taking into consideration the relevant role of  $\beta_2$ -Glycoprotein-I in induction of aCL synthesis, it is possible to suspect participation of AB to purine metabolism enzymes in conformational changes of  $\beta_2$ -Glycoprotein-I and/or cardiolipin structure, that promotes formation of "immune" complex of  $\beta_2$ -Glycoprotein-I with phospholipides and induces aCL synthesis in RA patients.

**PC13/71 IMMUNOLOGICAL PROFILE IN PATIENTS WITH OSTEOARTHRITIS**C. Tsigalou<sup>1</sup>, E. Konstantinidou<sup>1</sup>, T. Konstantinidis<sup>1</sup>, T. Gioka<sup>1</sup>, K. Zervogiannidou<sup>1</sup>, N. Galanopoulos<sup>2</sup>, G. Kampourimiti<sup>1</sup><sup>1</sup>University General Hospital of Alexandroupolis, Immunology Department of Microbiology Laboratory, Alexandroupolis, Greece, <sup>2</sup>University General Hospital of Alexandroupolis, Rheumatology Unit, Alexandroupolis, Greece

**Objectives:** To detect certain autoantibodies such as antinuclear antibodies (ANA) and antibodies against double stranded DNA (ds-DNA) and to study their prevalence in patients with osteoarthritis (OA).

**Methods:** 50 patients were included in the study, 3 (6%) male and 47(94%) female, aged 55-82, with osteoarthritis. The analyses included ANA (by IIFon Hep-2 cells), anti-dsDNA (by IIF-C.luciliae and elisa-AESCLISA), anticardiolipin (ACA) and anti-b2GPI IgG-IgM with elisa-AESCLISA and anti-CCPs elisa. Also C3, C4 and rheumatoid factor (RHF) determination was conducted by nephelometry (IMMAGE-BECMANN COULTER).

**Results:** Immunological screening revealed :

1/ 31 patients (62%) were found negative for ANA and 19 (38%) positive with titres 1/160 78,95% (15/19), 1/320 15.79% (3/19) and one patient with titre 1/640 5.26%(1/19).

2/ anti-dsDNA and ACA did not exist in any patient.

3/ Complement fractions C3, C4 were in normal ranges in all patients.

4/3 patients (6%) expressed anti-CCPs and two of them had RHF in low titres (37.9U/ml and 58.4 U/ml). In other 3 patients low titres of RHF were existing with negative anti-CCPs.

5/Only one patient developed low titres of anti-b2GPI IgM.

**Conclusions:** Clinicians should be aware that patients with OA may develop specific autoantibodies in follow up studies for 2-4 year and clinical features of rheumatic diseases. In our study no patient developed clinical and immunological features of such a disease. Previous reports have showed that the induction of non-organ-specific autoantibodies such as ANA (not in significant quantities) aren't have good prognostic value for developing autoimmune disorders. Our results were in line with previous reports.

**PC13/72 MACROPHAGE ACTIVATION SYNDROME IN LONG LASTING ADULT-ONSET STILL DISEASE**E. Verrecchia<sup>1</sup>, A. Soriano<sup>1</sup>, D.S. Giuliana<sup>1</sup>, C. Fannesu<sup>1</sup>, M. Gioviale<sup>1</sup>, M. Montalto<sup>1</sup>, R. Landolfi<sup>1</sup>, G.B. Gasbarrini<sup>1</sup>, R. Manna<sup>1</sup><sup>1</sup>Catholic University of Sacred Heart, Department of Internal Medicine, Rome, Italy

**Introduction:** Adult onset Still disease (AOSD) is a rare systemic inflammatory disorder of unknown etiopathogenesis, characterized by acute onset with high fever, arthralgia, arthritis and evanescent maculo-papular skin rash, often synchronous with fever, with neutrophilic leukocytosis and negative autoantibodies. The clinical course may present regular systemic exacerbations and frequent intervals disease-free. Severe manifestations of AOSD can be fatal and occasionally related



to macrophage activation syndrome (MAS). It is characterized by proliferation of mature non-neoplastic histiocytes with prevailing hemotofagocytic activity within the bone marrow, spleen and lymph nodes, and it is manifested by fever, lymphadenopathy, hepatosplenomegaly and cytopenia. Although MAS represents a secondary phenomenon, it can mask the underlying condition, generally a neoplastic or infective disease, thus making the patient management rather difficult.

**Methods and results:** We describe the case of a female, 56 y. o., admitted for recurrent fever (T. max 40° C), lasting 2 weeks every 15 days, with arthralgia, myalgia, which received diagnosis of AOSD according Yamaguchi criteria. HCV with normal transaminases and mild monoclonal gammopathy were present. Therapy with methylprednisolone (16 mg/day) was started with initial benefit. Following relapse of disease, metotrexate (7.5 mg/week), then hydroxychloroquine (200mg /die) were added to steroid without benefit. Blood tests documented pancytopenia with ferritin >1000 mg/ml and high levels of acute phase reactant. Other autoimmune examinations resulted negative. Serological analysis resulted negative except for transient positive hemocultures for *Staphylococcus epidermidis*, and lower urinary tract infections. A computed tomography showed lymphadenopathy and hepatosplenomegaly. Severe pancytopenia required blood transfusion. A bone marrow biopsy was performed showing increase in share reactive and presence of many macrophages laden with cellular debris. Thus, on the basis of clinical history and laboratory features, diagnosis of AOSD with MAS likely to infectious triggers was made. Intravenous steroids and cyclosporine (CyA) (200 mg/day) were administered with a gradual resolution of the clinical picture, with reduction of fever, and improvement of blood parameters during disease-free periods. No HCV activation was observed during CyA treatment.

**Conclusion:** CyA has been proven useful in AOSD and MAS treatment, without leading to a worsening of HCV-related liver disease.

#### PC13/73 CORRELATION OF THREE ANALYTICAL METHODS FOR DETERMINATION OF ANTIBODIES ANTI-CCP (ACCP)

D. Cembrero<sup>1</sup>, A. Hernández<sup>1</sup>, M.B. Aparicio<sup>1</sup>, J.C. Ramos<sup>1</sup>, M.B. García<sup>1</sup>, J. Navajo<sup>1</sup>

<sup>1</sup>Hospital Universitario Salamanca, Laboratorio Autoinmunidad, Salamanca, Spain

**Objectives:** Rheumatoid Arthritis (RA) to chronic inflammatory disorder of the synovial membrane, is one of the most common autoimmune diseases. RA clinical diagnosis depends on both manifestations and to multitude of laboratory test. The objective of our work is to make the comparative study by means of the statistical analysis being obtained the existing correlation between the results obtained after the determination of the levels of antibodies anti-CCP of 2 generation by means of enzymeimmunoassay (three ELISA) of kits commercial different.

**Methods:** 83 samples sent to our laboratory of patients coming from the Department of Rheumatology of our hospital were analyzed, of which 25 belong to healthy donors, 45 are samples of patients, with values of antibodies anti-CCP over the point of cut (positive) of the technique used then in our laboratory, (Innovates, ELISA) and the 13 rest also come from the service of Rheumatology of the hospital, but in this case they present/display values below the point of cut (negative) with he himself Kit used at this moment by our Laboratory (It innovates, ELISA). Three measurements with three different reagents are made:

- KIT QUANTA-LiteTM CCP IgG ELISA (Innova)
- KIT KALLESTADTM Anti-CCP II Microplate EIA (BioRad)
- KIT of PCC Immunoscan CCPlus® (Euro-Diagnose).

The statistical analysis was made using the computer science program Spss-12.

**Results:** The obtained results show different correlations to us based on the used reagent, obtaining the best correlation ( $r=0,976$ ) between the Kit KallestadTM Anti-CCP II Microplate EIA of BioRad and the Kit PCC Immunoscan CCPlus® de Euro-Diagnose. The rest of correlations by importance order would be: Kit KallestadTM Anti-CCP II Microplate EIA of BioRad and Kit QUANTA-LiteTM CCP IgG ELISA de Innova ( $r=0,856$ ) and finally, Kit PCC Immunoscan CCPlus® de Euro-Diagnose and Kit QUANTA-LiteTM CCP IgG ELISA Innova ( $r=0,834$ ).

**Conclusion:** The obtained results show that a good correlation between the different analytical methods for the determination from antibodies exists anti-CCP, with respect to the positivity and negativity of the results, in case of wanting to use them indifferently would have to apply a correction factor.

#### PC13/74 IMMUNE CELLS PRODUCE CATECHOLAMINES DURING ARTHRITIS: NEW ASPECTS OF NEURO-IMMUNOLOGICAL RESPONSE

S. Capellino<sup>1</sup>, K. Weber<sup>1</sup>, A. Faßold<sup>1</sup>, C. Wolff<sup>1</sup>, R.H. Straub<sup>1</sup>

<sup>1</sup>University Hospital Regensburg, Dept. of Internal Medicine I, Regensburg, Germany

**Objectives:** In our previous study we demonstrated that synovial cells produce catecholamines during chronic inflammation such as rheumatoid arthritis, and that catecholamine modulation reduced TNF release in primary synovial cells. Also *in vivo* on DBA/1J arthritis mice we confirmed the beneficial effect of catecholamine modulation on arthritis. Therefore, we aimed to understand whether catecholamine-producing cells differentiate in synovial tissue or not, and when they start to play a role in the inflammatory response.

**Methods:** Collagen type-II induced arthritis model was used in DBA/1J mice. Twenty mice were immunized and 20 mice served as controls. At day 0, 7, 14, 21, 28, 35, 42, 49, 60 and 80 after first immunization, two mice of each group were sacrificed. Bone marrow, paws and spleen were stained by immunofluorescence to detect vesicular monoamine transporter-2 (VMAT-2). Density of positive cells was averaged and expressed per square millimeter.

**Results:** Twenty-one days after first immunization, the amount of VMAT-2 positive cells in bone marrow started to be significantly higher in arthritic mice compared to controls. In the spleen, the amount of VMAT-2 positive cells was significantly higher in arthritic mice from day 49. First results on the paws showed that VMAT-2 positive cells appear later during the chronic phase.

**Conclusions:** These results demonstrate that catecholamines are produced and stored by immune cells in the early asymptomatic phase of arthritis. Thus, they might play a crucial role in immunomodulation.

#### PC13/76 IGE AND RF CORRELATION IN SYNOVIAL FLUID AND SERUM IN RHEUMATOID ARTHRITIS WITH REACTIVE SINOVITIS

L. Begolli<sup>1</sup>, G. Begolli<sup>2</sup>, L. Pajazit<sup>3</sup>, V. Topciu<sup>1</sup>, Z. Baruti<sup>3</sup>

<sup>1</sup>Medical Faculty, University of Prishtina, Biochemistry, Prishtina, Serbia and Montenegro, <sup>2</sup>Biochemical Laboratory 'BIOTICUS', Biochemistry, Prishtina, Serbia,

<sup>3</sup>Medical Faculty, University of Prishtina, Prishtina, Serbia and Montenegro

**Objective:** High prevalence of diseased with rheumatoid arthritis is present in our country. The etiology of RA is not clear yet. It is believed that there are some arthritogen factors which initiate the immunological changes at the predisposed persons and create auto antibodies. We consider reasonable that except other examinations of the diseased with reactive synovitis, the level of IgE in serum and synovial fluid and rheumatoid factor should be examined, too.

**Material and method:** 30 diseased with RA and reactive synovitis have been examined. They were of both sex and 30-60 years aged. After the clinic examination the laboratory investigations have been determined. The IgE level has been determined by using ELISA method, while the rheumatoid factor (RF) by agglutination.

**Results:** After statistical analysis of the obtained values, the IgE level in synovial fluid and serum was lower in RA  $p < 0.05$  compared with referent values. There was significant correlation with the values in serum, which increased in linear form with the values in synovial fluid. There were some cases with high values in serum, synovial fluid and high titers of rheumatoid factor (RF). The IgE level is in significant correlation with the RF titers.

**Conclusion:** Obtained results shows that the diseased with RA and reactive synovitis is serum and synovial fluid has low level of IgE.

The correlation between the IgE level in serum and synovial fluid is significant.

The rheumatoid factor is in direct correlation with the IgE level.

#### PC13/77 RANKL-RANK/OPG MOLECULAR COMPLEX – CONTROL FACTORS IN BONE REMODELING

G.R. Cioaca<sup>1</sup>

<sup>1</sup>University of Medicine and Pharmacy Victor Babes Timisoara, Immunology, Timisoara, Romania

Bone remodeling is a cyclic and continuous physiological process, which ensures the conservation and renewal of the bone matrix. Osteosynthesis of the bone matrix is achieved by osteoblasts and coordinated within this complex machinery of bone remodeling with resorption of extracellular bone matrix performed by osteoclasts.

The phenotype, function and osteogenesis of osteoblasts and osteoclasts are essentially different. Osteoblasts arise from bone-marrow stromal cells, and osteoclasts arise from hematopoietic myeloid progenitor cells-monocyte/macrophage. Both types of cells have a common target – the bone structure. The imbalance between the effector activities of osteoblasts and osteoclasts has clinical implications associated with the decrease or increase of bone mass mineral density, osteoporosis or osteopetrosis respectively.

Reports from 1977-1988 has offered new explanations that have changed the perception on bone metabolism and has opened new immunoclinical interpretations with therapeutic prospects. The balance of the trimolecular complex composed of Osteoprotegerin (OPG), RANKL (Osteoprotegerin-ligand) and RANK, which are control factors through osteoclastogenesis modulation, governs homeostasis of bone remodeling. These molecules, OPG, RANKL and RANK, function as receptors and ligand and belong to the superfamily of tumor necrosis factor (TNF).

#### PC13/78 SERUM LEVELS OF HUMAN ERYTHROPOIETIN, TUMOUR NECROSIS FACTOR-ALPHA AND INTERLEUKIN-10 IN PATIENTS WITH EARLY RHEUMATOID ARTHRITIS TREATED WITH INFILIXIMAB

S. Oranskiy<sup>1</sup>, L. Yeliseyeva<sup>1</sup>, Y. Vasinova<sup>1</sup>

<sup>1</sup>Kuban State Medical University, Krasnodar, Russian Federation

**Objectives:** Now it is actively studied the cytokine profile in patients with rheumatoid arthritis (RA) and its dynamics with anticytokine treatment. Less it is known about a problem of erythropoietin production and its relationships with cytokines in RA patients without anemia. The aim of this study is researching of the serum concentrations of human erythropoietin (EPO), tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-10 (IL-10) in 15 patients with early seropositive RA (less than 3 years) without anemia, treated with infliximab (I).

**Methods:** Cytokine and EPO levels were determined with commercially available ELISA kits. I was initially injected at a single dose of 3 mg/kg intravenously, followed by administration 2 and 6 weeks later, and then repeated every 8 weeks. Data presented as median (25; 75 percentiles).

**Results:** We find the initial high levels of TNF- $\alpha$  – 257 (172; 283) pg/ml than in control group – 110 (89; 143) pg/ml ( $p=0.03$ ) and IL-10 – 473 (267; 763) versus 183 (158; 202) pg/ml ( $p=0.02$ ) and low concentration of EPO – 4,2 (1,5; 6,1) versus 9 (5; 12) mIU/ml in controls ( $p=0.03$ ). We also find negative correlation between TNF- $\alpha$  and EPO levels ( $r = -0.42$ ;  $p=0.04$ ). After 6 weeks of 1 therapy TNF- $\alpha$  serum level was decreased to 175 (120; 201) ( $p=0.04$ ), IL-10 level did not changed significantly in comparison with pretreatment level. EPO concentration increased after 1 therapy to 10,1 (6; 14,3) mIU/ml ( $p=0.03$ ) without differences with control group.

**Conclusion:** In our study we demonstrated that patients with early RA without anemia may have depression of EPO that associated with TNF- $\alpha$ . Infliximab treatment normalized not only TNF- $\alpha$  levels, but also activate EPO production.

#### PC13/79 ANTI-CYCLIC CITRULLINATED PEPTIDE (ANTI-CCP3) AND ANTI-MODIFIED CITRULLINATED VIMENTIN (ANTI-MCV) ANTIBODIES: SENSITIVITY IN THE DIAGNOSIS OF RHEUMATOID ARTHRITIS (RA)

A. Vakaloudi<sup>1</sup>, V. Papakonstantinou<sup>1</sup>, V. Bliskas<sup>1</sup>, V. Galanopoulou<sup>1</sup>

<sup>1</sup>"Agios Pavlos" General Hospital, Biopathology Laboratory, Thessaloniki, Greece

**Objective:** To evaluate the sensitivity of anti-MCV in the diagnosis of rheumatoid arthritis (RA) and to compare it with the sensitivity of anti-CCP3.

**Methods:** Concentrations of anti-CCP3 and anti-MCV were determined in the sera of 53 patients of "Agios Pavlos" General Hospital, 39 females (mean age 58 years) and 14 males (mean age 60 years), with early and established RA. All individuals were positive for rheumatoid factor (RF). Anti-CCP3 and anti-MCV were measured with ELISA, while RF was measured with nephelometry.

**Results:** Anti-CCP3 were detected in the sera of 38 patients (sensitivity 71.7%), with a level range of 25 – 2430 IU/ml. Anti-MCV were detected in the sera of 39 patients (sensitivity 73.6%), with a level range of 21 – 851 IU/ml. 29 patients (54.7%) were positive for both anti-CCP3 and anti-MCV, whereas 9 patients (16.9%) were anti-CCP3-positive and anti-MCV-negative, 10 patients (18.8%) were anti-CCP3-negative and anti-MCV-positive and 9 patients (16.9%) were negative for both anti-CCP3 and anti-MCV. Finally, 44 patients (83%) were positive for at least one of the two antibodies.

**Conclusion:** Anti-CCP3 and anti-MCV present similar sensitivity in the diagnosis of RA. The combined use of the 2 markers leads to increased sensitivity.

### PC18 – NEUROMUSCULAR DISEASES, MULTIPLE SCLEROSIS AND PRIONS

#### PC18/1 THE CELLULAR PRION PROTEIN PROTECTS T LYMPHOCYTES TO OXIDATIVE STRESS DURING THEIR DEVELOPMENT IN THE THYMUS

P.N. Marche<sup>1</sup>, C. Aude-Garcia<sup>2,3</sup>, C.L. Villiers<sup>1</sup>, S. Candéias<sup>2,3</sup>, C. Bertrand<sup>2</sup>, V. Collin<sup>3</sup>, E. Jouvin-Marche<sup>1</sup>

<sup>1</sup>INSERM U823/University of Grenoble, Institut Albert Bonniot, Grenoble, France, <sup>2</sup>CNRS-UMR 5092, Grenoble, France, <sup>3</sup>CEA/DSV/IRTSV, Laboratoire Biochimie et Biophysique des Systèmes intégrés, Grenoble, France

The cellular prion protein (PrP<sup>C</sup>) is an ubiquitously expressed glycoprotein whose physiologic functions remain enigmatic, particularly its role in immune system. Here, we demonstrate both *in vitro* and *in vivo*, that PrP<sup>C</sup> is involved in T lymphocytes response to oxidative stress. In addition, we show that each thymocyte subpopulation, defined flow cytometry for the expressions of CD4, CD8, CD44 and CD25 markers, is characterized by a precise level of reduced glutathione that evolves throughout differentiation in the thymus. This indicates a marked evolution of the redox status of the T lymphocytes during their maturation in the thymus. By monitoring the level of reduced glutathione in thymocytes after H<sub>2</sub>O<sub>2</sub> exposure, we show that thymocytes from PrP<sup>C</sup> mice defective for PrP<sup>C</sup> expression, display a higher susceptibility to induced oxidative stress than thymocytes from control C57BL/6 mice. Then, by submitting PrP<sup>C</sup> and C57BL/6 mice to a restricted diet, known to increase the intracellular level of ROS, we find that developing thymocytes are more sensitive to oxidative stress in PrP<sup>C</sup> mice. Finally, PrP<sup>C</sup> is thought to act specifically on oxidative stress, since when other kinds of stress are applied, no significant differences are observed between PrP<sup>C</sup> and C57BL/6 exposed mice. Taken together, our results clearly ascribe to PrP<sup>C</sup> a protective function in T lymphocytes against oxidative stress.

#### PC18/2 INTRAVITAL IMAGING OF TH17-MEDIATED NEURONAL DAMAGE PROCESSES IN EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

V. Siffrin<sup>1</sup>, H. Radbruch<sup>2</sup>, R. Glumm<sup>2</sup>, E. Esplugues<sup>3</sup>, R. Niesner<sup>2</sup>, H. Luche<sup>4</sup>, J.L. Rinnenthal<sup>2</sup>, H.-J. Fehling<sup>4</sup>, F. Zipp<sup>2</sup>

<sup>1</sup>Charité – University Medicine Berlin, MDC Berlin-Buch, Cecile Vogt Clinic for Neurology, Berlin, Germany, <sup>2</sup>Charité – University Medicine Berlin, Berlin, Germany, <sup>3</sup>Yale University, New Haven, United States, <sup>4</sup>University Clinics Ulm, Ulm, Germany

**Objectives:** Experimental Autoimmune Encephalomyelitis (EAE) is the prototype inflammatory demyelinating disease of the central nervous system (CNS), which serves as a model for the human disease Multiple Sclerosis. Although, it is assumed that in autoimmune neuroinflammation, the primary immune response is directed against the myelin sheath, axonal and neuronal damage is prominent already in early disease stages and probably determines the long-term disability in patients.

**Methods:** We investigated here the role of immune cells in these early neurodegenerative processes *in vivo* by monitoring CNS inflammation using two-photon microscopy of living anaesthetized mice. Investigating *Thy-1-EGFP* and *Thy-1-CertNL15* transgenic and *Rosa26-tRFP* or *IL17-EGFP* bone marrow chimeric mice in EAE, we gathered real-time data on immune mediated interaction and damage processes in the CNS in correlation to the clinical disease manifestation.

**Results:** Indeed, we were able to detect *in vivo* both transient and long-lasting interactions of immune cells with neuronal processes in the brainstem of EAE affected animals. Contact formation led to severe disturbances of neuronal intracellular Calcium concentration as indicator of neuronal dysfunction. Quantification of contact formation showed distinct distribution pattern in the course of the disease. Direct interaction of immune and neuronal cells in the course of autoimmune neuroinflammation in demyelinated lesions could be attributed to IL-17 producing CD4 T helper cells, which indicates a special role of the TH17 subpopulation for neuronal dysfunction and clinical deficit in chronic neuroinflammation.

**Conclusion:** Our data demonstrate a direct attack of TH17 cells against neurons in chronic demyelinating inflammation of the brain.

#### PC18/3 PRIMARY OLIGODENDROPATHY IS NOT A TRIGGER OF CNS INFLAMMATION

S. Würtge<sup>1</sup>, F. Frommer<sup>1</sup>, T. Buch<sup>2</sup>, G. Lucatelli<sup>2</sup>, I. Bechmann<sup>3</sup>, K. Karram<sup>4</sup>, J. Trotter<sup>4</sup>, B. Becher<sup>2</sup>, A. Waisman<sup>1</sup>

<sup>1</sup>University Medical Center of the Johannes Gutenberg University Mainz, I. Medical Department, Mainz, Germany, <sup>2</sup>Institute of Experimental Immunology, University of Zurich, Department of Pathology, Zurich, Switzerland, <sup>3</sup>Johann Wolfgang Goethe-University, Institute of Clinical Neuroanatomy, Frankfurt am Main, Germany, <sup>4</sup>Johannes Gutenberg University of Mainz, Molecular Cell Biology, Mainz, Germany

Multiple sclerosis (MS) is an autoimmune disease thought to be initiated by an autoimmune Th1 and Th17 response specific to antigens of the central nervous system. Contrary to this, recent data suggest that neurodegenerative mechanisms are primary triggers of some forms of MS. In this scenario, a major role could be played by the release of potential self-antigens from dying oligodendrocytes (ODCs).

To test this hypothesis we developed a mouse model (MOGCre/iDTR) that allows us to selectively initiate ODC cell death in an inducible manner by transgenic expression of a diphtheria toxin receptor in ODCs and peripheral injection of diphtheria toxin (DT). Injection of DT resulted in pronounced demyelination, and was clinically characterized by ataxia, tremor, development of a hunchback and complete paralysis. Due to the massive demyelination at the peak of the disease a dramatic neuronal degeneration was observed. Surprisingly, some of the diseased mice recovered completely even after complete hind limb paralysis. The later may be a result of an accumulation of ODC precursors in the demyelinated brain areas, as observed by us. None of the sick animals showed signs of classical EAE or brain infiltration of immune cells, although the animals showed an activation of microglia followed by strong gliosis. Thus, we investigated whether and under which conditions dying ODCs release antigens that can prime T-cell responses towards myelin. Injection of CFSE-labeled, MOG-reactive T-cell receptor transgenic cells showed no or only moderate T-cell proliferation after ablation of ODCs. In addition, release of antigen by dying ODCs did not lead to tolerance induction.

We conclude that factors in addition to ODC-death lead to the neurological symptoms observed in EAE, and that at least in mice, dying of ODCs does not contribute to EAE induction or even tolerance.

#### PC18/4 EXPRESSION OF THE ORPHAN NUCLEAR RECEPTOR NR4A2 IS REQUIRED FOR IL-17 PRODUCTION BY TH17 CELLS

B.J.E. Raveney<sup>1</sup>, S. Oki<sup>1</sup>, T. Yamamura<sup>1</sup>

<sup>1</sup>National Institute of Neuroscience, NCNP, Department of Immunology, Kodaira, Japan

**Objectives:** To examine how NR4A2, an orphan nuclear receptor implicated in autoimmune disease, controls Th17 cell responses.

**Methods:** EAE and EAU were induced in susceptible mice by immunisation with MOG or IRBP peptides in CFA. Th1 and Th17 cells either infiltrating the target organ (CNS or retina) or from secondary lymphoid tissue were isolated based on cytokine secretion and gene expression by these cells was analysed by RT-PCR. Using siRNA, the influence of NR4A2 on Th1 and Th17 differentiation and function was examined *in vitro* and *in vivo*.

**Results:** We have previously shown that NR4A2 is upregulated in peripheral blood cells during the human autoimmune disease multiple sclerosis (MS) and in peripheral blood cells and CNS-infiltrating cells in EAE (Doi *et al.* PNAS 2008). We now show that NR4A2 is also associated with the onset of inflammation in a model of ocular autoimmunity, EAU. As MS, EAE, and EAU result from infiltration of both Th1 and Th17 cells into the target organ, we investigated whether NR4A2

expression was associated with these T cell subsets. The NR4A2 upregulation observed in CNS-infiltrating leukocytes is most apparent amongst IL-17-secreting CD4<sup>+</sup> T cells. Therefore, we examined the role of NR4A2 in the differentiation of these Th17 cells. Transfection of naïve T cells with NR4A2 siRNA precludes IL-17 secretion when cultured under Th17 polarising conditions. However, such cells do proliferate and upregulate ROR $\gamma$ t to the same extent as functional Th17 cells. Surprisingly, NR4A2 inhibition also reduced the expression of foxp3. Furthermore, NR4A2 siRNA treatment prevents the induction of EAE.

**Conclusion:** NR4A2 upregulation by CD4<sup>+</sup> T cells is associated with Th17-mediated inflammatory autoimmune disease of both the CNS and the retina. Upregulation of this gene is also required for *in vitro* generation of IL-17-producing CD4<sup>+</sup> T cells. The control of IL-17 production by NR4A2 is independent of both ROR $\gamma$ t upregulation and foxp3-mediated regulation of ROR $\gamma$ t. Our findings indicate that NR4A2 may play a critical role in Th17 cell function and may be essential for the initiation of organ-specific autoimmunity. Thus, NR4A2 is an attractive therapeutic target in the treatment of Th17-mediated autoimmune diseases.

#### PC18/5 ROLE OF CD8+ T CELLS IN THE PATHOGENESIS OF EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

A. Weishaupt<sup>1</sup>, N. Beyersdorf<sup>2</sup>, M. Camara<sup>2</sup>, M.J. Herold<sup>2,3</sup>, J. van den Brandt<sup>4</sup>, H.M. Reichardt<sup>4</sup>, T. Hünig<sup>2</sup>, T. Kerkau<sup>2</sup>

<sup>1</sup>University of Würzburg, Department of Neurology, Würzburg, Germany, <sup>2</sup>University of Würzburg, Institute for Virology and Immunobiology, Würzburg, Germany,

<sup>3</sup>The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia, <sup>4</sup>University of Göttingen Medical School, Department of Cellular and Molecular Immunology, Göttingen, Germany

Multiple Sclerosis (MS) and its animal model Experimental Autoimmune Encephalomyelitis (EAE) are T cell-mediated inflammatory diseases of the central nervous system. While CD4<sup>+</sup> T cells specific for myelin-derived antigens are believed to be critical for induction, progression and regulation of these autoimmune diseases, the role of CD8<sup>+</sup> T cells has not been clarified so far. Here we investigated the contribution of CD8<sup>+</sup> T cells to the immunopathogenesis of EAE in the Lewis rat by comparing disease activity in normal Lewis rats with that of CD8<sup>+</sup> T cell-deficient animals (CD8 $\alpha$ -deficient animals or animals depleted of CD8<sup>+</sup> T cells after application of the monoclonal antibody OX8). In both models we found diminished disease activity of EAE induced by immunization with guinea pig myelin basic protein (gpMBP). The reduction in the magnitude of the disease was accompanied by reduced infiltration of immune cells into the central nervous system (CNS). Activated gpMBP-specific CD4<sup>+</sup> T cells could be detected in the draining lymph nodes of CD8<sup>+</sup> T cell-deficient animals, although they did not produce inflammatory cytokines such as Interferon- $\gamma$ . These results suggest that CD8<sup>+</sup> T cells are needed for the induction of effector cell differentiation in the periphery. To assess whether CD8<sup>+</sup> T cells were also needed for CNS infiltration of already differentiated MBP-specific T cells, we transferred CD4<sup>+</sup> encephalitogenic T cells generated from normal Lewis rats into either wild type recipients or CD8<sup>+</sup> T cell-deficient recipients. Interestingly, Adoptive Transfer EAE induced in CD8-deficient recipient rats was indistinguishable from that in normal recipient rats. Taken together, we conclude that CD8<sup>+</sup> T cells are crucial for the differentiation of MBP-specific T cells, but dispensable once the encephalitogenic T cells have developed into effector T cells.

This work was supported by grants from the Interdisziplinäres Zentrum für Klinische Forschung Würzburg (IZKF A-52).

#### PC18/6 ENDOGENOUS ESTROGENS PROTECT FROM EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS THROUGH ESTROGEN RECEPTOR $\alpha$ -SIGNALING

K. Lélou<sup>1</sup>, L. Delpy<sup>2</sup>, V. Robert<sup>1</sup>, S. Laffont<sup>1</sup>, L. Pelletier<sup>1</sup>, J.-C. Guéry<sup>1</sup>

<sup>1</sup>INSERM U563, Toulouse, France, <sup>2</sup>CNRS, UMR6101, Limoges, France

**Objectives:** Sex hormones influence immune responses and the development of autoimmune diseases including multiple sclerosis (MS) and its animal model, experimental autoimmune encephalomyelitis (EAE). Indeed, we have previously shown that administration of 17 $\beta$ -estradiol (E2), through estrogen receptor  $\alpha$  (ER $\alpha$ ) protect from EAE in C57BL/6 mice (Garidou et al., J. Immunol. 2004 173:2435). However, it is still not clear whether basal levels of endogenous estrogens through classical estrogen receptors (ER) can significantly influence CNS autoimmunity.

**Methods:** In the present work, we investigated the effect of endogenous estrogens on the disease course of EAE in C57BL/6 (B6) mice by comparing disease development between ovariectomized (Ovx) and sham-operated mice. Two EAE models were used: i) active EAE induced by immunization with MOG35-55 in CFA and ii) passive EAE induced by adoptive transfer of MOG-specific CD4<sup>+</sup> T cells. The role of ER $\alpha$  signaling was also evaluated using ER $\alpha$ -mutant mice and bone marrow chimera experiments.

**Results:** Here we show that induction of active EAE with lower M. tuberculosis concentrations for immunization results in less severe disease course in normal but not in Ovx mice revealing a regulatory role for endogenous ovarian hormones in EAE. Although, gonadectomy accelerated the recruitment of inflammatory cells into the CNS, it did not modify autoantigen-specific T cell priming and differentiation into Th1 or Th17 effector cells in the periphery. Consistent with this observation, adoptive transfer of MOG-specific encephalitogenic CD4<sup>+</sup> T lymphocytes induced more severe EAE in Ovx mice as compared to normal female mice. Castration of estrogen receptor  $\alpha$  (ER $\alpha$ -/-) deficient female mice did not affect EAE development, demonstrating that endogenous estrogens down regulate EAE through ER $\alpha$ . Finally, using bone-marrow chimeras, we show that ER $\alpha$ -expression in hematopoietic cells was dispensable for the protective effect of endogenous estrogens.

**Conclusions:** Our data firmly establish that endogenous estrogens, through ER $\alpha$ -signaling, exert a strong regulatory effect on both active and passive EAE development and CNS inflammation in female mice. Altogether, these data suggest that endogenous estrogens could act by decreasing inflammatory cell recruitment into the CNS rather than by limiting effector T cell differentiation or cytokine production in the periphery.

#### PC18/7 CONSTITUTIVE ACTIVATION OF THE RAS-MAPK PATHWAY LEADS TO ONCOGENE-INDUCED PREMATURE SENESCENCE OF HUMAN THYMIC MEDULLARY EPITHELIAL CELLS OF PATIENTS WITH MYASTHENIA GRAVIS

V. Antonini<sup>1</sup>, A. Porzia<sup>2</sup>, M. Colombara<sup>1</sup>, A.P. Riviera<sup>3</sup>, O. Poffe<sup>1</sup>, N. Brutti<sup>3</sup>, S. Grasso<sup>1</sup>, G. Tridente<sup>1</sup>, F. Mainiero<sup>2</sup>, D. Ramarli<sup>3</sup>

<sup>1</sup>University of Verona, Pathology/Section of Immunology, Verona, Italy, <sup>2</sup>University 'La Sapienza', Experimental Medicine and Pathology, Rome, Italy, <sup>3</sup>Azienda Ospedaliera di Verona/Clinical Immunology, Verona, Italy

Myasthenia Gravis (MG) is an autoimmune disease characterized by muscular weakness resulting from the autoantibody-targeting to Acetylcholine receptors at the neuromuscular plaque. MG association with thymic pathology, mainly with thymic hyperplasia (TH) is unique among autoimmune diseases.

The pathogenic role of TH, occurring in 60% of patients, but in 100% of early onsets (EOMG) is an established fact. Thymectomy lowers the titre of anti-AchR antibodies and alleviates neurological symptoms. Thymic medulla is site of germinal centres that arise from infiltrated peripheral lymphocytes and comprise B cells producing anti-AchR antibodies.

Homing receptor/s enabling peripheral lymphocyte immigration to thymus remain/s elusive. Conversely, overproduction of IL1-beta, IL-6, CCL5, CCL21 and CXCL13 by medullary epithelial cells (mTEC) appears implicated in pathogenesis.

We have previously reported that in EOMG-mTECs the IL-6 and CCL5 overexpression relies on the constitutive activation of p38 and ERK1/2 MAPK. Constitutive activation of RAS-MEK-MAPK has been reported to lead to oncogene-induced senescence (OIS) in normal cells. According to this, our recent results of gene profiling and protein analysis demonstrate that EOMG-mTECs show the molecular phenotype of this type of senescence (OIS): constitutive RAS activation, increased NF- $\kappa$ B and NF-IL6 activity, increased transcription of B-RAF, MEK1/2 and cell-cycle inhibiting proteins (p57KIP2, p16INK4a, p15INK2B). Moreover, extended morphological and functional analysis of 350 clones obtained by limiting dilution of EOMG-mTECs and normal controls confirmed that terminally differentiated clones were significantly over-represented in EOMG-mTEC cultures and, remarkably, the those progressing from intermediate to terminal stage were dramatically outnumbered (79.5% $\pm$ 8.7 vs 22% $\pm$ 7.1 of control mTEC, p< 0.001). IL-6, CCL5 and IL-8 productions were tenfold increased in EOMG-mTEC senescent cells and apoptosis reduced to background, findings that are of pathogenic relevance in the light of our previous report showing that IL-6 and CCL5 support respectively survival and migration of EOMG peripheral lymphocytes.

Our comprehensive work demonstrates for the first time that cytokine/chemokine overproduction of EOMG-mTEC may depend on OIS and it implicates RAS activation in the misrled functions of MG-mTEC leading to peripheral lymphocytes immigration to MG thymus.

#### PC18/8 T CELL INTRINSIC SMAD7 DETERMINES TH1/TH17 DIFFERENTIATION AND SUSCEPTIBILITY TO AUTOIMMUNE ENCEPHALOMYELITIS

D. Lukas<sup>1</sup>, J. Song<sup>1</sup>, N. Yoge<sup>1</sup>, A. Croxford<sup>1</sup>, M. Hasan<sup>2</sup>, B. Neumann<sup>2</sup>, I. Kleiter<sup>3</sup>, A. Waisman<sup>1</sup>

<sup>1</sup>Med, Uniklinik Mainz, Mainz, Germany, <sup>2</sup>University Medical Center Regensburg, Regensburg, Germany, <sup>3</sup>University Medical Center Regensburg, Regensburg, Germany

Transforming growth factor (TGF)- $\beta$  is a cytokine of great importance for T cell differentiation, proliferation and survival. While blocking T helper (Th)1 and Th2 differentiation, TGF- $\beta$  is crucial for the generation of T regulatory (Treg) and Th17 cells. Signaling through the TGF- $\beta$ -R is in part regulated by Smad7, which in combination with ubiquitin ligases targets the TGF- $\beta$ -R for degradation. Thus, Smad7 is a potential regulator of T helper cell subset differentiation and autoimmunity via its adverse effects on TGF- $\beta$  signaling.

Since the function of Smad7 *in vivo* is not entirely understood, we generated a mouse model in which Smad7 could be conditionally deleted in T cells after Cre-mediated recombination.

The steady state analysis showed normal T cell development with decreased frequencies of CD69<sup>+</sup> T cells. During experimental autoimmune encephalomyelitis (EAE), a mouse model for multiple sclerosis, mice lacking Smad7 in T cells showed attenuated clinical disease and a subdued Th1 response. Depletion of Tregs in mice with Smad7-deficient T cells partially restored clinical development of autoimmune neuroinflammation.

We postulate that Smad7 controls Th1 and Th17 lineage commitment decisions, and that T cells lacking Smad7 are more sensitive to TGF- $\beta$  signalling. Through this mechanism, both pro- and anti-inflammatory cellular responses can be regulated through Smad7-dependant pathways.



**PC18/9 REGULATION OF AUTOIMMUNE NEUROINFLAMMATION BY NRF-DEPENDENT CYTOPROTECTIVE GENES EXPRESSED IN THE CENTRAL NERVOUS SYSTEM**A. Cunha<sup>1</sup>, R. Gozzelino<sup>1</sup>, I. Bechmann<sup>2</sup>, I. Marguti<sup>1</sup>, Á. Chora<sup>1</sup>, M.P. Soares<sup>1</sup><sup>1</sup>Instituto Gulbenkian de Ciência, Oeiras, Portugal, <sup>2</sup>Institute for Clinical Neuroanatomy, Johann Wolfgang Goethe University, Frankfurt/Main, Germany

Multiple sclerosis (MS) is a T cell-mediated autoimmune disease of the central nervous system (CNS). Oligodendrocyte cytotoxicity in the CNS is a central event for the pathogenesis of MS. The aim of this study is to test whether expression of cytoprotective genes in oligodendrocytes, regulated by the nuclear factor erythroid 2-related factor (Nrf) family of transcription factors, can modulate the pathogenesis of experimental autoimmune encephalomyelitis (EAE), an experimental model of MS. We have previously shown that Nrf-dependent genes, e.g. heme oxygenase-1 (HO-1/*Hmox1*), can inhibit EAE progression, as revealed by the more severe and often lethal form of EAE that develops in HO-1-deficient (*Hmox1*<sup>-/-</sup>) vs. control wild type (*Hmox1*<sup>+/+</sup>) mice (Chora *et al.*, 2007). This phenotype is also observed when HO-1 expression is deleted specifically in non-hematopoietic tissues (i.e. bone marrow chimeric mice), suggesting that expression of HO-1 in the CNS is protective against EAE. We have found that inhibition of Nrf transcriptional activity in oligodendrocytes overexpressing a dominant negative mutant form of Nrf2 (*Nrf2*<sup>DNM</sup>) renders these cells highly sensitive to TNF as well as Fas-ligand-mediated apoptosis *in vitro*. This suggests that expression of Nrf-dependent cytoprotective genes in oligodendrocytes prevents TNF and/or Fas-ligand-mediated apoptosis, a protective effect that should inhibit the pathogenesis of EAE (Hövelmeyer *et al.*, 2005). To test this hypothesis *in vivo* we have compared the progression and outcome of EAE in Nrf2-deficient (*Nrf2*<sup>-/-</sup>) vs. wild type (*Nrf2*<sup>+/+</sup>) mice. These experiments are under way and the results obtained will be described in detail. In addition, we have generated transgenic mice expressing the *Nrf2*<sup>DNM</sup> specifically in oligodendrocytes under the control of the myelin oligodendrocyte glycoprotein (MOG) or the proteolipid protein promoters, affording low and high *Nrf2*<sup>DNM</sup> expression, respectively. Characterization of these transgenic mice, including EAE progression, is under way and the results obtained will be described in detail.

**PC18/10 PI3K $\gamma$  DEFICIENCY DELAYS EAE ONSET AND AMELIORATES ITS CLINICAL OUTCOME**L. Berod<sup>1,2</sup>, S. Heink<sup>1</sup>, A. Escher<sup>3</sup>, R. Wetzker<sup>1</sup>, C. Stadelmann-Nessler<sup>3</sup>, J. Norgauer<sup>2</sup>, T. Kamradt<sup>1</sup><sup>1</sup>Friedrich Schiller University, Department of Immunology, Jena, Germany, <sup>2</sup>Friedrich Schiller University, Department of Dermatology, Jena, Germany, <sup>3</sup>Georg-August University, Institute for Neuropathology, Göttingen, Germany, <sup>4</sup>Friedrich Schiller University, Institute for Molecular Cell Biology, Jena, Germany

**Objectives:** Cell migration is a prerequisite for basic immune surveillance and adaptive immune responses; e.g. T cell migration to the CNS is necessary for the onset of EAE. Activation of the PI3K $\gamma$  pathway is a biochemical response to most chemokine receptors. To better understand the potential therapeutic utility that inhibition of this signalling pathway may have in controlling an inappropriate immune response, we evaluated the role of PI3K $\gamma$  in the pathogenesis of EAE.

**Results:** Following immunization with 200 $\mu$ g MOG<sub>35-55</sub> peptide and pertussis toxin on day 0 and 2, *wt* mice developed a severe, chronic encephalomyelitis with clinical signs of disease appearing around day 10 – 15 and peaking at day 18. In PI3K $\gamma$ <sup>-/-</sup> mice, the clinical symptoms started and peaked significantly later. Moreover, the overall duration of disease as well as its severity was reduced in PI3K $\gamma$ <sup>-/-</sup> mice. Histological examination of CNS inflammatory lesions during the acute phase showed defective or absent inflammatory infiltrates in PI3K $\gamma$ <sup>-/-</sup> mice compared to *wt* controls. On the contrary, when compared at the corresponding peaks of disease, PI3K $\gamma$ <sup>-/-</sup> mice showed similar infiltrates to *wt* mice. To gain mechanistic insights into the influence of PI3K $\gamma$  in EAE, lymph nodes and spleens were removed at different time points after immunization and analysed for proliferation and inflammatory cytokine production by FACS. During the induction phase, both proliferation and cytokine production were lower in PI3K $\gamma$ <sup>-/-</sup> mice than in the *wt* littermates. PI3K $\gamma$  deficiency delayed the appearance of CD4+CD154+ antigen specific T cells in the lymphoid organs of these mice as well as their arrival to the CNS, contributing to the late EAE onset. In addition, selective inhibition of PI3K $\gamma$  in normal *wt* mice was effective in reducing signs of the disease when mice were treated after clinical onset.

**Conclusion:** These results demonstrate that PI3K $\gamma$  is involved in the pathogenesis of EAE, probably by delaying T cell priming in the lymphoid organs and reducing migration of antigen specific cells to the CNS. Moreover, selective inhibition of this pathway reduced severity of EAE, suggesting that PI3K $\gamma$  might be a useful target in the treatment of MS.

**PC18/11 THE ENVELOPE OF HUMAN ENDOGENOUS RETROVIRUS IN NEURO-INFLAMMATION**H.-L. Dougier-Reyraud<sup>1</sup>, C. Lomparski<sup>1</sup>, C.L. Villiers<sup>1</sup>, A. Duperray<sup>1</sup>, C. Bernard<sup>2</sup>, E. Jouvin-Marache<sup>3</sup>, H. Perron<sup>3</sup>, P.N. Marche<sup>1</sup><sup>1</sup>INSERM 823, Université Joseph Fourier Grenoble, Grenoble, France, <sup>2</sup>Geneuro, Plan-les-Ouates, Switzerland, <sup>3</sup>Geneuro-Innovation, Lyon, France

Several viruses are known to interact with the host defences either to escape from the immune responses or to gain advantage of inflammatory mediators to survive. Human endogenous retroviruses (HERV) are integrated and are estimated represent up to 8% of the human genome. An exogenous virus from HERV.W family was initially isolated from brain cells of patients suffering of multiple sclerosis and named MSRV. Several lines of evidence support that its envelope protein (ENV) or its soluble extracellular subunit (ENV-SU) contributes to inflammation associated with the disease: 1) ENV promotes polyclonal expansion of T lymphocytes, 2) ENV-SU induces human monocytes and dendritic cells (DC) to produce inflammatory cytokines through engagement of CD14 and TLR4.

In order to study *in vivo* effects of MSRV, mice were treated for experimental allergic encephalitis (EAE) induction, a mouse model for MS, after antigenic myelin peptide immunisation either with complete Freund's adjuvant or ENV-SU. Clinical score showed significant EAE symptoms in both mice but no symptoms in control mice (no adjuvant or ENV). Administration of Anti-ENV-SU antibodies blocked EAE symptoms induced only in ENV-SU treated mice. Cultures of splenocytes from either ENV-SU or adjuvant treated mice, recalled with the myelin antigen, led to IFN- $\gamma$  production, suggesting T lymphocyte reactivity towards the myelin antigen. To characterise the mode of action of ENV in mice, DC were cultured from C57BL/6 bone marrow and incubated with ENV-SU. DC secreted amounts of IL-6 and IL-12p70 dose-dependant of the ENV-SU. Flow cytometry analysis for CMH-II, CD11c, B7.1 and B7.2 molecules DC demonstrated that DC were activated and underwent further differentiation upon ENV-SU exposure comparable to other known stimulations. DC derived from TLR4 deficient mice were unable to respond to ENV-SU stimulation, arguing that, as found in humans, TLR4 pathway is involved in DC responses in mice.

In conclusion, by promoting inflammatory response through CD14/TLR4 pathway, the envelope of MSRV/HERV contributes to EAE in mice and thus may be one of the key actors of MS etiology in humans.

**PC18/12 IN VITRO GENERATED REPETITIVELY ACTIVATED MOG-SPECIFIC TH17 CELLS ARE HIGHLY ENCEPHALITOGENIC AND KEEP THEIR PHENOTYPE IN VIVO**M. Paterka<sup>1</sup>, T. Leuenberger<sup>1</sup>, M. Smyth<sup>1</sup>, H. Waiczies<sup>1</sup>, J. Würfel<sup>1</sup>, C. Infante-Duarte<sup>1</sup>, F. Zipp<sup>1</sup>, V. Siffrin<sup>1</sup><sup>1</sup>Cecilie Vogt Clinic for Neurology, Charité – University Medicine Berlin and Max Delbrueck Center for Molecular Medicine Berlin-Buch, Berlin, Germany

**Objectives:** The induction of chronic autoimmune CNS inflammation has been shown to critically depend on IL-6 and IL-23 dependent mechanisms in experimental autoimmune encephalomyelitis (EAE). *In vitro* T cell differentiation experiments have shown that both of these cytokines are involved in the generation of Th17 cells. However, classical approaches to induce adoptive transfer EAE with myelin oligodendrocyte glycoprotein (MOG)-specific CD4 Th17 cells have yielded low encephalitogenicity. Here, we investigated requirements for the induction of stable encephalitogenic MOG-specific CD4 Th17 cells.

**Methods:** We evaluated the potential of *in vitro*-generated MOG-specific CD4 Th17 cells derived from T cell receptor transgenic mice (2d2 mice) to induce EAE after adoptive transfer in C57BL/6 wild type (*wt*) and *Rag1*<sup>-/-</sup> mice. We established restimulation protocols and correlated pre-transfer flow cytometric profiles of Th17 cells with *ex vivo* isolated (post-transfer) phenotype of the respective cell subsets to define the optimal *in vitro* stimulation conditions to generate a stable phenotype and high disease incidence.

**Results:** Chronic stimulation protocols of initially naïve MOG-specific CD4 T cells are essential to induce a stable encephalitogenic Th17 population. Adoptive transfer of repetitively stimulated IL-17-secreting CD4 T cells into *wt* recipients led to severe, non-remitting clinical EAE in about 50% of transferred animals. In *Rag1*<sup>-/-</sup> C57BL/6 mice adoptive transfer of CD4 Th17 cells led to fulminant and severely progressive EAE, which resulted in death in almost all animals. Titration of Ag-specific Th17 cells showed that even low cell numbers can induce severe disease in the *Rag1*<sup>-/-</sup> mice but fail to do so in the *wt* mice. Only repetitive *in vitro* stimulation ensured disease induction, which correlated with enrichment of transferred 2d2 Th17 cells still secreting high amounts of IL-17 in the CNS of affected animals. Utilizing magnet resonance imaging blood-brain barrier breakdown was detected by contrast-agent enhancement throughout the myelinated areas of the brain of affected animals and could be correlated to lymphocyte recruitment to the CNS using immunohistochemistry.

**Conclusion:** Taken together, chronic activation of *in vitro*-generated MOG-specific Th17 cells is necessary to yield a highly encephalitogenic Th17 subset, which is a potent inducer of EAE in an adoptive transfer model.

**PC18/13 DYSREGULATED CONTROL OF THE EXPRESSION OF CD46 CYTOPLASMIC ISOFORMS IN T CELLS FROM PATIENTS WITH MULTIPLE SCLEROSIS**S. Ni Choileain<sup>2</sup>, N.J. Weyand<sup>2</sup>, M. So<sup>2</sup>, B. Weller<sup>3</sup>, D. Photiou<sup>1</sup>, A.L. Astier<sup>1,4</sup><sup>1</sup>University of Edinburgh, Centre for Inflammation Research/Centre for MS Research, Edinburgh, United Kingdom, <sup>2</sup>University of Arizona, BIO5 Institute, Tucson, United States, <sup>3</sup>Western General Hospital, Division of Clinical Neuroscience, Edinburgh, United Kingdom, <sup>4</sup>University of Edinburgh, Institute of Immunology and Infection Research, Edinburgh, United Kingdom

CD46 induces Tr1 cells secreting large amounts of IL-10. This pathway is altered in patients with multiple sclerosis, as IL-10 production is impaired. The two cytoplasmic isoforms of CD46 produced by alternative splicing, Cyt1 and Cyt2, are co-expressed in human cells. They exhibit antagonistic roles on inflammation when expressed in transgenic mice. Our previous study suggested increased CD46-Cyt2 mRNA levels in CD46-activated T cells from several MS patients compared to healthy controls. CD46-Cyt1 mRNA levels were unchanged. Hence, the altered ratio of these two isoforms might regulate inflammation in MS.

Here, we demonstrate the regulation of CD46 expression upon T cell activation. CD3/CD46 costimulation led to the disappearance of cell surface CD46. In contrast, both CD3 activation and CD28-costimulation increased surface CD46 expression. Stimulation of CD46 alone led to loss of surface CD46, however, this loss

was faster with TCR co-engagement. The disappearance of CD46 is unlikely to be due to its endocytosis, as no CD46 was detected by intracellular staining with anti-ectodomain antibodies. Furthermore, flow cytometry analyses demonstrate that CD46-costimulation led to a decrease in Cyt1 protein expression, followed later by a decrease in Cyt2 expression. In contrast, a significant increase in Cyt1 expression was observed upon CD28 co-activation, implying a crosstalk between CD28 and CD46.

We then compared CD46 expression in T cells from healthy donors and MS patients. Loss of surface CD46 was observed in CD46-activated T cells from both groups. However, a differential pattern was observed for the cytoplasmic tails. Firstly, we observed an increase in Cyt2 protein levels in both CD46- and CD28-coactivated T cells from patients. Secondly, no decrease in Cyt1 or Cyt2 protein levels was observed in MS. CD46 can be cleaved by matrix metalloproteases, and Cyt1 and Cyt2 are further cleaved by GMSA-Secretase (Weyand *et al.*, submitted). Our data in human T cells are consistent with this model, and suggest that the defects in CD46 pathways observed in MS are at least partly due to a defect in CD46 processing. Further identification of mechanisms involved in these pathways might provide new means to control T cell activation with potential clinical outcomes.

#### PC18/14 M2 ACTIVATED MONOCYTES CURE ACUTE RELAPSING EAE. APPLICATION PERSPECTIVES FOR MULTIPLE SCLEROSIS

C. Boiziau<sup>1</sup>, J. Mikita<sup>1</sup>, N. Dubourdieu-Cassagno<sup>1</sup>, M. S. A. Deloire<sup>1</sup>, B. Brochet<sup>1</sup>, J.-M. Franconi<sup>2</sup>, K. G. Petry<sup>1</sup>

<sup>1</sup>Bordeaux 2 University, EA2966 Neurobiology of Myelin Disorders, Bordeaux Cedex, France, <sup>2</sup>Bordeaux 2 University, CNRS UMR 5536, RMSB, Bordeaux Cedex, France

Proinflammatory « M1 » monocytes participate in general host defence by the production of pro-inflammatory mediators. In contrast, immunomodulatory « M2 » monocytes have homeostatic functions to help recovery after an inflammation event. Multiple Sclerosis (MS) is an autoimmune disease of central nervous system (CNS), with major leucocytes' infiltrations. Involvement of M1 cells was long described during acute relapses, both in MS and rodent models of Experimental Autoimmune Encephalomyelitis (EAE). The role of M2 cells remains unclear.

We investigated in relapsing EAE in Dark Agouti rats whether the alteration of the balance between M1 and M2 monocytes plays a role in the relapsing disease activity. Therefore, we compared monocyte profiles in blood samples and CNS infiltrates at different time points of severe relapsing and mild monophasic EAE disease progress. At disease onset, relapsing disease severity was predicted by strong macrophage CNS infiltration using *in vivo* MRI-USPIO marker. This predictive discrimination allowed comparative histological analysis of M1 and M2 infiltrates in severe vs mild EAE, in particular at onset and first clinical attack of EAE, although the relapsing disease course was not yet completed.

**Results:** During the second phase of disease progression characterised by a relapse in severe EAE and by spontaneous recovery in mild EAE, expression patterns of M1 and M2 monocytes/macrophages reveals that M1/M2 equilibrium in blood and CNS favours mild EAE, while imbalance towards M1 promotes relapse. Consequently, in MRI-USPIO predicted severe EAE rats, *ex vivo* activated M2 monocytes were administered to induce recovery from acute clinical attack: these cells suppress ongoing severe EAE while administration of non-activated monocytes has no beneficial effect.

To evaluate whether the expression of M1 and M2 profiles of circulating monocytes is modulated in RR-MS patients in remission we phenotyped blood samples from 52 MS patients and 10 healthy controls by real-time RT-PCR: immunomodulatory M2 markers are strongly suppressed in circulating monocytes of patients.

**Conclusion:** Imbalance of monocyte activation profiles and impaired M2 expression are key factors in development of relapses and progressive MS. The maintained suppression even in RRMS patients in remission might indicate that the relapsing disease progresses.

#### PC18/15 EFFECT OF NOGO-NOGO RECEPTOR INTERACTIONS IN EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

K. Steinbach<sup>1</sup>, R. Martin<sup>1</sup>

<sup>1</sup>Institute for Neuroimmunology and Clinical MS-Research (inims), ZMNH, Hamburg, Germany

The myelin-proteins Nogo-A and MAG are considered to mediate inhibition of nerve regeneration in the adult central nervous system (CNS) via Nogo receptor (NgR)-Signaling. Blocking strategies targeting this pathway have been successfully applied in several nerve injury models, suggesting Nogo-A and functionally related proteins like LINGO-1 as therapeutic targets to enhance regeneration in Multiple Sclerosis (MS).

Especially anti-Nogo-A strategies have ameliorated the clinical outcome of experimental autoimmune encephalomyelitis (EAE), the established animal model of MS. However, if NgR-Signalling is really mediating Nogo-A related inhibition of neuroregeneration is currently under debate. Therefore, we aimed to elucidate a potential function of NgR-Signalling in autoimmune CNS inflammation and in regeneration of the damaged CNS. We induced EAE in NgR1-/-, NgR2-/- and MAG-/- deficient mice, but could not demonstrate an effect of NgR1- or MAG-deficiency on the clinical outcome of EAE.

Interestingly, an additional role for NgR-Signalling in leukocyte trafficking into injured nervous tissue has recently been described. Therefore, we established bone marrow chimeras of the above mentioned mouse strains to dissect potential immunological functions of NgRs from their role in neuroregeneration. In summary, our data argues against an involvement of NgR-Signalling in the development of progressive autoimmune CNS inflammation like in MS.

#### PC18/16 MONOCLONAL ANTIBODY PRODUCTION BY IMMORTALIZATION OF B CELLS FROM THE THYMUS OF MYASTHENIA GRAVIS PATIENTS

K. Vrolix<sup>1</sup>, J. Fraussen<sup>2</sup>, J. Van Den Broeck<sup>1</sup>, E. Meulemans<sup>3</sup>, M. Phernambucq<sup>1</sup>, V. Somers<sup>2</sup>, M. Losen<sup>1</sup>, M. De Baets<sup>1</sup>, P. Martinez-Martinez<sup>1</sup>

<sup>1</sup>Maastricht University, Department of Neuroscience, Maastricht, Netherlands, <sup>2</sup>Hasselt University, Biomedical Research Institute, Diepenbeek, Belgium, <sup>3</sup>Academic Hospital Maastricht, Maastricht, Netherlands

**Introduction:** More than 80% of patients with myasthenia gravis (MG) have auto-antibodies against the acetylcholine receptor (AChR) at the neuromuscular junction. MG is frequently associated with thymic abnormalities and the myasthenia often improves after thymectomy. Immortalization of thymic B cells by Epstein Barr Virus (EBV) transformation provides a tool to characterize the auto-antibodies in MG patients.

**Methods:** Memory B lymphocytes were isolated from the thymus of 3 MG patients and immortalized by EBV transformation. To overcome the low efficiency of EBV-based immortalization, the polyclonal B cell activator CpG 2006 was added to the B cells. IgG-positive B cell clones were cultured for the production and characterization of monoclonal auto-antibodies.

**Results:** Screening of the monoclonal IgG-positive B cell clones by immunohistochemistry on muscle tissue showed immunoreactivity to striated muscle proteins in about 25%. However, shown by ELISA, this immunoreactivity was not directed against muscle proteins myosin, actin, alpha-actinin, nor titin which have been previously described to be produced by MG thymus. 3 out of 250 B cell clones produced anti-AChR antibodies, detected by radioimmunoassay. To further study the specificity of the antibodies we performed protein array techniques using extracts of different human tissues. Finally, the sequences of the IgG heavy chains were analyzed to characterize the auto-antibody specificity. Interestingly strong sequence homology was found between an antibody tested positive by radioimmunoassay against the AChR and an anti-rabies virus antibody (SC4022).

**Conclusion:** The increased immortalization efficiency of EBV transformation by the addition of a polyclonal B cell activator allowed us to immortalize memory B cells from MG patients and to produce monoclonal antibodies. With this improved method further insights into the pathology of this autoimmune disease might be obtained by identifying autoantibodies in both seropositive and double seronegative MG patients.

#### PC18/17 ASSOCIATION BETWEEN THE NOD2-ISOFORM VAL955ILE AND MYELIN BASIC PROTEIN-INDUCED T CELL RESPONSES IN PATIENTS WITH MULTIPLE SCLEROSIS

C. J. Hedegaard<sup>1</sup>, C. Enevold<sup>1</sup>, K. Bendtzen<sup>1</sup>, C. H. Nielsen<sup>1</sup>

<sup>1</sup>Copenhagen University Hospital, Rigshospitalet, 7541, Institute for Inflammation Research, Copenhagen O, Denmark

**Objective:** Following stimulation of the nucleotide oligomerization domain 2 (NOD2) receptor with muramyl dipeptide (MDP), monocytes secrete interleukin (IL)-23 and IL-1, which, in turn, promote IL-17 secretion in memory T cells. IL-17 plays a deleterious role in multiple sclerosis (MS). Mutations in the NOD2 gene (CARD15) confer susceptibility to certain chronic inflammatory disorders. Three polymorphisms in CARD15, resulting in Pro268Ser, Arg703Cys and Val955Ile substitutions, are all more frequent than 1% in Caucasians, but it has not been investigated whether they are associated with MS.

**Methods:** Genotyping of the three polymorphisms was carried out on bead-based SNP-analysis using the Luminex platform on 24 patients with MS. CFSE-labelled mononuclear cell (MNC) cultures were stimulated with myelin basic protein (MBP). The consequent production of IL-17 was measured by Luminex technology, and CD4+ T cell proliferation was measured flow cytometrically as dilution of the cellular CFSE content.

**Results:** None of the patients were homozygous for the Ile955 isoform, while five were heterozygous. MBP induced CD4+ T cell proliferation MNC cultures from all of the heterozygous patients versus only four of the 19 patients with wild type ( $P < 0.003$ ). Moreover, the 955Ile allele was associated with MBP-induced production of IL-17 ( $P < 0.03$ ), which was displayed by eight of the nine patients with CD4+ T cell proliferation.

**Conclusion:** We hypothesize that the Ile955 isoform of NOD2 plays a role in determining MBP-elicited CD4+ T cell proliferation and IL-17 production in MS patients.

#### PC18/18 B CELL HOMEOSTATIC PERTURBATION DURING ACUTE AND REMISSION PHASES OF EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

C. Lee Chang<sup>1</sup>, D. Lefranc<sup>1</sup>, C. Faveeuw<sup>2</sup>, P. Vermersch<sup>1,3</sup>, L. Prin<sup>1</sup>, B. Oxombre-Vanteghem<sup>1</sup>

<sup>1</sup>Centre Hospitalier Régional et Universitaire de Lille, Groupe Autoimmunité – Laboratoire d'Immunologie EA2686, Lille, France, <sup>2</sup>Institut Pasteur de Lille, Inserm U547, Lille, France, <sup>3</sup>Centre Hospitalier Régional et Universitaire de Lille, Pôle de Neurologie – Service de Neurologie D, Lille, France

B cells appear to play an important effector role in the pathogenesis of multiple sclerosis (MS) and its murine experimental autoimmune encephalomyelitis (EAE). They are also involved in regulatory mechanisms through different ways such as cytokines secretion, antibodies production and the ability to function as antigen

presenting cell (APC) to suppress encephalitogenic T cells responses. The approach of the present study was to analyze in cervical lymph nodes (CLN) and spleen the distribution of regulatory B and T cells subsets over EAE clinical manifestations. Special interest was focused on B1a, transitional 2 (T2) and marginal zone (MZ) B cell subsets, strong candidates to operate as regulatory B cells (Bregs), as well as Foxp3+ regulatory T cell (Foxp3+ Tregs). To better understand the involvement of regulatory lymphocytes in the autoimmune process underlying EAE we used two mice strain, the sensible to EAE induction SJL/J mice and resistant B10.S mice. In fact, immunized SJL/J Foxp3+ Tregs, T2 and especially MZ B cell subset suffered dramatic homeostatic perturbations over EAE clinical states. In the other hand, B1a B cells proportions were maintained over EAE disease, except at late remission moment where proportions were greater. Same approach carried out in MS patient will be discussed. All together, the loss of regulatory T and B cell homeostasis are relevant in EAE and MS pathogenesis.

#### PC18/19 T CELL RECEPTORS OF CLONES FROM PROBABLE MULTIPLE SCLEROSIS PATIENTS AUTOACTIVE T-CELLS DERIVE THEIR VARIABLE BETA CHAIN ALLELES FROM A DISEASE PRONE HOTSPOT IN THE BV GENE LOCUS

M. Mandel<sup>1</sup>, G. Lavie<sup>1</sup>, T. Tuller<sup>2</sup>, T. Barliya<sup>1</sup>, A. Achiron<sup>3</sup>

<sup>1</sup>Sheba Medical Center, Blood Center, Ramat-Gan, Israel, <sup>2</sup>Tel Aviv University, Computer Sciences, Tel Aviv, Israel, <sup>3</sup>Sheba Medical Center, Multiple Sclerosis Center, Ramat-Gan, Israel

**Objectives:** Characterize the clonal composition of myelin reactive, disease-related autoreactive T cell lines (TCLs) derived from first attack probable multiple sclerosis (pMS) patients. Compare phenotypic expression patterns of memory cell determinants and α1b1 (VLA-1) integrin in patient derived lines to myelin antigen-reactive healthy donor (HD) TCLs.

**Methods:** TCLs were propagated from PBMC of pMS patients and HD in response to immunodominant myelin epitopes as part of a T-cell vaccination trial. Co-expression of CD45RO memory and CD49a was examined by FACS. Spectratyping (Immunoscope) and Bio-Rad iScript™ Select cDNA Synthesis Kit were used for V-beta typing in the TCR V-beta gene usage analyses. The ABI PRISM 7700 sequence detection application program (PE, Applied Biosystems) was used to measure PCR-amplified product fluorescence. Hamming distances, defined as numbers of V-beta alleles in two lines that are different, were calculated to measure dissimilarities between lines. Pearson correlation was used to measure similarities between clone combinations comprising lines from different patients/controls (as groups). Non-parametric Kolmogorov-Smirnov statistical analyses and 2-sample *t* tests were performed.

**Results:** Generated TCLs homogeneously co-express CD45RO memory cell and CD49a (VLA-1 adhesion molecule). These cells resemble T cell subsets proposed to transigrate into tissues in rheumatoid arthritis and induce systemic and local inflammation.

The combinations of T-cell oligoclones comprising pMS, disease-related TCLs use unique sets of TcR beta-chain variable allele (BV-genes) combinations, forming "disease-specific cluster patterns". TCLs derived from different patients and stimulated to proliferate with different myelin epitopes display striking similarities in BV-gene allele clusters, 60% of which are derived primarily from a disease-prone hotspot in the BV gene locus, residing between Vb6 – Vb9. Conversely, healthy subject control TCLs use different BV gene allele sets, forming "healthy responder usage formats" absent from the probable MS patient V-beta gene allele combinations.

**Conclusions:** Hierarchical V-beta gene combination clustering, point to existence of three pMS patient groups, or up to three disease-related immune response patterns. These subgroup patterns may reflect different disease subclasses or suggest immune reactivity to different etiologic agents. Clonal-cluster pattern analyses may aid in characterizing disease-related autoreactive T cell populations, sub-classification of MS and in characterization of etiologic agents of MS.

#### PC18/20 THE KINETICS OF INTERFERON-GAMMA PRODUCING NATURAL KILLER CELLS IN EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

M. Momčilović<sup>1</sup>, Ž. Miljković<sup>2</sup>, D. Miljković<sup>1</sup>, M. Mostarica-Stojković<sup>2</sup>

<sup>1</sup>Institute for Biological Research 'Siniša Stanković', University of Belgrade, Department of Immunology, Belgrade, Serbia, <sup>2</sup>Institute of Microbiology and Immunology, School of Medicine, University of Belgrade, Belgrade, Serbia

**Objectives:** Experimental autoimmune encephalomyelitis (EAE) is a T cell-mediated autoimmune inflammatory demyelinating disease inducible in susceptible strains of experimental animals. Natural killer (NK) cells, being part of the innate immune system, have been implicated in regulating adaptive immune responses, in particular organ-specific autoimmune diseases. Increasing evidence suggests both regulatory and effector role of NK cells in autoimmune demyelinating diseases. Interferon (IFN)-gamma is a cytokine produced by T lymphocytes and NK cells, considered to play dual (proinflammatory and regulatory) role in the pathogenesis of neuroinflammatory diseases. In order to elucidate the role of NK cells in EAE the kinetics of NK cells and IFN-gamma producing NK cells in the CNS during actively induced EAE in Dark Agouti (DA) rats was examined.

**Methods:** For the induction of EAE in DA rats spinal cord homogenate and complete Freund's adjuvant were used. Spinal cords were isolated at various time points after the immunization and spinal cord infiltrating cells were subsequently separated on the Percoll gradient. Flow cytometry was used for phenotypization of spinal cord infiltrating cells and quantitation the number of NK cells and IFN-gamma producing cells in spinal cord infiltrating cells at various time points after the immunization.

**Results:** Considerable number of NK cells were found in the spinal cord of DA rats at the early stage of EAE and the number of NK cells increased as the disease progressed. Interestingly, the number of NK cells peaked in the recovery phase of the disease. Also, the number of NK cells among IFN-gamma producing cells gradually increased in the course of the disease and reached maximal values in the recovery phase.

**Conclusion:** In this study, we demonstrated that NK cells and especially IFN-gamma producing NK cells increased in the CNS during EAE suggesting that these cells are involved in the recovery from the disease and might play a suppressive (regulatory) role in acute EAE. The results of this study also implicate that IFN-gamma production might be potential regulatory mechanism of NK cells. Thus, the therapeutic modulation of NK cell number and function may be an effective and immunospecific therapy for autoimmune demyelinating diseases.

#### PC18/21 EPITOPE SPREADING OF RESPONSE TO PLP178-191 DURING EAE ACTIVATES A TCR REPERTOIRE THAT OVERLAPS WITH THAT INDUCED BY ACTIVE IMMUNIZATION

G. Di Sante<sup>1</sup>, C. Nicolò<sup>1</sup>, R. Penitente<sup>1</sup>, A. Giglio<sup>1</sup>, F. Ria<sup>1</sup>

<sup>1</sup>Università cattolica del Sacro Cuore di Roma, Patologia Generale, Rome, Italy

Spontaneous spreading of T cell response to subdominant epitopes during EAE is deemed to be relevant in the determination of the relapsing-remitting course of the disease and, by extension, of Multiple Sclerosis. We compared TCR usage during responses to PLP178-191 (p178) induced by active immunization or after spontaneous spreading in SJL mice undergoing to EAE induced by PLP139-151. Studies were performed by CDR3 BV-BJ spectratyping (Immunoscope). Results showed that both responses are characterized by a largely individual usage of TCRs. We identified seven semiprivate rearrangements, only two of which appear specifically associated with active immunization with p178. Thus, immunization with p178 and spontaneous spreading to this epitope activate T cell repertoires that overlap to a large extent. These observations can be sustained either by a severe limitation of the p178-specific TCR repertoire due to control tolerance, or/and by the fact that presentation of PLP from the inflamed CNS involves cellular machineries similar to those involved in presentation of antigens from other peripheral organs. Intriguingly one of the shared rearrangements specific for p178-191 was the result of recombination of BV10 with BJ1.1, yielding a product indistinguishable in the chromatograph from that previously described for the CD4+ IFNγ+ BV10-BJ1.1+ encephalitogenic cells specific for PLP139-151 (Nicolò et al, Int Immunol, 2006). However, the two cells displayed clear distinct antigen specificity. Sequencing of these TCR-beta chains (that is currently being performed) will provide information on the relative role of beta- and alpha- chains of the TCR in the recognition of CNS antigens. These observations offer new tools to examine the relative contribution of spreading of response to subdominant epitopes in the pathogenesis of relapses of EAE.

#### PC18/22 INTERLEUKIN-15 IN MULTIPLE SCLEROSIS: PERIPHERAL SOURCES, CENTRAL SOURCES AND IMPACT ON CD8 T CELL CYTOTOXICITY

R. Schneider<sup>1</sup>, P. Saikali<sup>2</sup>, C. Pittet<sup>1</sup>, P. Duquette<sup>1</sup>, N. Arbour<sup>1</sup>

<sup>1</sup>Université de Montréal, Médecine-CHUM, Montreal, Canada, <sup>2</sup>McGill University, Montreal, Canada

Increasing evidences suggest that CD8 T cells partake in multiple sclerosis (MS) pathogenesis, but whether cytokines contribute to activate these cells is not elucidated. Interleukin-15 (IL-15) is pivotal in the generation and maintenance of memory CD8 T cells. Antigen presenting cells are the main sources expressing a biologically active IL-15/IL-15Rα complex on their surface. Our goal is to determine whether IL-15 contributes to enhancing CD8 T cell responses in MS patients. We compared ex-vivo levels of IL-15/IL-15Rα on human peripheral blood monocytes in healthy controls and MS patients using flow cytometry. Most monocytes were IL-15Rα positive whereas a subset expressed surface IL-15 (10-62%). More monocytes expressed the IL-15/IL-15Rα complex in relapsing-remitting MS patients than in chronic progressive MS patients or healthy controls. We assessed whether IL-15 has more impact on CD8 T cell effector functions in MS patients compared to healthy controls. Purified CD8 T cells were shortly stimulated in vitro with anti-CD3 in the presence or absence of IL-15 and then analyzed using flow cytometry-based assays for proliferation (CFSE) and effector functions (lytic enzyme i.e. Granzyme B). Addition of IL-15 led to a significant dose-dependent increase of proliferation, and boosted IFN-γ and Granzyme B production by CD8 T cells. Moreover, the impact of IL-15 on Granzyme B production by CD8 T cells was greater in MS patients compared to healthy controls. Our results confirmed the reported increased amounts of IL-15 in MS patients' blood, but also underline that enhanced IL-15 is provided as biologically active surface IL-15/IL-15Rα complex on monocytes. We showed that CD8 T cells from MS patients displayed augmented cytotoxic capacity (Granzyme B) compared to healthy controls in response to IL-15, thus underscoring the pro-inflammatory impact of IL-15 on CD8 T cells in the context of MS. The role of IL-15 in different disease stages is currently under investigation.

Financial support: Multiple Sclerosis Society of Canada (MSSC). Fellowship to RS from German Academic Exchange Service (DAAD), to PS from Canadian Institutes of Health Research (CIHR), to CP from Neuroinflammation Training Program (CIHR). NA holds Awards from MSSC and FRSQ.



**PC18/23 THE APPEARANCE OF OLIGOCLONAL IGM BANDS IN CSF ASSOCIATES WITH A HIGH CD5+ B CELL PERCENTAGE IN PERIPHERAL BLOOD AND RAISED CXCL13 LEVELS IN CSF**

M. Espiño<sup>1</sup>, E. Roldán<sup>1</sup>, M. C. Sadaba<sup>2</sup>, P. González-Porqué<sup>2</sup>, T. Gasalla<sup>2</sup>, J. C. Álvarez-Cermeño<sup>2</sup>, L. M. Villar<sup>1</sup>

<sup>1</sup>Hospital Ramón y Cajal, Immunology, Madrid, Spain, <sup>2</sup>Hospital Ramón y Cajal, Madrid, Spain

**Background and objectives:** Local synthesis of anti myelin lipid IgM bands (M bands) within the central nervous system conditions an aggressive multiple sclerosis (MS) course. These antibodies are produced by CD5+ B cells present in the central nervous system (CNS). Our purpose was to study if the presence of these cells in CSF of MS patients is related to the presence of higher percentages in peripheral blood or to an increased traffic of these cells into the CNS.

**Patients:** We studied 146 patients with relapsing remitting MS (44 with M bands (M+) and 102 without them (M-)), 19 with other inflammatory neurological diseases (OIND) and 32 patients with non inflammatory neurological diseases (OND).

**Methods:** M bands were analyzed by isoelectric focusing and immunoblotting. Chemokines were quantified by ELISA. Cerebrospinal fluid (CSF) and peripheral blood (PB) cells were labeled with conjugated mAbs and analyzed on a standard FACSCanto instrument (BD). Results were analyzed with the Kruskal-Wallis test with Dunn's post-hoc test for comparisons between groups.

**Results:** The percentages of CD5+ B cell subsets in CSF were  $1.96 \pm 0.36$  for M+ MS patients, and  $0.77 \pm 0.09$ ,  $0.31 \pm 0.12$  and  $0.46 \pm 0.13$  for M- MS, OIND and OND patients, respectively ( $p < 0.0001$ ). Differences were due to M+ group. In PB, CD5+ B cell percentage was  $5.22 \pm 0.46$  in M+ MS patients,  $3.77 \pm 0.24$  in M- ones,  $2.95 \pm 0.54$  in OIND and  $2.99 \pm 0.43$  in OND. ( $p = 0.002$ ). Differences were also due to M+ group.

We next studied CXCL12 and CXCL13 levels in CSF and serum. No differences were found in serum. CSF CXCL12 values were higher in MS but we did not found significant variations between M+ and M- groups. CSF CXCL13 values were  $28.59 \pm 7.87$  pg/ml M+ MS patients,  $13.37 \pm 2.94$  in M- ones,  $1.05 \pm 1.05$  in OIND and  $1.59 \pm 1.52$  in NIND ( $p = 0.0008$ ). Differences were due to M+ MS samples that showed higher values than OIND ( $p < 0.05$ ) and OND ( $p < 0.01$ ).

**Concluding remarks:** The production of M bands in CSF of MS patients is closely related to a high percentage of peripheral blood CD5+ B cells and to raised levels of CXCL13 in CSF.

**PC18/24 MESENCHYMAL STEM CELLS AS POTENTIAL THERAPEUTIC VEHICLES FOR MULTIPLE SCLEROSIS – THE IMMUNOMODULATORY ROLE OF INDOLEAMINE 2,3-DIOXYGENASE-1 (IDO1)**

C. Opitz<sup>1,2</sup>, T. V. Lanz<sup>3,4</sup>, U. Litztenburger<sup>2</sup>, P. P. Ho<sup>4</sup>, L. Steinman<sup>4</sup>, W. Wick<sup>1,2</sup>, M. Platten<sup>1,2</sup>

<sup>1</sup>University Hospital Heidelberg, Heidelberg, Germany, <sup>2</sup>DKFZ, Heidelberg, Germany, <sup>3</sup>University Hospital Tübingen, Tübingen, Germany, <sup>4</sup>Stanford University School of Medicine, Stanford, United States

Mesenchymal stem cells (MSC) display unique suppressive properties on T-cell immunity, thus representing an attractive vehicle for the treatment of conditions associated with harmful T-cell responses such as graft-versus-host disease. Here we show that toll-like receptors (TLR) expressed on human bone marrow-derived MSC enhanced the immunosuppressive phenotype of MSC. Immunosuppression mediated by TLR was dependent on the production of immunosuppressive kynurenes (kyn) by the tryptophan (trp) degrading enzyme indoleamine-2,3-dioxygenase-1 (IDO1). Induction of IDO1 by TLR involved an autocrine interferon- $\beta$  (IFN- $\beta$ ) signalling loop, which was dependent on protein kinase R (PKR), but independent of IFN- $\gamma$ . In contrast, IDO1 is inducible in bone marrow-derived MSC (mMSC) by proinflammatory stimuli such as interferon- $\gamma$  (IFN- $\gamma$ ) and TLR ligands, but it does not lead to catabolism of trp *in vitro*, despite functional TLR expression. While mMSC are capable of suppressing the activation of antigen-specific, myelin-oligodendrocyte glycoprotein (MOG)-reactive T cell receptor (TCR) transgenic T helper (TH) cells in coculture experiments, neither pharmacological inhibition nor genetic ablation of IDO1 reversed this suppressive effect. Systemic administration of both, IDO1-proficient and phenotypically identical IDO1-deficient mMSC equally resulted in amelioration of experimental autoimmune encephalomyelitis (EAE). Due to their immunosuppressive properties MSC represent a promising tool for cell-based therapies of autoimmune diseases such as multiple sclerosis (MS). But there are important mechanistic differences between human and mouse bone marrow-derived MSC with respect to IDO1-mediated suppression of antigen-specific T cell responses in autoimmune neuroinflammation. mMSC as therapeutic vehicles in mouse models of autoimmune diseases such as EAE should be used with caution as preclinical models for human disorders as they lack an important immunosuppressive phenotype of hMSC.

**PC18/25 ANTIBODIES TO AQUAPORIN-4 IN NEUROMYELITIS OPTICA: BIOLOGICAL RELEVANCE AND USE AS BIOMARKERS**

S. Mader<sup>1</sup>, C. Rainer<sup>1</sup>, B. Kuenz<sup>1</sup>, T. Berger<sup>1</sup>, W. Kristoferitsch<sup>2</sup>, M. Reindl<sup>1</sup>, ARGE NMO Austria

<sup>1</sup>Innsbruck Medical University, Dept. of Neurology, Innsbruck, Austria, <sup>2</sup>SMZO-Donauspital, Dept. of Neurology, Vienna, Austria

**Objectives:** Neuromyelitis optica (NMO) is a devastating neurological inflammatory disease, which is clinically characterized by optic neuritis and longitudinally extensive transverse myelitis (LETM). Recently Vanda Lennon and colleagues discovered autoantibodies in the serum of NMO patients which target aquaporin 4 (AQP4), a membrane spanning water channel protein mainly localized in the brain and spinal cord. Being highly expressed in the polarized plasma membrane of astrocytic endfeet, AQP4 is a key constituent of the blood brain and blood CSF barrier. Recently various assays with differing sensitivity and specificity have been developed for detecting NMO-IgG antibodies in patient's sera.

**Methods:** We established an anti-AQP4 antibody live cell staining immunofluorescence (IF) assay. Human embryonic kidney HEK cells were transiently transfected with AQP4, fused at its N terminus with a green fluorescence protein (EmGFP). Subsequently, preabsorbed serum samples were added to the AQP4-EmGFP expressing HEK cells, detecting anti-AQP4 IgG with a secondary in red fluorescence labeled antibody. Patients with clinically definite NMO as well as patients with estimated diagnosis for Devic disease were recruited from several neurological centers in Austria. Together with numerous samples of MS patients, healthy controls and other neurological diseases, they were screened for the occurrence of NMO-IgG antibodies. Further, we have also screened seropositive samples with different AQP4-EmGFP fusion proteins containing different isoforms and intra- and extracellular domains of AQP4 to define the antibody binding sites.

**Results:** Our results show the presence of anti-AQP4 specific IgG in the sera of NMO patients and patients with longitudinally extensive transverse myelitis. Furthermore, NMO-IgG antibodies were detected in patients with systemic lupus erythematosus and Sjögren Syndrome, which are reported to be NMO accompanying autoimmune diseases. All control samples were proven to be AQP4-IgG negative, confirming the sensitivity and specificity of our developed AQP4 IF assay. Further, our results indicate that autoantibodies to AQP4 mainly target conformational epitopes of the protein. The biological relevance of these antibodies is currently tested *in-vitro* and *in vivo*.

**Conclusions:** To summarize, serum autoantibodies to AQP4 are a highly specific biological marker and pathogenic factor in NMO.

**PC18/26 ANTIBODY-PRODUCING MONOCLONAL B CELL LINES FROM MULTIPLE SCLEROSIS PATIENTS OBTAINED BY B CELL IMMORTALIZATION**

J. Fraussen<sup>1</sup>, K. Vrolix<sup>2</sup>, P. Martinez-Martinez<sup>2</sup>, R. Hupperts<sup>3</sup>, A. Van Diepen<sup>4</sup>, R. Medaer<sup>1</sup>, B. Vanwijmeersch<sup>1</sup>, E. Meulemans<sup>5</sup>, M. De Baets<sup>1,2,3</sup>, P. Stinissen<sup>1</sup>, V. Somers<sup>1</sup>

<sup>1</sup>Hasselt University, Biomedical Research Institute, Diepenbeek, Belgium, <sup>2</sup>Maastricht University, Department of Psychiatry & Neuropsychology, Maastricht, Netherlands, <sup>3</sup>Academic Hospital Maastricht, Department of Neurology, Maastricht, Netherlands, <sup>4</sup>Atrium Heerlen, Department of Neurology, Heerlen, Netherlands, <sup>5</sup>Academic Hospital Maastricht, Department of Pathology, Maastricht, Netherlands

**Objectives:** B cells and oligoclonal antibodies are present in the cerebrospinal fluid (CSF) of patients with multiple sclerosis (MS) but their target antigens remain unknown. The focus of this study was to produce and characterize autoantibodies in MS based on B cell immortalization.

**Methods:** Antibodies were produced from CSF and peripheral blood of 7 MS patients and 7 control patients with non-inflammatory or other inflammatory neurological disease (NIND/OIND). Peripheral blood mononuclear cells (PBMC) or CSF cells (500 or 5,000 respectively) were infected with Epstein-Barr virus (EBV) in the presence of irradiated allogeneic PBMC, T cell inhibitory and B cell stimulating factors to obtain continually dividing B cell lines. This was verified by screening the culture supernatant for the presence of immunoglobulin G (IgG). IgG-positive immortalized B cell cultures were maintained for antibody production and clonality was verified using a B cell spectratyping procedure (Identiclon<sup>TM</sup> IGH gene clonality assay, Invivoscribe).

**Results:** We obtained 37 immortalized B cell lines from 7 MS patients, 7 originating from CSF of 1 MS patient and 30 from PBMC of 6 MS patients. From 5 NIND and 2 OIND patients 10 B cell lines have been isolated, 2 derived from CSF cells of 1 patient and 8 from PBMC of 5 other patients. B cell spectratyping analysis for 39 of the immortalized B cell lines showed that 35 were monoclonal and 4 were biconal. Preliminary screening demonstrated intracellular binding of the antibodies obtained from 8 immortalized B cell lines to a human oligodendrogloma (HOG) cell line. The B cell immortalization procedure has been further optimized, including the comparison of two different approaches to obtain the highest possible immortalization efficiency. Using this optimized procedure, more than 30 immortalized B cell lines were recently isolated from a new patient.

**Conclusion:** B cell immortalization has proved to be a useful method for the production of antibodies. The obtained monoclonal antibodies will be further analyzed for autoreactivity by detecting antibody binding to healthy and EAE brain tissue from rat and rhesus monkey and to some viruses such as cytomegalovirus (CMV) and EBV.

**PC18/27 DC-SIGN CONTROLS IMMUNE HOMEOSTASIS IN THE BRAIN**

J.J. Garcia-Vallejo<sup>1</sup>, W.J. Unger<sup>1</sup>, H. Kalay<sup>1</sup>, S. Chamorro<sup>1</sup>, B. 't Hart<sup>2</sup>, Y. van Kooyk<sup>1</sup>

<sup>1</sup>VU University Medical Center, Molecular Cell Biology & Immunology, Amsterdam, Netherlands, <sup>2</sup>Biomedical Primate Research Centre, Immunobiology, Rijswijk, Netherlands

C-type lectin receptors (CLR) have long been known as pattern-recognition receptors implicated in the glycan mediated recognition of pathogens by the innate immune system. However, evidence is accumulating that many CLR are also able to recognize endogenous "self" ligands and that this recognition event often plays an important role in immune homeostasis. CLR-targeting experiments have shown that these receptors efficiently contribute to antigen internalization, processing,

and presentation in a tolerogenic fashion. However, co-administration of a TLR ligand results in the induction of immunity, indicating the existence of a TLR-CLR balance controlling tolerance and (auto)immunity. It remains to be established whether the endogenous glycosylated CLR ligand would induce tolerance as well. Myelin/oligodendrocyte glycoprotein (MOG) is a major autoantigen in multiple sclerosis (MS). MOG is a low abundant glycoprotein of CNS myelin post-translationally modified with one N-glycan. Immunization of mice, rats, or marmosets with MOG results in the development of experimental autoimmune encephalomyelitis (EAE), an animal model for MS. A more severe EAE is induced when non-glycosylated MOG is used, indicating a potential role of glycosylation in the maintenance of peripheral tolerance. To test the hypothesis of the involvement of CLR in this process, we first investigated the expression of these receptors in the brain. Amongst several CLR identified on microglia, the brain APC, was DC-SIGN. Using monocyte-derived DC as a model for a DC-SIGN expressing APC, we demonstrated that DC-SIGN is able to bind myelin and contributes to its internalization. The ligand recognized on myelin is MOG and binding results in the activation of signaling pathways leading to the production of IL-10 and the inhibition of proliferative responses in mixed leukocyte reactions. This effect can be blocked by modifying the glycosylation of MOG to avoid DC-SIGN recognition, using an anti-DC-SIGN blocking antibody, or pre-treating the monocyte-derived DC with an inhibitor of DC-SIGN signaling. Our data underscores the importance of glycosylation specific C-type lectin interactions in the human brain and provides new evidence supporting a role for these receptors in the maintenance of peripheral tolerance.

#### PC18/28 MODULATION OF DENDRITIC CELL FUNCTION BY TOLL-LIKE RECEPTOR AGONISTS IN MULTIPLE SCLEROSIS

L. Sanvito<sup>1</sup>, K. O'Brien<sup>1</sup>, C. Constantinescu<sup>1</sup>, B. Gran<sup>1</sup>

<sup>1</sup>Division of Clinical Neurology, University of Nottingham, Nottingham, United Kingdom

**Background and aims:** Multiple Sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system. Myeloid dendritic cells (mDCs) regulate the adaptive immune response and display a pro-inflammatory profile in MS. Toll-like receptors (TLRs) are innate immune receptors that can modulate the function of several cell types including mDCs. In experimental autoimmune encephalomyelitis, an animal model of MS, TLR agonists can either exacerbate or suppress disease through modulation of DCs and other immune cells, depending on the TLR signalling pathway involved (MyD88-dependent versus independent pathways). The functional response of mDCs to TLR stimulation in MS patients has not been fully investigated. Our aim was to establish the pattern of cytokine production induced by MyD88-dependent and -independent TLR pathways in mDCs from MS patients and healthy controls.

**Patients and methods:** We recruited untreated patients with relapsing-remitting MS and healthy controls (HC). We cultured blood monocyte-derived DCs with TLR3 (MyD88-independent), TLR7 (MyD88-dependent), and TLR4 (dependent on both) agonists (polyI:C, Imiquimod and LPS respectively). After 24-hour culture we measured the production of interleukin (IL)-1beta, IL-12, IL-23, IL-18, IL-10, Interferon (IFN)-beta, and the chemokine CCL2 in cell culture supernatants by ELISA and flow cytometry.

**Results:** CCL2 responses were increased in MS as compared to HC, whereas LPS-induced IL-10 responses were weaker in samples from MS patients. There were no differences between the two groups in the levels of IFN-beta, IL-12 and IL-6, which were all induced by LPS and polyI:C but not Imiquimod. IL-23 and IL-1beta were elicited mainly by LPS without differences between patients and controls.

**Conclusions:** mDC from MS patients displayed increased production of the chemokine CCL2 in response to TLR3, 7 and 4 stimulation. IL-10 response to TLR4 stimulation was reduced in MS. The DC response to TLR agonists may play a role in MS pathogenesis and warrants further investigations. We plan to extend our study to other TLRs and innate immune receptors.

#### PC18/29 INDUCTION OF IL-27 BY IFN- $\beta$ TREATMENT IN MULTIPLE SCLEROSIS

C.M. Sweeney<sup>1</sup>, R. Loneragan<sup>2</sup>, K. Kinsella<sup>2</sup>, N. Tubridy<sup>2</sup>, J.M. Fletcher<sup>1</sup>, K.H.G. Mills<sup>1</sup>

<sup>1</sup>Trinity College, School of Biochemistry and Immunology, Dublin, Ireland, <sup>2</sup>St Vincent's University Hospital, Department of Neurology, Dublin, Ireland

Recombinant IFN- $\beta$ , the most commonly used therapy for multiple sclerosis (MS), is effective at reducing relapses by approximately 30%. However, the precise mechanism of action and the reasons for the high number of non-responders to IFN- $\beta$  is still unclear. Innate immune cells, in particular dendritic cells (DC), play a crucial role in directing T cell responses via the production of regulatory cytokines, including IL-12 family members and IL-10. IL-12 promotes induction of IFN- $\gamma$ -secreting T helper (Th) 1 cells, IL-10 promotes IL-10 secreting regulatory T (Treg) cells, and IL-23 promotes the expansion of Th17 cells, which play a pathogenic role in autoimmune disorders, including MS. Furthermore, IL-27, another IL-12 family member, has recently been shown to be protective in experimental autoimmune encephalomyelitis (EAE), a mouse model of MS, by enhancing IL-10 and suppressing Th17 cells.

In this study we examined the effect of IFN- $\beta$  on cytokine production by DC and the consequence for directing the induction of different T cell subsets. Peripheral blood mononuclear cells (PBMC) from relapsing remitting MS patients undergoing IFN- $\beta$  treatment and normal controls were stimulated with IFN- $\beta$  and expression of IL-27 and IL-10 was analyzed by real time PCR. Furthermore, DC stimulated with TLR agonists in the presence or absence of IFN- $\beta$  were used to polarise T cells and cytokine production was measured by ELISA.

We found that IFN- $\beta$  induces the expression of IL-27 in PBMC from MS patients and from healthy controls. In addition, IFN- $\beta$  decreased the production of IL-23 and IL-12p40 from DC in response to TLR activation. Furthermore, IFN- $\beta$  treatment of DC inhibited the secretion of IL-17 by CD4<sup>+</sup> T cells but did not effect the production of IL-10 or IFN- $\gamma$ . In addition, recombinant IL-27 inhibited the production of IL-17 by CD4<sup>+</sup> T cells, thus it appears that IFN- $\beta$  may inhibit activation of Th17 cells via induction of IL-27. These data suggest that IFN- $\beta$  treatment induces the expression of the protective cytokine IL-27 in MS patients, which may account for some of its therapeutic effects in MS and if confirmed could be used to predict IFN- $\beta$  non-responsive patients.

#### PC18/30 EXPLORING THE MECHANISMS OF AXONAL AND NEURONAL DAMAGE CAUSED BY CD8 T CELLS

G. Chevalier<sup>1,2</sup>, E. Suberbielle<sup>1,2</sup>, T. Scheikl<sup>1,2</sup>, R. Liblau<sup>1,2</sup>, D. Gonzalez-Dunia<sup>1,2</sup>

<sup>1</sup>INSERM U563, Centre de Physiopathologie de Toulouse Purpan, Toulouse, France, <sup>2</sup>Toulouse III University, Toulouse, France

Multiple Sclerosis (MS) is an inflammatory demyelinating disorder of the central nervous system (CNS) characterized by multifocal demyelination, loss of oligodendrocytes and axonal damage. Observations from animal models and results from genetic studies have demonstrated the implication of several immune effectors in the physiopathology of MS. The implication of CD4<sup>+</sup> T cells has long been demonstrated, but recent data have pointed out the possible importance of CD8<sup>+</sup> T cells in MS pathogenesis. Since damage to neurons and transection of axons is an important feature of CNS lesions in MS, one hypothesis is that autoreactive cytotoxic T lymphocytes (CTLs) may contribute to this process. However, the assumption that neurons could represent a target for CTLs has not formally been demonstrated. Therefore, a better understanding of the mechanisms underlying neuronal and axonal attack by CTLs is much wanted.

Given this background, our objective is to bring new information about the mechanisms of neuronal injury caused by CTLs.

To address this question, we have developed new experimental systems, giving the opportunity to assess immune attack of neurons ex vivo and to study the underlying mechanisms. These models are based on transgenic mice expressing an alloantigen and on the model of neuroinflammation induced by neurotropic Borna disease virus and offer the possibility to combine CD8<sup>+</sup> T lymphocytes with defined antigenic specificity with neurons expressing the target antigen. This allows the analysis of the dynamics and consequences of CTL interaction with primary cultures of neurons, using live-cell imaging and time-lapse microscopy techniques. Our first results have demonstrated the ability of ex vivo purified CTLs to specifically interact with neurons, in an MHC class I-dependent manner. After their incubation with primary neurons, we observed a strong antigen-dependent reduced mobility of the CTLs in the culture. Moreover, analysis using lysotracker red and calcein staining revealed the polarization of the CTL lytic granules after neuronal contact, accompanied with changes in neuronal permeability. Surprisingly, neurons appeared to resist quite well to this "lethal hit", suggesting that the dynamics of CTL-neuron interaction may be quite different to that of a classical CTL target.

#### PC18/31 ANTIGEN PRESENTATION IN THE CENTRAL NERVOUS SYSTEM

M. Løbner<sup>1</sup>, T. Owens<sup>1</sup>

<sup>1</sup>Medical Biotechnology Center/University of Southern Denmark, Odense, Denmark

**Objective:** The objective of the study is to investigate the capacity of glial cells to induce T cell immune responses within the brain parenchyma.

**Hypothesis:** Differential activation status of microglia determines their relative role in animal models of demyelination.

**Background:** A fundamental issue in the etiology of autoimmune diseases of the central nervous system (CNS) such as multiple sclerosis concerns the identity of cells within the CNS capable of presenting autoantigen to the T cells that mediate these diseases. Dendritic cells (DC) are considered the major antigen presenting cells (APC) for primary T cell response. But in the CNS dendritic cells are restricted to extra-parenchymal sites such as the perivascular and subarachnoid space and meninges where they are likely contribute to control of T cell entry. Microglia represent a highly reactive intraparenchymal CNS cell populations which respond rapidly to infections and inflammation and they have been shown to be potent APC. In both experimental autoimmune encephalomyelitis (EAE) and a model of primary demyelination (cuprizone) microglia are activated and a subpopulation express the dendritic cell marker CD11c as well as molecules required for antigen presentation. In this study we have compared in vivo activated CD11c positive microglia to CD11c negative and resting microglia in terms of expression of molecules needed for antigen presentation and ability to induce primary and secondary T cell response.

**Results:** CD11c positive microglia from both EAE spinal cord and cuprizone-treated brain express a higher levels of MHC I, CD86, ICAM-1 and CD24 than CD11c negative microglia. CD11c positive microglia furthermore show a stronger ability to induce a secondary T cell response than either CD11c negative or resting microglia. At a high microglia to T cell ratio CD11c positive microglia are capable of inducing a primary T cell response.

**Conclusion:** A subpopulation of activated microglia from EAE spinal cord and from cuprizone treated mice show a dendritic like phenotype by expressing CD11c and molecules needed for antigen presentation. This microglia subpopulation are highly effective in presenting antigen for a secondary T cell response, and capable of inducing primary T cell response at a high microglia to T cell ratio.

# PC18/32 THERAPEUTIC EFFECT OF THE ALKYL-LYSOPHOSPHOLIPID EDELFOSINE ON IMMUNE CELLS AND EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

P. Abramowski<sup>1</sup>, K. Steinbach<sup>1</sup>, F.A. Ayuk<sup>2</sup>, R. Martin<sup>1</sup>, A.R. Zander<sup>2</sup>

<sup>1</sup>University Medical Center Hamburg-Eppendorf, Center for Molecular Neurobiology, Institute for Neuroimmunology and Clinical Multiple Sclerosis Research, Hamburg, Germany, <sup>2</sup>University Medical Center Hamburg-Eppendorf, Center of Oncology, Clinic for Stem Cell Transplantation, Hamburg, Germany

**Objectives:** The cytostatic drug edelfosine is a synthetic analog of lysophosphatidylcholine. Edelfosine is incorporated by cells and it acts on cellular membranes rather than DNA by selectively activating the cell death receptor Fas/CD95. Edelfosine may also interfere with the rate-limiting step of phosphatidylcholine synthesis catalyzed by CTP:phosphocholine cytidyltransferase inducing apoptosis by oxidative stress as well as accumulation of ceramide. Edelfosine is incorporated to effective intracellular concentrations by highly proliferating cells, like activated immune cells and tumor cells. Our aim was to study the effect of edelfosine on the immune system in the context of experimental autoimmune encephalomyelitis (EAE), an animal model for multiple sclerosis.

**Methods:** In order to analyze the anti-inflammatory properties of edelfosine we have used two models, C57BL/6 mice immunized with the peptide 35-55 of the myelin oligodendrocyte glycoprotein (MOG 35-55) and SJL mice immunized with the peptide 139-151 of the proteolipid protein (PLP 139-151). The effect of edelfosine on EAE disease course was evaluated by clinical and histological analysis.

**Results:** We found that daily treatment of C57BL/6 mice with 10mg/kg edelfosine by gavage from the day of immunization led to a reduction of the maximum mean EAE score of 2.8 in controls (gavage of PBS) to 1.2 in a 0 to 5 scale. In SJL mice the i.p. edelfosine treatment mediates the reduction of the maximum mean EAE score of 2.9 in control animals (PBS administration) to 1.7 (10mg/kg edelfosine administration). In vitro investigation of edelfosine impact on T cell activation and proliferation was studied on murine lymph node cells. 1µg/ml edelfosine appeared to be the lowest effective concentration with an effect on T cell proliferation. 5µg/ml edelfosine exerted the most profound proliferation impairment.

**Conclusions:** These results point to a beneficial effect of edelfosine on EAE disease severity and suggest that it interferes with immune cell infiltration into the central nervous system as indicated by flow cytometric and histological analysis. Further experiments to explore the therapeutic effects of edelfosine, when started at disease onset, are ongoing.

# PC18/33 THE EFFECT OF METHYLPREDNISOLONE ON IFN- $\gamma$ AND IL-17 EXPRESSION AND PRODUCTION BY CELLS INFILTRATING CNS IN EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

Z. Miljkovic<sup>1</sup>, M. Momčilović<sup>2</sup>, D. Miljkovic<sup>2</sup>, M. Mostarica-Stojkovic<sup>1</sup>

<sup>1</sup>Institute of Microbiology and Immunology, School of Medicine, University of Belgrade, Belgrade, Serbia, <sup>2</sup>Institute for Biological Research "Siniša Stanković", University of Belgrade, Department of Immunology, Belgrade, Serbia

**Objectives:** Glucocorticoids have been shown effective in the treatment of autoimmune diseases of the CNS such as multiple sclerosis and its animal model, experimental autoimmune encephalomyelitis (EAE). The aim of this study was to investigate the *in vivo* effect of synthetic glucocorticoid methylprednisolone (MP) on the expression and production of interferon (IFN)- $\gamma$  and interleukin (IL)-17, cytokines involved in autoimmune damage, by T lymphocytes infiltrating CNS tissue in EAE.

**Methods:** The disease was induced in Dark Agouti (DA) rats by immunization with rat spinal cord homogenate with Complete Freund's adjuvant (CFA) and in NOD mice with MOG<sub>35-55</sub> peptide with CFA and B. pertussis. Commencing on the day when first EAE signs appeared animals were injected daily for 3 days with MP (50 mg/kg body weight). Mononuclear cells were isolated from the spinal cord (SCC) three hours after the last injection of MP and IFN- $\gamma$  and IL-17 gene expression was determined by real time PCR. The production of cytokines by SCC was measured by ELISA in cell culture supernatants. The proportion of CD4<sup>+</sup> cells and the level of apoptosis among SCC were determined by flow cytometry.

**Results:** MP treatment ameliorated EAE in animals that recovered without relapses. Further, IFN- $\gamma$ , but not IL-17 expression and production was inhibited in cells isolated from the spinal cord of MP treated DA rats and NOD mice. The observed effect of MP *in vivo* treatment was not mediated through modulation of CD4<sup>+</sup> T cells proportion among CNS infiltrating cells, or through induction of their apoptosis within the CNS.

**Conclusion:** These results demonstrate that amelioration of EAE by exogenous glucocorticoids might be, at least partly, ascribed to the limitation of effector cell functions in the target tissue. Also, it seems that exogenous glucocorticoids exert different effects on CNS-infiltrating T helper-1 and T helper-17 cells.

This work was supported by the Serbian Ministry of Science (grants 143029 and 145066).

# PC18/34 BENEFICIAL EFFECT OF CORTISTATIN AND GHRELIN ON EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

L.S. Moreira<sup>1</sup>, V. Delgado-Maroto<sup>2</sup>, M. Delgado<sup>2</sup>, E. Gonzalez-Rey<sup>1</sup>

<sup>1</sup>University of Seville, Seville, Spain, <sup>2</sup>Institute of Parasitology and Biomedicine Lopez-Neyra, CSIC., Granada, Spain

**Objectives:** Multiple sclerosis (MS) is a disabling inflammatory autoimmune disease of the central nervous system (CNS), characterized by inflammation and CD4 Th1/Th17-mediated immunity. The autoimmune attack against myelin cause important neurological disorders. Current curative therapies are unavailable and ineffective for MS. Cortistatin (CST) and Ghrelin (GHR) are novel neuropeptides synthesized by immune cells that have various characteristics to be considered as possible therapeutic agents for MS. They are potent anti-inflammatory agents, which downregulate a broad spectrum of pro-inflammatory mediators, inhibit Th1 immune response following antigenic stimulation, and favour differentiation and generation of regulatory T cells (Treg). The therapeutic effect of CST and GHR on models of rheumatoid arthritis and Crohn's disease, two inflammatory and Th1-mediated autoimmune diseases, demonstrates their efficacy to treat this kind of disorders.

**Methods:** We investigated the therapeutic effect of CST and GHR in an established model for MS, experimental autoimmune encephalomyelitis (EAE), with which shares immune, histopathologic and clinical features. We determined CST/GHR effect on clinical score, disease incidence, histopathology, as well as cellular and molecular mechanisms involved.

**Results:** CST and GHR treatment of EAE mice following disease onset prevented and ameliorated the clinical signs of the disease. From a histopathologic point of view, neuropeptides treatment diminished the infiltration of inflammatory cells in the brain and spinal cord parenchyma and the subsequent demyelination characteristic of EAE. CST and GHR-treated mice showed decreased expression of proinflammatory mediators (cytokines and chemokines) in CNS parenchyma in comparison with untreated EAE mice.

Lymph node T cells from CST/GHR-treated mice are much less autoreactive than those from control EAE mice, and showed less antigen-specific proliferation and cytokine production. In addition, neuropeptides induce CD4+CD25+Foxp3+ Treg on EAE mice. In this sense, transfer of lymph node T cells from CST/GHR-treated mice to EAE mice controlled the disease progression.

**Conclusion:** In this study, we showed that CST and GHR are attractive candidates to treat MS. Both of them were beneficial for EAE incidence and progression by restoring immune tolerance and downregulating its two pathologic components: inflammation and autoimmunity.

# PC18/35 BONE MARROW-DERIVED MESENCHYMAL STEM CELLS SUPPRESS MYELIN-STIMULATED CD4+ AND CD8+ EFFECTOR-MEMORY T CELLS PROLIFERATION IN MULTIPLE SCLEROSIS PATIENTS

M. Zafranskaya<sup>1</sup>, G. Khulup<sup>1</sup>, D. Nizheharodava<sup>1</sup>, N. Milanovich<sup>2</sup>, M. Kolobova<sup>1</sup>, S. Bagatka<sup>1</sup>, A. Fedulov<sup>3</sup>

<sup>1</sup>Belarusian Medical Academy for Post-Graduate Education, Minsk, Belarus, <sup>2</sup>9th Municipal Hospital, Minsk, Belarus, <sup>3</sup>Belarusian State Medical University, Minsk, Belarus

Multiple sclerosis (MS) is a complex autoimmune disease leading to demyelination of axonal sheaths, degradation of nerve tissue, and eventually to irreversible damage. Our previous data have shown that autoreactive response in MS patients is driven by both CD4+ and CD8+ memory T cells. Mesenchymal stem cells are multipotent progenitor cells with great promise for pathogenic therapy of MS due to their immunomodulatory and neuroprotective properties.

In the present study, we characterized the antigen-specific response of CCR7-CD45RO+ effector-memory T cells from relapsing-remitting MS patients and immunosuppressive effect of autologous bone marrow-derived mesenchymal stem cells (bMSCs) on recombinant myelin oligodendrocyte glycoprotein (MOG)-stimulated lymphocyte proliferation *in vitro*. bMSCs (1<sup>st</sup>-4<sup>th</sup> passages) were phenotypically characterized (CD34+/31-/45-/90+/105+/44+/CCR7+) and considered to be able to differentiate into adipocytes and osteocytes thus proving their pluripotency. For detecting CFDA SE (Molecular Probes) labeling cells proliferation flow cytometry FC 500 (Beckman Coulter) was used.

We showed that bMSCs from MS patients, as well as bMSCs from healthy donors significantly reduced myelin-stimulated T cells proliferation *in vitro*; the inhibition varied from 22 to 60% and depended on responding cells expression. While the suppression level of bMSCs from MS patients as well as in CD4+CCR7-CD45RO+ and CD8+CCR7-CD45RO+ effector-memory T cells being similar, in healthy donors bMSCs inhibited mostly CD8+CCR7-CD45RO+ T cells population.

The demonstration of bMSCs immunosuppressive properties provides us with better understanding of further clinical implementation of MSC in pathogenic therapy of multiple sclerosis.



# PC18/36 CONTACTIN-2/TAG-1 DIRECTED AUTOIMMUNITY IS IDENTIFIED IN MULTIPLE SCLEROSIS PATIENTS AND MEDIATES GRAY MATTER PATHOLOGY IN ANIMALS

D. Derfuss<sup>1,2</sup>, K. Parikh<sup>3</sup>, S. Velhin<sup>1</sup>, M. Braun<sup>1</sup>, E. Mathey<sup>3</sup>, M. Krumbholz<sup>1</sup>, T. Kumpfel<sup>2</sup>, A. Moldenhauer<sup>4</sup>, C. Rader<sup>5</sup>, P. Sonderegger<sup>5</sup>, W. Pöllmann<sup>6</sup>, C.R. Tiefenthaler<sup>7</sup>, J. Bauer<sup>7</sup>, H. Lassmann<sup>7</sup>, H. Wekerle<sup>1</sup>, D. Karageorgos<sup>8</sup>, R. Hohlfeld<sup>1</sup>, C. Linington<sup>9</sup>, E. Meinl<sup>1,2</sup>  
<sup>1</sup>Max Planck Institute of Neurobiology, Martinsried, Germany, <sup>2</sup>Institute of Clinical Neuroimmunology, Univ. of Munich, Munich, Germany, <sup>3</sup>University of Aberdeen, Aberdeen, United Kingdom, <sup>4</sup>Institute of Transfusion Medicine, Berlin, Germany, <sup>5</sup>Institute of Biochemistry, Zurich, Switzerland, <sup>6</sup>MS Clinic, Berg, Germany, <sup>7</sup>Center for Brain Research, Vienna, Austria, <sup>8</sup>IMBB, University of Crete Medical School, Heraklion, Greece, <sup>9</sup>Division of Clinical Neuroscience, Univ. of Glasgow, Glasgow, United Kingdom

Gray matter pathology is increasingly recognized as an important feature of multiple sclerosis (MS), but the nature of the immune response that targets the gray matter is poorly understood. Starting with a proteomics approach, we identified contactin-2/TAG-1 as a candidate autoantigen recognized by both autoantibodies and Th1/Th17 T cells in MS patients. Contactin-2 and its rat homologue TAG-1 (transiently-expressed axonal glycoprotein 1) are expressed by various neuronal populations and sequestered in the juxtaparanodal domain of myelinated axons both at the axonal and myelin side. The pathogenic significance of these autoimmune responses was then explored in experimental autoimmune encephalitis (EAE) models in the rat. Adoptive transfer of TAG-1 specific T-cells induced an encephalitis characterized by a preferential inflammation of gray matter of the spinal cord and cortex. Co-transfer of TAG-1 specific T cells with a myelin oligodendrocyte glycoprotein (MOG)-specific mAb generated focal perivascular demyelinating lesions in the cortex and extensive demyelination in spinal cord gray and white matter. This study identifies contactin-2 as a novel autoantigen targeted by T cells and autoantibodies in MS. Our findings suggest that a contactin-2 specific T cell response contributes to the development of gray matter pathology.

# PC18/37 MULTIPLE SCLEROSIS ASSOCIATES WITH LILRA3 DELETION IN SPAIN

D. Ordóñez<sup>1</sup>, A. Sánchez<sup>2</sup>, J.E. Martínez Rodríguez<sup>3</sup>, E. Cisneros<sup>1</sup>, E. Ramil<sup>2</sup>, N. Romo<sup>4</sup>, M. Moraru<sup>1</sup>, M. López-Botet<sup>4</sup>, J. Roquer<sup>3</sup>, J.A. Gracia-Merino<sup>2</sup>, C. Vilches<sup>1</sup>  
<sup>1</sup>Hospital Universitario Puerta de Hierro – Majadahonda, Immunogenetics, Majadahonda (Madrid), Spain, <sup>2</sup>Hospital Universitario Puerta de Hierro – Majadahonda, Madrid, Neuroimmunology, Majadahonda (Madrid), Spain, <sup>3</sup>Neurology, Hospital del Mar-IMIM, Barcelona, Spain, <sup>4</sup>Universitat Pompeu Fabra & IMIM, Molecular Immunopathology Unit, Barcelona, Spain

The genetic susceptibility to multiple sclerosis (MS) is only partially explained, and it shows geographic variations. We report here that relapsing MS (R-MS) is associated in Spain with a 6.7-kb gene deletion affecting the complete coding region of the hypothetically secreted Leukocyte Immunoglobulin-like Receptor A3 (LILRA3, 19q13.4), in agreement with a previous finding in German patients. The distribution of homozygous and heterozygous genotypes in two independent groups of R-MS patients and healthy controls from Madrid and Barcelona, determined by a novel single-tube PCR method, reveals an apparently protective role for LILRA3. We will also present: (i) a study on the genotypic diversity of Killer-cell Ig-like Receptors (KIR, encoded only ~400 Kb telomeric to LILRA3, and implicated in autoimmunity and defence against viruses and tumours) as possible risk factors for MS, either independent or in linkage disequilibrium with LILRA3 deletion; and (ii) an analysis of the interaction between LILRA3 deletion and HLA-DRB1\*1501, the classical risk factor for MS.

# PC18/38 DEMONSTRATION OF MYELIN BASIC PROTEIN-REACTIVE ANTIBODIES IN HEALTHY INDIVIDUALS AND PATIENTS WITH MULTIPLE SCLEROSIS USING A NOVEL, HIGHLY SENSITIVE ASSAY

C.J. Hedegaard<sup>1</sup>, N. Chen<sup>1</sup>, K. Bendtzen<sup>1</sup>, C.H. Nielsen<sup>1</sup>

<sup>1</sup>Copenhagen University Hospital, Rigshospitalet, 7541, Institute for Inflammation Research, Copenhagen O, Denmark

**Objective:** Autoantibodies to myelin basic protein (MBP) are considered to be absent from sera from healthy individuals, but their presence has been reported in sera from some patients with multiple sclerosis (MS). We developed a novel assay for anti-MBP antibodies (MBPAb) to examine the occurrence of disease-associated MBPAb and “natural” MBPAb in MS patients and healthy individuals, respectively.

**Methods:** MBP, tetanus toxoid (TT) and human serum albumin (HSA) were attached to separate bead sets, which were incubated with sera from 17 healthy individuals and 17 MS patients for 16 hours at 4°C. The beads were subsequently assessed for IgM- and IgG-uptake using biotinylated anti-human IgM and -IgG class-specific antibodies, respectively, and streptavidin-phycoerythrin as the probing reagents.

**Results:** Sera from 17 MS patients and 17 healthy individuals contained IgM and IgG reactive with MBP as well as with TT. The levels of IgM MBPAb were similar in the two groups, while the levels of IgG MBPAb were non-significantly higher in the patient group. The IgG-/IgM-binding depended on the density of MBP on the beads and the concentration of serum.

**Conclusion:** Using a novel, highly sensitive method, we show here that MBPAb of IgM and IgG isotypes are generally present in sera from healthy individuals, as well as in sera from MS patients. At least in healthy individuals these antibodies may be classified as natural autoantibodies. We are currently investigating the influence of beta-interferon therapy on the MBPAb levels in MS patients.

# PC18/39 HLA-DR1 AND HLA-DQ1 GENOTYPES PREDICT RESPONSE TO GLATIRAMER ACETATE TREATMENT IN MULTIPLE SCLEROSIS

M. Gurevich<sup>1</sup>, T. Gritzman<sup>1</sup>, A. Achiron<sup>1,2</sup>

<sup>1</sup>Sheba Medical Center, Multiple Sclerosis Center, Ramat-Gan, Israel, <sup>2</sup>Tel Aviv University, Sackler School of Medicine, Tel-Aviv, Israel

**Objective:** To analyze whether HLA type polymorphism can be used as a marker to predict clinical response to glatiramer acetate (GA) treatment in relapsing-remitting multiple sclerosis (RRMS) patients.

**Methods:** 31 RRMS patients treated with 20 mg GA, 25 females, age: 37.8 ± 1.7 years, disease duration: 3.3 ± 0.7 years, Expanded Disability Status Scale (EDSS) score: 2.3 ± 0.3, annual relapse rate: 1.4 ± 0.2 were genotyped for major histocompatibility complex (MHC) class II loci HLA-DRB1\* and HLA-DQB1\* by PCR sequence specific primer (SSP). Clinical response to GA was defined by the change in annual relapse rate before and after treatment. Statistical analysis was performed by student's t-test and p < 0.05 was considered significant.

**Results:** GA treatment significantly reduced annual relapse rate in MS patients. Annual relapse rate before initiation of GA was 1.6 ± 0.1 and decreased significantly within the first year of treatment to 1.1 ± 0.2, p = 0.05. HLA-DRB1\*04 and HLA-DQB1\*03 were found to be associated with better clinical outcome. In HLA-DRB1\*04 (N = 9) or HLA-DQB1\*03 (N = 5) positive patients, annual relapse rate decreased from 1.7 ± 0.2 to 0.56 ± 0.1, p = 0.02 or from 2.3 ± 0.2 to 0.2 ± 0.1, p = 0.01, respectively.

**Conclusion:** HLA-DR1\*04 and HLA-DQ1\*03 loci are associated with better response to GA treatment in relapsing-remitting MS patients.

# PC18/40 METALLOTHIONEINS AND TISSUE METALS IN CHRONIC RELAPSING FORM OF EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS IN RATS

D. Grebić<sup>1</sup>, H. Jakovac<sup>1</sup>, M. Tota<sup>2</sup>, V. Barac-Latas<sup>1</sup>, I. Mrakovčić-Šutić<sup>1</sup>, C. Milin<sup>2</sup>, B. Radosevic-Stasic<sup>1</sup>

<sup>1</sup>Medical Faculty, University of Rijeka, Department of Physiology and Immunology, Rijeka, Croatia, <sup>2</sup>Medical Faculty, University of Rijeka, Department of Chemistry and Biochemistry, Rijeka, Croatia

**Aim:** In the genetically susceptible Dark Agouti rats (DA) experimental autoimmune encephalomyelitis may be induced in chronic relapsing form (CR-EAE) that resembles to multiple sclerosis, which is an inflammatory, demyelinating disease the central nervous system. It is caused by erroneous activation of self-reactive T cells specific for myelin antigen, but the severity of symptoms depends on the balance between the aggressive and the localized, or systemic protective tissue reaction to infection or injury.

**Methods:** Since in the pathogenesis of disease a significant role may have the metallothioneins (MTs), which are the antioxidant proteins with high metal-binding properties, in this study we estimated the expression of MTs I+II, as well as the tissue concentrations of Zn<sup>2+</sup> and Cu<sup>2+</sup> in the brain, spinal cord and liver during the first and second attack (on the 12<sup>th</sup> and 22<sup>nd</sup> post-immunization day, respectively) and during the remission phases (on the 18<sup>th</sup> and 28<sup>th</sup> day) of CR-EAE, induced in DA rats by subcutaneous injection of bovine brain homogenate in complete Freund's adjuvant. Controls consisted of rats treated only with CFA. Clinical assessment was performed according to the standard criteria, while MTs and metal ions were analyzed by immunocytochemistry and inductivity coupled plasma spectrometry, respectively.

**Results:** The data showed that during the first attack MTs I+II were upregulated in the brain (subpial region, perivascular space and parenchymal astrocytes) and in the spinal cord (glial cells and neurons). Simultaneously, increased the concentration of Zn<sup>2+</sup> in the spinal cord and Zn<sup>2+</sup> and Cu<sup>2+</sup> in the liver. During the second attack a very high, new overexpression was found in the molecular layer of cerebellum, in sulcus hippocampi, on several neurons and oligodendrocytes in spinal cord, and particularly on hepatocytes around the central vein. Simultaneously, in the brain and spinal cord increased the concentration of Cu<sup>2+</sup>.

**Conclusions:** The data point to neuroprotective role of MTs and to important regulatory role of essential metals and hepatic MTs for the recovery from acute attacks of EAE.

# PC18/41 SUPERANTIGEN ENCEPHALITIS IN THE LEWIS RAT INDUCED BY STAPHYLOCOCCAL ENTEROTOXIN A (SEA)

A. Emmer<sup>1</sup>, K. Gerlach<sup>1</sup>, M.E. Kornhuber<sup>1</sup>, M.S. Staeger<sup>2</sup>

<sup>1</sup>Martin-Luther-University Halle-Wittenberg, Department of Neurology, Halle (Saale), Germany, <sup>2</sup>Martin-Luther-University Halle-Wittenberg, Department of Pediatrics, Halle (Saale), Germany

**Objectives:** Superantigens have been suggested to play a role in the pathogenesis of different autoimmune diseases including multiple sclerosis (MS). Previously it was demonstrated that local expression of the superantigen Staphylococcal Enterotoxin-A (SEA) in the brain of rats may lead to encephalitis which was amplified by intravenous injection of Concanavalin-A (ConA)-activated splenocytes.

**Methods:** In the present investigation gene expression analysis of the rat brain (Affymetrix rat genome U34A microarrays) was performed 8 days after injection of 50 µl of 1mg/ml SEA or saline and 5 days after intravenous injection of 1x10<sup>7</sup> ConA-activated spleen cells and immunohistochemical sections were analyzed 12 hours, 3 and 5 days after intravenous injection of 1x10<sup>7</sup> ConA-activated spleen cells.

**Results:** Out of 8800 investigated genes 106 were significantly and at least 3-fold increased with SEA while 29 genes were decreased at least 3-fold. Increased gene expression was compatible with an intracerebral inflammatory response mediated by antigen presenting cells and CD8+ T-lymphocytes. Elevated chemokines included RANTES (CCL5), Osteopontin, MCP-1 (CCL2) and CXCL10. Further genes with elevated expression were assigned to the extracellular matrix, microglia/macrophage cell elements, astrocytes (GFAP) and phagocytosis. The immunohistochemical investigation showed a CD8+ T-cell dominated inflammation predominantly in the right (SEA-injected) hemisphere with a maximum 12 h after intravenous injection of activated spleen cells. The most intensive inflammation was detected three days after intravenous injection of activated spleen cells. CD3+ T-cell levels were lower than the levels of CD4+ and CD8+ T-cells during time course.

**Conclusion:** Our data is in accordance with the concept that T-cell superantigen expressed locally in the central nervous system induces an inflammatory response. There was a considerable similarity between previously reported gene expression profiles and immunohistochemical investigations for experimental autoimmune encephalomyelitis (EAE) or MS and our findings. This similarity between the three different states of inflammation may be due to the fact that a T-cell-driven pathogenesis is common to all of them.

#### PC18/42 CCR5-DELTA32 ALLELE IS ASSOCIATED WITH THE RISK OF DEVELOPING MULTIPLE SCLEROSIS IN THE IRANIAN POPULATION

M. Shahbazi<sup>1</sup>, H. Ebadi<sup>2</sup>, D. Fathi<sup>2</sup>, D. Roshandel<sup>3</sup>, M. Mahammadhoseini<sup>3</sup>, A. Rashidbaghan<sup>3</sup>

<sup>1</sup>Medical Cellular & Molecular Research Center, Golestan University of Medical Sciences, Molecular Medicine & Genetics, Gorgan, Iran, Islamic Republic of, <sup>2</sup>5 Azar Hospital, Golestan University of Medical Sciences, Neurology, Gorgan, Iran, Islamic Republic of, <sup>3</sup>Medical Cellular & Molecular Research Center, Golestan University of Medical Sciences, Gorgan, Iran, Islamic Republic of

Multiple sclerosis (MS) is a progressive demyelinating disorder in the central nervous system (CNS) of young people. Until recently, no distinct mechanism had been proposed for the aetiology or pathogenesis of MS, but it now appears that immune dysregulation plays an important role in the MS development.

It is widely accepted that susceptibility to MS involves a complex interaction between several genes and the environment. Studies of MS-twin pairs have revealed that the estimated concordance for MS was 3-5% for dizygotic and 25-30% for monozygotic twins.

Chemokines and their receptors are vital modulators of the immune response. The CCR5-delta-32 deletion may slow the rate of MS disease progression. These findings lend credence to the hypothesis that the 32-bp deletion have a protective role against immune disorders.

We have amplified the fragment including the CCR5-delta32 polymorphism DNA of 254 patients and 380 controls. Data were analysed using Fisher's exact test. Bonferroni's post hoc analysis was performed for correction of P-values of genotype distribution differences and statistical significance was defined as corrected P-values. The CCR5-delta32 allele was more frequent among MS patients than healthy individuals (P< 0.0001). Furthermore, 43 MS patients (19%) were homozygous for the delta32 deletion, which was significantly higher than controls (P< 0.0001). The delta32 allele was more frequent in MS patients compared to controls (OR=2.3, P< 0.0001). Also, we found a significant difference in the frequency of the delta32/delta32 genotype among patients and controls (OR=10, P< 0.001). The CCR5-delta32 deletion might be a predisposing factor for the development of MS in the Iranian population. Diverse findings about the effect of CCR5-delta32 on the development and clinical course of MS might be due to differences in the genetic background of different populations. Hence, genetic interactions have been described in many disorders, and the complexity of a genetic basis for MS is a definite possibility, as was observed for the HLA-DR4 molecule. There is no doubt regarding the importance of chemokine receptors and their ligands in MS. However, more studies are needed to reveal the exact role of chemokine network and its association with other factors in the pathogenesis of this disease.

#### PC18/43 EVALUATION OF ANTI-EPSTEIN BARR VIRUS ANTIBODIES AND PRODUCTION OF IFN-γ, IL-4 AND IL-12 BY MITOGEN-ACTIVATED PERIPHERAL BLOOD MONONUCLEAR CELLS IN PATIENTS WITH MULTIPLE SCLEROSIS

M. Shekarabi<sup>1</sup>, B. Laribi<sup>1</sup>, A. H. Zarnani<sup>2</sup>, M. Ghaffarpour<sup>3</sup>, M. Ghabaei<sup>4</sup>, S. Nourbakhsh<sup>2</sup>, M. Bakhshayesh<sup>5</sup>

<sup>1</sup>Iran University of Medical Sciences, Research Center of Immunology, Tehran, Iran, Islamic Republic of, <sup>2</sup>Iran University of Medical Sciences, Tehran, Iran, Islamic Republic of, <sup>3</sup>Tehran University of Medical Sciences, Department of Neurology, Iranian Center of Neurological Research, Tehran, Iran, Islamic Republic of, <sup>4</sup>Tehran University of Medical Sciences, Department of Neurology, Iranian Center of Neurological Research, Tehran, Iran, Islamic Republic of, <sup>5</sup>IBB-Universidad Autonoma de Barcelona, Tehran, Iran, Islamic Republic of

**Introduction:** Multiple sclerosis is the most common inflammatory disease of central nervous system, which is caused by an autoimmune process leading to destruction of myelin sheath. In this disease, CD4+T lymphocytes mostly of T<sub>H</sub>1 phenotype play important roles in destruction of neuronal tissues. Because of the probable etiologic participation of EBV virus in immunopathogenesis of MS, the aim of this study was to evaluate the relationship between serum levels of anti-Epstein Barr virus antibodies and production of T<sub>H</sub>1 and T<sub>H</sub>2 cytokines.

**Methods:** 68 patients with MS and 20 normal individuals who were matched in sex and age with patients were selected as the control group. Blood samples were taken and serum levels of anti-EBNA-1 and VCA antibodies were determined by ELISA method. Then, peripheral blood mononuclear cells (PBMC) were isolated by Ficoll Hypaque gradient and stimulated with PHA in optimal culture media. The levels of IFN-γ, IL-12 and IL-4 cytokines in culture supernatants were measured by ELISA method.

**Results:** The mean levels of anti EBNA-1 and VCA antibodies were significantly higher in patients compared to controls (p=0.04, p=0.001 respectively). Concentrations of IFN-γ, IL-4 & IL-12 were also significantly higher in MS patients than healthy individuals (p=0.001, p=0.005, p=0.002 respectively). Although cytokines ratios of IFN-γ/IL-4 and IL-12/IL-4 were higher in MS patients than healthy individuals, but this increase is not statistically significant (p>0.05). Statistically significant correlation was found between anti EBNA-1 and VCA antibodies and IL-12 production (p=0.02, r=0.27 & p=0.04, r=0.25 respectively); whereas no significant correlation was found between the antibody levels and production of IFN-γ and IL-4 in MS patients.

**Conclusion:** significant correlation between the level of anti-Epstein Barr virus antibodies with IL-12 production in MS patients imply the probable role of this cytokine in skewing of immune responses toward the T<sub>H</sub>1 phenotype. It is possible that this putative viral agent in combination with other etiological agents involves in etiology and progression of disease, which requires further investigations.

**Key words:** Multiple sclerosis, Epstein Barr virus, Anti-EBNA-1, Anti-VCA, T<sub>H</sub>1.

#### PC18/44 OLIGOCLONAL M BANDS IN THE CEREBROSPINAL FLUID IN NEUROMYELITIS OPTICA SPECTRUM DISORDERS

M. J. Magraner<sup>1</sup>, M. Simó-Castelló<sup>1</sup>, I. Bosà<sup>1</sup>, F. Coret<sup>2</sup>, J. C. Álvarez-Cermeño<sup>3</sup>, L. M. Villar<sup>3</sup>, B. Casanova<sup>1</sup>

<sup>1</sup>Hospital Universitari La Fe, València, Spain, <sup>2</sup>Hospital Clínic/Facultat de Medicina, València, Spain, <sup>3</sup>Hospital Ramón y Cajal, Madrid, Spain

**Background:** Neuromyelitis optica (NMO) is an idiopathic demyelinating disease characterized by attacks of optic neuritis and myelitis. Pathologic studies had demonstrated deposition of immunoglobulins (mainly of the IgM type) co-localizing with products of complement activation cascade around the vessels in the early lesions. With this background, we hypothesized that NMO-patients could have oligoclonal IgM bands in the cerebrospinal fluid (CSF).

**Objective:** To describe the presence of Oligoclonal IgG (OCGB) and IgM (OCMB) bands in the CSF of patients with NMO.

**Methods:** 7 patients fulfilling the Wingerchuk's criteria to NMO and 2 patients with recurrent long extensive transverse myelitis; and 12 patients with definitive MS according McDonald criteria, have been studied. In all patients paired samples of serum and CSF were analyzed by isoelectrofocusing and immunodetection of OCGB and OCMB. IgG and IgM present in the CSF and serum were quantified by nephelometry in a Siemens nephelometer.

**Results:** NMO patients: there were 8 women (88.8%), mean age at the beginning 29.4 years-old (range 6-43), mean evolution time 6.5 years (sd 4.5). OCGB were present in 2 cases (22.2%), and OCMB were present in 7 cases (77.7%). MS patients: there were 10 women (83.3%), mean age at the beginning 37.2 years-old (range 20-54). OCGB were present in all cases and OCMB were present in 4 patients (33.3%). Differences were significant between groups for the OCGB, p< 0.000 (Fisher exact test), and for OCMB p=0.05 (Fisher exact test). Moreover, the CSF pattern showing only the presence of OCMB was specific of NMO (5 patients, 55.6%), and no patients with NMO shown the CSF pattern of OCGB present with absence of OCMB, that was specific for the MS patients (8 patients, 66.7%).

**Conclusions:** OCMB in the CSF fluid of NMO-patients are twice as frequent as in MS patients and the CSF pattern showing only OCMB seems to be specific for NMO. These preliminary results need to be confirmed with more patients, but appoint to the idea that determination of OCMB in the CSF in NMO patients can help us in the diagnosis of NMO and the understanding of the pathogenic basis of these diseases.

#### PC18/45 NEUROTROPIC AUTOANTIBODIES IN THE BLOOD SERUM OF CHILDREN FIRST 2 YEARS OLD WITH CNS PATHOLOGY, CAUSED BY PERINATAL HYPOXIA-ISCHEMIA

T. Mardovina<sup>1</sup>

<sup>1</sup>National Research Practical Center Mother and Child, Minsk, Belarus

**Objectives:** Under supervision there were 131 children first 2 years old: 55 children with delay of motor development; 46 children with cerebral palsy, hydrocephaly and 30 health children for control. Auto-Abs (A1) to brain proteins (GFAP- glial fibrillary acidic protein, S100, MBP- myelin basic protein, NGF - nerve growth factor.) and antiidiotypic Abs (A2) level were analyzed.

**Methods:** We used ELISA method for determination autoantibodies to brain proteins.

**Results:** The obtained data indicates that the generalized and balanced increased production of neurotropic A1 and A2 is typical for children with cerebral palsy, hydrocephaly. Children with delay of motor development had neurotropic A1 and A2 level not differ from health children. Children with cerebral palsy, hydrocephaly had statistically significant increasing level of neurotropic A1 and A2 compare with children with delay of motor development: S 100 A1 (78,9 ± 28,36 and

111.6 ± 43.18,  $p < 0.001$ ), A2 (78.3 ± 29.09 and 110.8 ± 45.41,  $p = 0.027$ ); GFAP A1 (77.2 ± 31.48 and 105.5 ± 42.03,  $p < 0.001$ ), A2 (84.1 ± 44.50 and 110.8 ± 46.99,  $p = 0.016$ ); MBP A1 (76.5 ± 34.92 and 117.5 ± 50.32,  $p < 0.001$ ), A2 (83.8 ± 40.33 and 118.4 ± 55.88,  $p = 0.001$ ); NGF A1 (95.3 ± 38.74 and 146.1 ± 60.04,  $p < 0.001$ ), A2 (94.4 ± 36.22 and 141.2 ± 59.15,  $p < 0.001$ ).

**Conclusions:** The neural system dysfunctions are usually accompanied by the immune changes; in particular, changes in the serum contents of natural auto-antibodies against nervous cells proteins (Poletaeu). It is proved newborns after perinatal CNS damage had increased level of auto-Abs against nervous cells proteins and such level depend on severity of CNS pathology. Some scientists thought that temporary elevation of neurotropic level auto-antibodies are compensatory mechanisms after acute pathology process (dysfunction of blood-brain barrier after hypoxia-ischemia). But well known that proof increasing of auto-antibodies level can cause and support autoimmune diseases. Children with cerebral palsy and hydrocephaly had increasing level of neurotropic autoantibodies and antidiabetic antibodies. It talks that pathological process caused by perinatal hypoxia-ischemia proceeds, with engaging in it of the immune system.

#### PC18/46 DRY OLIVE LEAF EXTRACT AMELIORATES EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

D. Miljkovic<sup>1</sup>, D. Dekanski<sup>2</sup>, Z. Miljkovic<sup>3</sup>, M. Momcilovic<sup>1</sup>, M. Mostarica Stojkovic<sup>3</sup>

<sup>1</sup>Institute for Biological Research 'Sinisa Stankovic', University of Belgrade, Belgrade, Serbia, <sup>2</sup>R&D Institute – Biomedical Research, Galenika a. d., Belgrade, Serbia, <sup>3</sup>Institute of Microbiology and Immunology, School of Medicine, University of Belgrade, Belgrade, Serbia

**Objectives:** Experimental autoimmune encephalomyelitis (EAE) is an animal model of CNS inflammatory and demyelinating disease multiple sclerosis. Mediterranean diet, which is based on olive products, is associated with lower incidence of multiple sclerosis in South European population. Dry olive leaf extract (DOLE) is rich in polyphenolic compounds, flavonoids and tannins which could contribute to the observed beneficial effect of the Mediterranean diet. Therefore, the influence of DOLE on EAE course was investigated. Also, its influence on the production of interferon (IFN)-gamma and interleukin (IL)-17 in EAE was determined, as it has been known that these two cytokines are of primary importance for pathogenesis of neuroinflammatory disorders.

**Methods:** Spinal cord homogenate and complete Freund's adjuvant were used for the induction of EAE in Dark Agouti rats. DOLE was applied intragastrically once per day, starting from the day of the immunization. Real time PCR and ELISA were used for the determination of IFN-gamma and IL-17 gene expression and production, respectively.

**Results:** DOLE reduced various parameters of EAE severity in DA rats, including cumulative disease index, maximal clinical score and disease duration. Also, DOLE decreased cellularity of the draining lymph nodes and production of IFN-gamma and IL-17 by the cells infiltrating spinal cord of EAE rats.

**Conclusion:** The results of this investigation strongly suggest that DOLE-enriched diet has a beneficial effect in EAE. Thus, DOLE could be a useful supplementary dietetic for the patients suffering from multiple sclerosis and other neuroinflammatory disorders.

#### PC18/47 THE INHIBITORY NEUROTRANSMITTER GABA HAS ANTI-INFLAMMATORY EFFECTS ON MACROPHAGE FUNCTION IN VITRO

S. Carmans<sup>1</sup>, J. Hendriks<sup>1</sup>, K. Thewissen<sup>1</sup>, J.-M. Rigo<sup>1</sup>, P. Stinissen<sup>1</sup>, N. Hellings<sup>1</sup>

<sup>1</sup>Hasselt University, Biomedical Research Institute and Transnational University Limburg, School of Life Sciences, Diepenbeek, Belgium

Gamma-aminobutyric acid (GABA), the major inhibitory neurotransmitter of the central nervous system (CNS), participates in the communication between the nervous and the immune system. Outside the CNS, the GABA receptor is expressed in various tissues and immune cells. Moreover, GABA is present at sites of inflammation where it may modulate the ongoing immune response. Such an immunomodulatory role can be of importance for neuroinflammatory diseases such as multiple sclerosis (MS). Although GABA levels are changed in the CNS during the course of experimental autoimmune encephalomyelitis (EAE), the animal model of MS, the exact role of GABA in the disease process is unclear.

**Objectives:** In this study the effect of GABA on macrophages, the most important effector cells in MS, was investigated.

**Methods:** The expression of the GABA<sub>A</sub>-receptor on rat peritoneal macrophages was investigated by immunocytochemistry. Moreover, rat peritoneal macrophages were pre-incubated with different concentrations of GABA for 24 hours. Subsequently, lipopolysaccharide was added for another 24 hours and the effect of GABA on the production of nitric oxide (NO) and tumor necrosis factor-alpha (TNF-alpha) was examined by means of the Griess assay and ELISA, respectively. Macrophage viability was assessed by the MTT assay. Flowcytometry was used to study myelin phagocytosis and production of reactive oxygen species (ROS) by macrophages after 24 hours incubation with GABA.

**Results:** The GABA<sub>A</sub>-receptor was clearly expressed on rat peritoneal macrophages. Moreover, GABA dose dependently diminished the production of the pro-inflammatory mediators NO, TNF-alpha and ROS without affecting macrophage viability. Furthermore, the capacity to phagocytose myelin was dose dependently reduced after incubation with GABA.

**Conclusion:** These experiments demonstrate an anti-inflammatory effect of GABA on macrophage function in vitro. Moreover, these findings suggest that GABA expression during a neuroinflammatory insult may suppress macrophage activity in the CNS and in this way modulate inflammatory lesions in MS.

#### PC18/48 INCREASED RISK OF MULTIPLE SCLEROSIS IN WOMEN WORKING IN MEDICAL GROUPS

B. Laribi<sup>1</sup>, M.A. Bahar<sup>1</sup>, H. Yaghooti<sup>2</sup>, M.H. Ahrar<sup>3</sup>, R. Karimzadeh<sup>2</sup>

<sup>1</sup>Iran University of Medical Sciences, Department of Immunology, Research Center of Immunology, Tehran, Iran, Islamic Republic of, <sup>2</sup>Iran University of Medical Sciences, Tehran, Iran, Islamic Republic of, <sup>3</sup>Tehran University of Medical Sciences, Tehran, Iran, Islamic Republic of

**Objective:** Multiple sclerosis is the most common chronic autoimmune inflammatory disease of the CNS, which is seen more among young adults particularly in women. It seems that in addition to genetic and autoimmune backgrounds, other factors such as infectious agents and occupational stressors are causative in this disease. We aimed the study on the female physicians, nurses and midwives working in Tehran who are exposed to the mentioned risk factors.

**Material & methods:** Statistical data were provided by MS society of Iran. Prevalence of the disease was calculated for each group. Chi square analysis was performed to show the significant dependence of the increased prevalence on the mentioned occupations.

**Result:** 192 MS patients were identified with the mentioned occupations. These include 67 physicians, 49 nurses and 76 midwives. Total number of these health care providers in Tehran is 31867 which are 16200, 9267 and 6400 female physicians, nurses and midwives respectively. The prevalence is significantly higher in each group and is fully occupation dependent ( $P$  value  $< 0.001$ ). 1.1% of all midwives, comparing 0.5% and 0.4% in nurses and physicians respectively, developed MS.

**Conclusion:** Considerable high MS prevalence in medical staffs proposed alternative risk factors for MS development. Pathogens such as Epstein – Barr virus, Human T leukemia Virus 1, Herpes Virus and Chlamydia are suspected to cause MS development. Medical staffs are generally more exposed to infectious agents, but different occupations such as physicians, nurses or midwives are in contact with different amount and types of infectious agents.

#### PC18/49 ANTIBODIES TO $\beta$ 2-GLYCOPROTEIN I IN SCHIZOPHRENIC PATIENTS: PREVALENCE AND CLINICAL ASSOCIATION

K.S. Halacheva<sup>1</sup>, S. Dimova<sup>2</sup>, D. Dimov<sup>3</sup>, M. Nikolova<sup>3</sup>, T. Tolev<sup>3</sup>

<sup>1</sup>Trakia University, Faculty of Medicine, Immunology, Stara Zagora, Bulgaria, <sup>2</sup>Trakia University, Faculty of Medicine, Pathophysiology, Stara Zagora, Bulgaria, <sup>3</sup>State Psychiatric Hospital, Radnevo, Bulgaria

Many studies report a presence of antiphospholipid antibodies (aPL) in patients with schizophrenia. Beta2-glycoprotein I ( $\beta$ 2GPI) is one of the main antigenic targets for aPL and the antibodies specific are shown to be pathogenic in some autoimmune diseases.

**Objective:** To assess the levels of anti- $\beta$ 2GPI antibodies in sera of schizophrenic patients and to evaluate their possible relationship with psychopathology.

**Methods:** The study group consisted of 55 drug-free patients who met the DSM-IV diagnostic criteria for schizophrenia and were admitted to the hospital following an exacerbation of psychosis. Severity of psychopathology was assessed with the Positive and Negative Syndrome Scale (PANSS). Blood samples were taken from the patients on the day after admission. The control group consisted of 32 healthy donors. The sera were separated on the same day and stored at  $-70^{\circ}\text{C}$  until analysed. All sera were tested for 3 isotypes (IgM, IgG and IgA) of anti- $\beta$ 2GPI antibodies with quantitative commercial ELISA kits (The Binding Site, UK). Following the manufacturer's instructions, values exceeding 20 U/ml for IgG anti- $\beta$ 2GPI and 10 U/ml for IgM and IgA anti- $\beta$ 2GPI were regarded positive.

**Results:** Among the patients tested four (7.3%) were positive for IgM anti- $\beta$ 2GPI antibodies and sixteen (29%) were positive for IgA anti- $\beta$ 2GPI. Patient sera had normal levels of anti- $\beta$ 2GPI of IgG-isotype. In patients IgA anti- $\beta$ 2GPI positivity significantly exceeded that in controls (Fisher's exact  $p = 0.001$ ). The anti- $\beta$ 2GPI antibody serum levels of three isotypes (IgM, IgG and IgA) tested were significantly higher in patients than in controls (Mann-Whitney  $U = 421$  and  $p = 0.001$  for IgM; Mann-Whitney  $U = 628.5$  and  $p = 0.025$  for IgG and Mann-Whitney  $U = 309$  and  $p = 0.001$  for IgA respectively). A significant correlation between IgA anti- $\beta$ 2GPI antibody levels and the total PANSS score (Spearman  $R = 0.530$ ;  $p = 0.026$ ) and the score of negative symptoms (Spearman  $R = 0.510$ ;  $p = 0.038$ ) in IgA-positive patients was found.

**Conclusion:** On the basis of our data we may conclude that anti- $\beta$ 2GPI antibodies of three isotypes (IgG, IgM and IgA) are elevated in schizophrenic patients, with the marked prevalence of IgA anti- $\beta$ 2GPI isotype. Only IgA anti- $\beta$ 2GPI antibodies seem to associate with psychopathology of schizophrenia.

#### PC18/50 QUANTITATIVE ANALYSIS OF DIFFERENTIALLY EXPRESSED PHOSPHORYLATED BRAIN PROTEINS IN EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

A. Vanheel<sup>1</sup>, R. Daniels<sup>1</sup>, B. Hoedemaekers<sup>1</sup>, K. Baeten<sup>1</sup>, J. Hendriks<sup>1</sup>, J.-P. Noben<sup>1</sup>, P. Stinissen<sup>1</sup>, N. Hellings<sup>1</sup>

<sup>1</sup>Hasselt University, Biomedical Research Institute and Transnational University Limburg, School of Life Sciences, Diepenbeek, Belgium

**Objectives:** Multiple sclerosis (MS) is an inflammatory autoimmune disease of the central nervous system. The underlying molecular processes remain poorly understood, but are crucial in the search for new therapeutic options. Protein phosphorylations and glycosylations are post-translational modifications (PTMs) that



may be involved in the pathology of MS and experimental autoimmune encephalomyelitis (EAE), an animal model of MS. To analyze differentially expressed phosphorylated proteins, brain proteins extracted from EAE animals were fluorescently stained and detected on two-dimensional gels. Association with a previous 2D-DIGE experiment allows quantitative comparison at different disease stages (control, onset, top and recovery, three biological replicates each).

**Methods:** Protein extracts from 'blood-free' brain stems of control and EAE Lewis rats were separated by 2D-gel electrophoresis. In order to detect phosphorylated proteins, these gels were stained with a fluorescent dye, Pro-Q diamond phosphoprotein gel stain (Invitrogen). The Ettan DIGE Imager (GE Healthcare) was used for scanning.

**Results:** The Pro-Q diamond phosphoprotein gel staining was optimized on 2D-gels. During optimization the proteins, detected with a post-gel stain, were compared with the spotmap of a Cydyne-labeled protein sample. Post-gel total protein staining is used as quality control for protein loss and thus normalization of the phosphoprotein signals. Moreover total protein staining is useful for matching different gels. Finally, the PTM-specific signal will be compared to the corresponding protein staining for the spot. Phosphorylated proteins are identified by mass spectrometry. Furthermore, we are able to study the expression profile of the identified phosphoproteins throughout the disease process via matching of the PTM-specific 2D-spotmaps with the spotmaps of a previous 2D-DIGE experiment, a quantitative study of the brain proteome dynamics during the disease course of EAE in which 36 differentially expressed proteins were identified (significance: ANOVA  $\leq 0.01$  and at least 1.5 fold regulated).

**Conclusion:** The identification of a panel of differentially expressed phosphorylated proteins in the disease course, could provide information about the global disease processes in EAE and MS. Protein phosphorylation is of great importance to the protein function. This global overview of phosphorylation during disease invites new studies to unravel the complicated molecular biological processes in the pathology of MS.

#### PC18/51 CYTOKINE LEVELS IN B CELL CULTURE SYSTEM OF MYASTHENIA GRAVIS PATIENTS

V. Yilmaz<sup>1</sup>, P. Oflazer<sup>2</sup>, F. Aysal<sup>3</sup>, Y.G. Parman<sup>2</sup>, H. Direskeneli<sup>4</sup>, F. Deymeer<sup>2</sup>, G. Saruhan Direskeneli<sup>1</sup>

<sup>1</sup>Istanbul University, Istanbul Medical Faculty, Physiology, Istanbul, Turkey, <sup>2</sup>Istanbul University Istanbul Medical Faculty, Neurology, Istanbul, Turkey, <sup>3</sup>Bakirkoy Research and Training Hospital for Psychiatric and Neurological Diseases, Department of Neurology, Istanbul, Turkey, <sup>4</sup>Marmara University, Faculty of Medicine, Division of Rheumatology, Istanbul, Turkey

**Objectives:** In myasthenia gravis (MG) 85-90% of patients have autoantibodies (Abs) to acetylcholine receptor (SP). Among seronegative patients (SN), a subgroup has Abs against muscle-specific kinase (MP). The antibody production in disease subgroups with or without complement fixing Abs is probably regulated by cytokines from T and B cells. In this study regulating cytokine production of B cells is evaluated in MG.

**Methods:** 71 MG (31 SP, 19 MP and 21 SN, F/M: 49/22), 20 rheumatoid arthritis (RA) (F/M: 17/3) patients and 27 healthy controls (HC) (F/M: 13/14) were included. Isolated B cells from peripheral blood were cultured with CD40L-transfectants and stimulated with polyclonal immunoglobulin (poly-Ig), CpG or non-CpG and IL-10, IL-6, IL-12p40, TNF-alpha and TNF-beta levels were measured in culture supernatants.

**Results:** Spontaneous IL-10 production levels were lower in MG (17.6 pg/ml,  $p=0.01$ ) and RA (12.9 pg/ml,  $p=0.02$ ) compared to HCs (38.8 pg/ml). SN (17.7 pg/ml,  $p=0.04$ ) and MPs (23.1 pg/ml,  $p=0.04$ ) also had lower IL-10 levels. In poly-Ig stimulation, B cells secreted lower levels of IL-10 in MG (113 pg/ml) in SN (105.8 pg/ml,  $p=0.01$ ) as well as in MPs (111.6 pg/ml,  $p=0.02$ ), as compare to HCs (140.7 pg/ml). CpG stimulated IL-10 secretion was also lower in MG (151.3 pg/ml,  $p=0.05$ ) or SP group (124.6 pg/ml,  $p=0.045$ ) than in HCs (235.1 pg/ml). High levels of spontaneous IL-6 production were detected in MG (14.0 ng/ml), in RA patients (10.2 ng/ml) and HCs (20.8 ng/ml), with a lower IL-6 level in RA only ( $p=0.008$ ). When stimulated with poly-Ig, B cells produced less IL-6 in MG (25.9 ng/ml,  $p=0.015$ ) compared to HCs (38.3 ng/ml). Poly-Ig stimulated TNF-alpha secretion was also lower in MG than HC (57.7 vs. 94.5 pg/ml,  $p=0.026$ ). However among the subgroups only, TNF-beta was induced at higher levels in SN (2214.1 pg/ml) than SP (1044.1 pg/ml,  $p=0.003$ ) and MP (1489.4 pg/ml,  $p=0.03$ ) with poly-Ig, and higher level (3045.1 pg/ml) in SN than SP (1876.5 pg/ml,  $p=0.02$ ) with CpG induction of B cells.

**Conclusion:** These results may implicate differential cytokine activities of B cells against non-specific stimulations in MG which may be related to differences in antibody production. This study is supported by TUBITAK.

#### PC18/52 EFFECT OF IFN- $\beta$ THERAPY ON THE FREQUENCY AND FUNCTION OF CD4<sup>+</sup>CD25<sup>+</sup> REGULATORY T CELLS AND FOXP3 GENE EXPRESSION IN RELAPSING- REMITTING MULTIPLE SCLEROSIS (RR-MS)

A. Namdar<sup>1</sup>, B. Nikbin<sup>1</sup>, M. Ghabai<sup>2</sup>, A. Bayati<sup>2</sup>, M. Izad<sup>1</sup>

<sup>1</sup>Tehran University of Medical Sciences, Faculty of Medicine, Immunology, Tehran, Iran, Islamic Republic of, <sup>2</sup>Iranian Center of Neurological Research, Imam Khomeini Hospital, Tehran, Iran, Islamic Republic of

Interferon- $\beta$  (IFN- $\beta$ ) is an immunomodulatory drug of choice to control relapsing-remitting multiple sclerosis (RR-MS), although its function is still unclear. A reduced suppressive function of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells (Treg) has been shown in RR-MS patients. In this study, to understand the effect of IFN- $\beta$  on CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells, we analyzed the frequency and function of these cells and Foxp3 gene expression before and after treatment.

We evaluated the frequency and function of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells by flowcytometry and co-culture inhibition test respectively and gene expression of Foxp3 by real-time PCR in longitudinal follow-up study in 18 relapsing-remitting MS patients.

Our data revealed that IFN- $\beta$  significantly improved frequency and suppressive function of Treg ( $P < 0.05$ ) but not gene expression of Foxp3 after 6 months. Our results suggest that IFN- $\beta$  therapy could restore function of regulatory T cells and control autoimmune reactions in RR-MS patients.

#### PC18/53 QUANTITATIVE ANALYSIS OF DIFFERENTIALLY EXPRESSED BRAIN PROTEINS IN AN ANIMAL MODEL OF MULTIPLE SCLEROSIS

R. Daniels<sup>1</sup>, A. Vanheul<sup>1</sup>, K. Baeten<sup>1</sup>, J. Hendriks<sup>1</sup>, D. Dumont<sup>1</sup>, J. Robben<sup>2</sup>, J.-P. Noben<sup>1</sup>, P. Stinissen<sup>1</sup>, N. Hellings<sup>1</sup>

<sup>1</sup>Hasselt University, Biomedical Research Institute and Transnational University Limburg, School of Life Sciences, Diepenbeek, Belgium, <sup>2</sup>K.U. Leuven Biochemistry, Molecular and Structural Biology, Leuven, Belgium

**Objectives:** Multiple sclerosis is a chronic inflammatory autoimmune disease of the central nervous system. The exact cause and working mechanism of the disease is not known yet, which makes specific treatment difficult. The aim of this study is to identify disease-related proteins that help us unravel the fundamental disease processes. To identify differentially expressed proteins in the brain stem of EAE-animals and control rats in different stages of the disease, a quantitative study by two-dimensional difference in-gel electrophoresis (2D-DIGE) was performed.

**Methods:** Three rats were CFA injected controls, the other 9 rats were immunized with MBP to induce acute EAE. The EAE animals were divided into three groups, representing onset, top and recovery of the disease (3 animals in each group). 'Blood-free' brain stems of all 12 Lewis-rats were collected after perfusion with PBS. Detergent-soluble brain protein extracts were used for the 2D-DIGE analysis. Differential proteins were selected and thereafter identified using mass spectrometry.

**Results:** We found 36 differentially expressed protein spots over the four conditions (control, onset, top and recovery) which are at least 1.5 fold regulated (1-ANOVA  $\leq 0.01$ ). With these proteins, we were able to discriminate between early and late groups (control and onset samples versus top and recovery samples). Moreover, a set of the 16 most discriminating proteins was selected from the discriminant analysis. This classifier was able to discriminate all four groups. Partition cluster analysis makes it possible to study proteins that are regulated in the same way. Apart from commonly identified differentially expressed proteins, also known brain markers and new candidates or interesting pathways were identified to be differentially expressed. Currently, a number of proteins are further studied to unravel their role in the disease process.

**Conclusion:** Quantitative analysis of differentially expressed brain proteins in an animal model of Multiple sclerosis was the first successful step to identify interesting disease-related proteins that may be targets for further fundamental research.

#### PC18/54 SUPPRESSION OF EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS BY NEUROSTEROID DERIVATIVES

M. Aggelakopoulou<sup>1</sup>, M. Semitekolou<sup>1</sup>, E. Kourepini<sup>1</sup>, A. Gravanis<sup>2</sup>, V. Panoutsakopoulou<sup>1</sup>

<sup>1</sup>Biomedical Research Foundation of the Academy of Athens, Cellular Immunology, Athens, Greece, <sup>2</sup>School of Medicine-University of Crete, Pharmacology, Heraklion, Greece

The neurosteroid dehydroepiandrosterone (DHEA) is the most abundant steroid in humans and is synthesized by neurons and glia in the central nervous system (CNS). Increasing evidence indicates that DHEA, apart from its neuroprotective actions, has potent immunoregulatory functions. Multiple sclerosis (MS) is an organ-specific autoimmune disease which results from infiltration of the CNS by destructive autoreactive T lymphocytes. Here, we investigated whether three different spiro-derivatives of DHEA, which do not metabolize to androgens and estrogens (BNN93, BNN124 and BNN50), as well as DHEA, were protective against experimental autoimmune encephalomyelitis (EAE), the mouse model for MS. We induced acute EAE, in C57BL/6 mice immunizing them against the pathogenic myelin oligodendrocyte glycoprotein (MOG) peptide (amino acids 35-55). We administered the spiro-analogs or DHEA or PBS daily, from the day of EAE induction, until day 26, when mice were sacrificed. Administration of DHEA and the spiro-analogs conferred significant protection against EAE and contributed to decreased clinical score and incidence, delayed disease onset and decreased inflammation in the CNS. Lymph node cells from mice treated with DHEA and all spiro-analogs showed substantially decreased proliferation to MOG<sub>35-55</sub> peptide and produced significantly increased amounts of IFN- $\gamma$  and IL-10, which are considered to have regulatory properties. Notably, the IFN- $\gamma$ /IL-17 ratio was very high. Furthermore, when we cultured isolated CD4<sup>+</sup> T cells with specific antigen or via their TCR with  $\alpha$ -CD3/ $\alpha$ -CD28, we noticed significantly decreased proliferation and secretion of IL-2 and IFN- $\gamma$  in the DHEA- and the spiro-analogs-treated cells, demonstrating that they directly suppress T cell responses. The decreased proliferative response of CD4<sup>+</sup> T cells was not due to increased cell death, as measured by FACS analysis. Collectively, our data demonstrate that DHEA and the spiro-analogs BNN93, BNN124 and BNN50 protect from MOG peptide-induced EAE and suppress T cell responses *ex vivo*, indicating a broad immunosuppressive effect.

**PC18/55 MULTIPLE SCLEROSIS RELAPSES: CORRELATION BETWEEN PSYCHOSOCIAL STRESS AND CYTOKINES**R. Thomaz<sup>1</sup>, A. L. L. Bachi<sup>2</sup>, R. R. Novaes e Brito<sup>2</sup>, M. Vaisberg<sup>2</sup>, C. Tilbery<sup>2</sup><sup>1</sup>Santa Casa de São Paulo, CATEM – Centro de Atendimento e Tratamento da Esclerose Múltipla, São Paulo, Brazil, <sup>2</sup>Universidade Federal de São Paulo – UNIFESP, São Paulo, Brazil**Objective:** The aim of the study was to evaluate the correlations between multiple sclerosis relapses severity, psychosocial stress and sera levels of pro and anti-inflammatory cytokines.**Methods:** Were collected peripheral venous blood of 11 patients during relapses, 10 control patients (without relapses at the same moment) and 11 normal subjects and we obtained sera to compare the systemic cytokines concentration in these groups. The patients during relapses were classified in mild, moderate and severe relapses according to Neurologic Rating Scale and Expanded Disability Status Scale. All subjects were submitted to neuropsychological assessment to stress, considering stress from vital events, coping and analyzed by Holmes and Rahe Scale.**Results:** The preliminary results demonstrate that pro-inflammatory cytokines levels, such as interleukin-6 [258.2±151.1pg/mL, relapses group, 112.77±37.86pg/mL, control group and 61.81±21.28pg/mL, normal group], interleukin-12p70 [15.02±10.15pg/mL, relapses group, 6.87±5.74pg/mL, control group and 2.46±1.31pg/mL, normal group] and interleukin-1 beta [137.67±47.63pg/mL, relapses group, 27.2±8.8pg/mL, control group and 39.8±21.25pg/mL, normal group], but not tumor necrosis factor – alpha [236.44±40.42pg/mL, relapses group, 330.1±3.1pg/mL, control group and 274.5±23.9pg/mL, normal group] were increased in patients during relapses group in relation of control and normal cohorts.**Conclusion:** Apparently, our preceding results showed that higher levels of pro inflammatory cytokines are related with relapses in comparison with control patients (without relapses at same moment) or normal subjects.**PC18/56 ARTIFICIAL ENZYMES AND CATALYTIC VACCINES**A. Gabibov<sup>1</sup><sup>1</sup>Shemyakin & Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Biocatalysis, Moscow, Russian Federation

Discovery of catalytic antibodies (abzymes) was a revolutionary event that created new junctions between chemistry, biochemistry, immunology and pathology. Creation of abzymes as a new class of biocatalysts is based upon the intrinsic properties of immunoglobulin superfamily to produce complementary “molecular imprints” using the hypervariability of CDRs. These “catalytic imprints” could be made from the stable chemical analogs of transition state of the enzyme reaction or on the basis of the immunological network hypothesis. The last approach allowed us to create abzymes with acetylcholinesterase and protease activities. This method stimulated our attempts to make antibody-like acceptors for phosphorus-based poisons. Recombinant antibodies with such functions were obtained recently in this lab using chemical selection of “naïve” phage-display library. Another advantage of abzyme field is the opportunity to make “catalytic vaccines”. One of the targets for the novel therapeutic approach may be viral envelope protein gp120. The specific cleavage of this protein by abzymes can lead to the dramatic changes in the immune response toward virus and decrease binding of HIV to CD4 receptor.

A novel approach for creating catalytic antibodies against pathogens involves utilizing the autoimmune disorder of SJL mice induced by myelin basic protein (MBP) as a background for raising a protein-specific catalytic response toward gp120. Site-specific abzyme-mediated cleavage of gp120 is demonstrated. This approach developed in this laboratory can be considered as a general strategy to obtain a catalytic vaccine to proteins of interest.

We firstly showed that catalytic antibody formation has the strong intrinsic and still enigmatic links with autoimmune diseases. The existence of DNA-specific abzymes in scleroderma, SLE, rheumatoid arthritis and AIDS was described in this laboratory. Very recently the input of abzyme activity in neurodegeneration process was demonstrated. AutoAb to MBP from humans with multiple sclerosis (MS) and SJL mice with EAE exhibited site-specific antigen degradation. AutoAb from patients with the secondary progressive MS and highest scores on the expanded disability status scale demonstrated augmented catalysis. An established MS therapeutic Copaxone® inhibited reaction *in vitro*. AutoAb catalysis thus appears to be a specific feature associated with MS pathogenesis and potential marker of disease progression.**PC18/57 ROLE OF REGULATORY T CELLS IN A SPONTANEOUS EAE MOUSE MODEL**M. Koutoulos<sup>1</sup>, M. Mues<sup>1</sup>, G. Krishnamoorthy<sup>1</sup>, H. Wekerle<sup>1</sup><sup>1</sup>Max Planck Institute of Neurobiology, Neuroimmunology, Martinsried, Germany**Background and objectives:** The importance of T<sub>reg</sub> cells has been extensively studied in actively induced EAE models. In this study, we investigated the functional role of T<sub>reg</sub> cells in a spontaneous opticospinal EAE mouse model, which develops following interactions between MOG specific T and B cells.**Methods:** We used a double-transgenic (TCR<sup>MOG</sup> × IgH<sup>MOG</sup>) mouse strain, which spontaneously develops opticospinal EAE (“OSE” mouse) at high frequency. We bred “OSE” mice a) with a transgenic strain expressing a diphtheria toxin (DTx) receptor-eGFP fusion protein (DEREG mice) in T<sub>reg</sub> cells, allowing selective depletion of T<sub>reg</sub> cells by DTx injection, and b) with a fluorescent T<sub>reg</sub> reporter mouse (Foxp3-GFP.KI) which expresses eGFP under the control of Foxp3 promoter to track T<sub>reg</sub> cells and their interaction with other immune cells *in vivo*.**Results:** First, we monitored whether the frequency of GFP<sup>+</sup> T<sub>reg</sub> cells changes during the spontaneous EAE by FACS. We found that in the peripheral immune system the frequency of MOG-specific T<sub>reg</sub> cells remained stable irrespective of the disease status. However, strikingly, FACS analyses indicated that numerous T<sub>reg</sub> cells accumulated in the CNS infiltrates, with numbers rising over time. At peak of the disease and later they equaled the numbers of effector T cells. These findings were confirmed by immunocytochemistry and imaging of “acute” spinal cord slices. Treatment of T<sub>reg</sub> cells in OSE × DEREG mice with DTx led to profound depletion of T<sub>reg</sub> cells, but unexpectedly did not affect the incidence of spontaneous EAE.**Conclusion:** We show that, in a spontaneous EAE model, the frequency of MOG-specific T<sub>reg</sub> cells remained stable in periphery but increased in the CNS, indicating a possible role of these cells within the target tissue. However, depletion of the T<sub>reg</sub> cells before the onset of the disease does not affect the disease incidence, suggesting a limited role of the T<sub>reg</sub> cells in spontaneous EAE.**PC18/58 GENE EXPRESSION AND SERUMS LEVELS OF IFN $\gamma$ , IL-6 AND EXPRESSION OF FOXP3 IN MULTIPLE SCLEROSIS PATIENTS BEFORE AND AFTER STEM CELL THERAPY**M. Mohajeri<sup>1</sup>, M. Mohyeddin Bonab<sup>2</sup>, A. Farazmand<sup>1</sup><sup>1</sup>Tehran University, Department of Cell and Molecular Biology, School of Biology, Faculty of Science, Tehran, Iran, Islamic Republic of, <sup>2</sup>Tehran University of Medical Sciences, Immunology, Tehran, Iran, Islamic Republic of**Objectives:** Multiple sclerosis (MS) is a neurodegenerative disease characterized by inflammation of the central nervous system (CNS) causing loss of feeling, vision problems, fatigue and weakness, gradually leading to complete disability. Despite the poor knowledge of pathogenesis of MS, there is a common hypothesis that autoimmune mechanisms through T and B cells, Macrophages and their secreted cytokines are causative factors in MS progression. Inflammatory cytokines cause inflammation and result in exacerbation. Recently many researchers focus on Stem Cell (SC) as therapeutic method in MS.**Methods:** The effects of SC therapy in MS patients was assessed by measuring the serum levels of IFN- $\gamma$  and IL-6 cytokines and the expression of their genes plus FOXP3 among 7 patients before treatment and after 1, 3 and 6 months. The real time PCR was the method of choice for expression study and the ELISA method for measuring cytokines serum levels.**Results:** The results show that in most cases expression of IL-6 and FOXP3 genes increased following injection.But the expression of INF- $\gamma$  remains almost unchanged, Serum levels of both cytokines increased slightly after injection. Data were analyzed by the classic Nonparametric Wilcoxon test.**Conclusion:** Our data show that SC therapy affects on FOXP3 expression as a modulator of immune responses by increasing its expression in all patients. As for IL-6, stem cells seem to promote its immunomodulatory role. However, IFN- $\gamma$  data does not seem to change considerably in these cases and may require longer follow up.

Key Words: Multiple Sclerosis, Stem Cell, Cytokine, Real Time PCR, ELISA

**PC18/59 PRION PROTEIN OLIGOMERS AND C1Q RECOGNITION**P. Erlich<sup>1,2</sup>, C. Dumestre-Perard<sup>1,2,3</sup>, L. Wai Li<sup>2,4,5</sup>, C. Lemaire-Vieille<sup>1,2</sup>, G. Arlaud<sup>2,4,5</sup>, J. Gagnon<sup>1,2</sup>, J.-Y. Cesbron<sup>1,2,3</sup><sup>1</sup>Laboratoire Adaptation et Pathogénie des Micro-organismes CNRS UMR 5163, Grenoble, France, <sup>2</sup>Université Joseph Fourier, Grenoble, France, <sup>3</sup>Laboratoire d'Immunologie CHU Grenoble, Grenoble, France, <sup>4</sup>Institut de Biologie Structurale Jean-Pierre Ebel UMR CNRS 5075, Grenoble, France, <sup>5</sup>Commissariat à l'Energie Atomique, Grenoble, FranceTransmissible Spongiform Encephalopathies (TSEs) are neurodegenerative diseases are characterized by accumulation in the central nervous system (CNS) of the misfolded, partially protease-resistant prion protein (PrP<sup>Sc</sup>). The widely supported protein-only hypothesis stipulates that the transmitted agent can replicate and propagate by converting the natively folded prion protein (PrP<sup>C</sup>). Results recently published suggest that the minimal “infectious” particles able to initiate TSE disease that are non-fibrillar aggregates, with molecular masses equivalent to 14–28 PrP molecules. To elucidate the conversion mechanism, we have used a model where *in vitro* formed soluble oligomers can acquire PrP<sup>Sc</sup>-like characteristics such as ability to form soluble beta-sheet rich oligomers (approximately 300 kDa) and partial resistance to proteolysis.The C1q component of the innate immune complement system has been described to be involved in the pathogenesis of TSEs, since C1q deficient mice display partial resistance to prions after peripheral infection. We have shown that C1q can bind *in vitro* recombinant PrP, but only when it is converted into soluble oligomers. Moreover, this recognition has a functional relevance because it triggers the activation of the classical cascade of the complement (Dumestre-Perard et al, Cell Microbiol, 2007, 9:2870). Here, we describe how C1q could participate in the aggregation process of PrP. This interaction leads to the formation of a stable complex

with the globular region of C1q. We have investigated the structural and biochemical features of this interaction by thioflavin T fluorescence, size exclusion chromatography, electron microscopy and complement activation assays.

Our data underline the concept that PrP-C1q complex could be involved in local inflammation and subsequent recruitment of the immune cells that prion initially infects.

#### PC18/60 CELL-MEDIATED IMMUNITY IN THE MANAGEMENT OF PRION DISEASES

S. Iken<sup>1</sup>, V. Bachy<sup>1</sup>, P. Gourdain<sup>1</sup>, C. Carnaud<sup>1</sup>

<sup>1</sup>INSERM U938 St Antoine Research Center, Immune Systeme and Conformational Disease Laboratory, Paris, France

Main obstacles to preventive (in animals) or curative (in humans) immunotherapy against TSE are i) a strong immune tolerance to PrP, ii) a lack of clear definition of the effectors that are most effective (humoral, cell-mediated, innate) and iii) the risk of generating adverse autoimmune responses. To study those issues, we have developed adoptive transfer models, whereby lymphocytes primed against PrP under optimal conditions (i.e. in PrP-null mice) are transferred into compatible scrapie-sensitive mice further infected.

A first series of data shows that primed CD4+ T cells transferred into PrP+ wt hosts are not deleted nor anergized by surrounding expression of PrP. Furthermore, they delay extraneural and neural invasion by prions, provided that they are iteratively recalled with antigen. This happens in the absence of evident T-B cooperation and Ab production (data in press).

To increase the resolution of our models, we have generated a Tg mouse expressing the b-chain of a TCR directed against a dominant T cell epitope of murine PrP. The result is a mouse with a strongly biased, but nevertheless full blown T cell repertoire. Cell-mediated responses against PrP are 10 to 100-fold higher than in conventional PrP-null mice and can be demonstrated both in Tg mice with a PrP-null or a PrP+ background, provided the latter are depleted of their Treg. A first adoptive transfer of T cells from PrP-null Tg mice into wt infected recipients has been launched. It will be completed and discussed at the meeting. Other experiments are on their way, including *in vivo* deletion of Treg cells in Tg PrP+ mice in order to evidence putative autoimmune manifestations and transfer of polarized T cells from PrP-null Tg donors, to define the most effective and less harmful T-helper profile.

#### PC18/61 PRION PROTEIN PEPTIDES AS VACCINES

T. Vranac<sup>1</sup>, M. Ghielmetti<sup>1</sup>, V. Čurin Šerbec<sup>1</sup>

<sup>1</sup>Blood Transfusion Centre of Slovenia, Department for the Production of Diagnostic Reagents and Research, Ljubljana, Slovenia

**Objectives:** The misfolded isoform of the cellular prion protein (PrP<sup>C</sup>), designated as PrP<sup>Sc</sup> (scrapie), is the sole cause of the inevitably lethal, transmissible spongiform encephalopathies (TSEs). Investigations into the immunogenicity of the prion protein are ongoing. To combat its pathological isoform without affecting the role of cellular protein is a challenge in prion research. Immunization of animals with prion protein peptides was a frequently used method that successfully evoked humoral and/or cellular anti-PrP immunity. For the first time these data have been summarised in our comparative study<sup>1</sup> with the aim to determine the most immunogenic parts of the prion protein.

**Results:** Our study showed that immunogenic PrP peptides that elicit antibodies that cross-react with the whole PrP arise from the central and C-terminal parts of PrP. More precisely, they tend to fall into one of the three amino acid clusters: 90-120, 140-170 or 200-230. These regions correspond in general to the hydrophilic parts of PrP, while a very hydrophilic part located in the N-terminus (amino acids 20-50) does not appear to be immunogenic. Interestingly, T-cell epitopes that should not be influenced by hydrophilicity are also located mostly in the central and C-terminal parts of PrP. A possible explanation for the low immunogenicity of the N-terminus is a better digestion and presentation of this highly mobile part during lymphocyte development, which results in more complete immune tolerance.

**Conclusions:** We conclude that active vaccination with PrP could offer a solution for the currently incurable TSEs. It is important, however, that the peptides are carefully chosen on the basis of their immunogenicity as well as their ability to mimic PrP<sup>Sc</sup> conformational epitopes. The application of various epitopes represented by a mixture of PrP peptides or by a multivalent vaccine should also be considered in future studies.

<sup>1</sup> Ghielmetti M., Vranac T., Čurin Šerbec V. Prion protein peptides as vaccines. *Mini Rev Med Chem.*, 2009, in press.

#### PC18/62 FROM PRIONS TO PRIONIC DISEASES

G. Catalui<sup>1</sup>

<sup>1</sup>Titu Maiorescu University, Faculty of Medicine, Bucharest, Romania

A prion is a protein that is capable of self replication, thereby altering a cell's metabolism. The prion was defined as an infectious pathogen that requires a protein for infectivity yet is highly resistant to procedures that modify or destroy nucleic acids. The physiologic roles attributed to cellular prion protein (PrP) are rather disparate and include:

- a) function as a membrane receptor;
- b) regulator of apoptosis;
- c) carrier or binding protein for copper ions;
- d) effectors in signal transduction mechanisms;
- e) regulator of synaptic transmission; and
- f) transcription factor.

Prions differ from bacteria, viruses, and viroids by their new structure and properties. PrP is normally expressed at the cell surface. The level of expression of PrP is known to influence susceptibility to infection. Prion diseases are transmissible neurodegenerative conditions characterized by the accumulation of protease-resistant forms of the PrP, termed PrP<sup>res</sup>, in the brain. Insoluble PrP<sup>res</sup> tends to aggregate into amyloid fibrils. These diseases are characterized by the accumulation of large amounts of protease-resistant aggregates of an altered isoform of the prion protein. Up to very recently amyloid formation was considered a very slow process of deposition of an abnormal protein due to genetic abnormalities or post-translational modification of the deposited protein. These diseases have enormous variability in their incubation periods, ranging from a few months to forty years. Despite the considerable uncertainty about the mechanism of pathogenesis in prion diseases, there is a considerable demand for both diagnostic techniques and potential therapies.

### PC24 – VETERINARY IMMUNOLOGY

#### PC24/1 MECHANISMS OF SUPPRESSION BY PORCINE FOXP3<sup>+</sup> REGULATORY T CELLS

T. Käser<sup>1</sup>, W. Gerner<sup>1</sup>, A. Saalmüller<sup>1</sup>

<sup>1</sup>University of Veterinary Medicine Vienna, Institute of Immunology, Vienna, Austria

Regulatory T cells (Tregs) are potent regulators of various immune reactions and a lot of effort was put in the analysis of their maturation, phenotype, activation requirements and mechanisms of suppression. Most studies were performed in mice and humans while in other species the identification and characterisation of Tregs was impeded by the lack of applicable reagents. Characterisation of Tregs in swine is still at the beginning. Their existence was demonstrated by their Foxp3 expression, their distribution in several lymphoid tissues, and functionally by their IL-10 production and their suppressive activity *in vitro*. In this study we focussed on the analysis of their mechanisms of suppression. Therefore we studied the induction and production of a panel of cytokines (IL-2, IL-4, IL-10, IFN- $\gamma$  and TGF- $\beta$ ) and effector molecules (perforin) supposed to be involved in suppression of T-helper cell reactivity by Tregs. Additionally, contact- and IL-2-dependency of this suppression was investigated. Furthermore we analysed the suppression of proliferation of various subsets of lymphocytes during co-cultivation of Tregs and CFSE-stained PBMC by multi-colour flow cytometry. Therefore this study provides the first results about the mechanisms of Treg suppression in swine, an important large animal model for several diseases and xenotransplantation.

#### PC24/2 A NEW ORAL POLYBACTERIAL PREPARATION "NATSTIM" FOR IMMUNOPROPHYLAXIS OF NEONATAL AND POST-WEANING DIARRHEA IN PIGS

R. Y. Kostadinova-Yankova<sup>1</sup>, V. Mateva<sup>2</sup>, P. Nenkov<sup>3</sup>, B. Petrunov<sup>1</sup>, S. Marinova<sup>1</sup>

<sup>1</sup>National Center of Infectious and Parasitic Diseases /NCIPD/, Immunology and Allergology, Sofia, Bulgaria, <sup>2</sup>Agrosloviyanin Ltd., Stara Zagora, Bulgaria, <sup>3</sup>Bul Bio-NCIPD Ltd., Sofia, Bulgaria

EU veto for use of antibiotics as forage additives and growth stimulators in farm-breeding imposes the development of new alternative products for prevention and control of infections, as immunostimulators. *E. coli* infections in pigs lead to considerable damages for farming industry in global aspect. In suckling piglets the main protective factor of immunity are colostrum/milk antibodies but after weaning an active systemic and especially intestinal immunity is required. Natstim is an oral preparation composed of killed whole cells and lysates from *E. coli* and *S. aureus*, intended for use in veterinary practice to stimulate natural resistance and specific immunity against bacterial infections. In this study the effect of N on humoral systemic and mucosal immunity and on some veterinary/economic parameters in pigs was assessed. Natstim was applied as follows: in pregnant dams for 30 consecutive days before farrowing; in piglets – 40 consecutive days (from 5. day after birth). The specific antibacterial (against preparation components) and anti-LPS IgA, IgM, IgG antibodies (Abs) in serum and colostrum/milk, measured by ELISA, were evaluated before and after Natstim application. The results received showed:

1). Statistically significant increase of serum antibodies in all experimental groups compared to negative control, with production mainly of IgM and IgA (key role in mucosal protection);



2). Considerable rise of Abs in large percent of experimental animals after treatment compared to their basic levels (auto-control): anti-*E. coli* IgM – in 60%, IgA – 77%; anti-*S. aureus* IgM – 81%, IgA – 79%; Abs against purified *E. coli* LPS – IgM – 64%, IgA – 35%;

3). Production of specific mucosal, locally secreted Abs (IgA, IgM, IgG) in colostrum and milk.

At the same time a positive effect from the supplementing with Natstim on growth and health parameters was achieved: improved health status of piglets (lower morbidity/mortality and medicine cost); improved forage utilization; higher growth intensity. In conclusion our data demonstrating the stimulating effect of new veterinary immunopreparation Natstim on specific humoral systemic and mucosal immunity, as well as undoubted health and economic benefits give grounds to assert that it represents a successful alternative of nutritive antibiotics in pigs.

#### PC24/3 IMMUNOGLOBULIN LIGHT CHAIN GENE POOL IN DOMESTIC CATTLE

A. Iivanainen<sup>1</sup>

<sup>1</sup>University of Helsinki, Department of Basic Veterinary Sciences, Helsinki, Finland

**Objectives:** The foundation of the primary antibody repertoire lies in the various V, D and J immunoglobulin genes which are recombined during B lymphocyte differentiation. This study aims at extracting the bovine light chain gene pool from the genome sequence. The analysis was based on Btau-3.1 assembly of the genome and was conducted as a part of a community effort to annotate the bovine genome.

**Methods:** An iterative blast search was made against Btau-3.1 at [www.ensembl.org](http://www.ensembl.org). The extracted genes were analysed for open reading frames, splice signals and recombination signal sequences. The IMGT numbering was used for translations. The peptide sequences were further analysed for framework elements and CDR lengths. The genomic sequences corresponding to regions spanning from FR1 up to but excluding CDR3 were aligned using a global alignment strategy in the MAFFT package [NAR 30:3059-3066]. Evolutionary distances were computed and phylogenetic trees constructed using neighbour joining algorithm in PHYLIP [Cladistics 5:164-166].

**Results:** 63  $\lambda$  and 22  $\kappa$  variable genes were identified from Btau-3.1. The specified phylogenetic relationships are congruent with the established ruminant light chain variable gene families or subgroups [JI 155:3068-3078, JI 159:3093-3095, Int Immunol 10:1251-1259]. In addition, 5 novel ruminant subgroups were identified. 25 IGLV, 8 IGKV, 2 IGLJ and 2 IGKJ genes were classified as potentially functional. These were clustered in IGLV subgroup I (16 genes) and IGKV subgroup II (7 genes).

**Conclusion:** The number of potentially functional variable genes is moderate in relation to that in man or mouse. Thus, the genomic data available at this point is compatible with the expansion of the recombinational repertoire by SHM during foetal and neonatal life [Cell 80:115-25]. However, there are gaps in the assembly so the number of genes might change in the future versions of the genome sequence. Also, the heavy chain genes are mostly missing from Btau-3.1 and their contribution could not be assessed.

#### PC24/4 INNATE IMMUNE RESPONSE IN EXPERIMENTAL COAGULASE-NEGATIVE STAPHYLOCOCCAL BOVINE MASTITIS

T. Salomäki<sup>1</sup>, H. Simojoki<sup>2</sup>, J. Laakkonen<sup>1</sup>, S. Pyörälä<sup>2</sup>, A. Iivanainen<sup>1</sup>

<sup>1</sup>University of Helsinki, Faculty of Veterinary Medicine, Department of Basic Veterinary Medicine, Helsinki, Finland, <sup>2</sup>University of Helsinki, Faculty of Veterinary Medicine, Department of Production Animal Medicine, Saarentaus, Finland

**Objectives:** Mastitis in dairy cows causes great economical losses world wide. The role of coagulase-negative *Staphylococcus* (CNS) strains in bovine mastitis has expanded during past years. While the number of mastitis caused by CNS strains has increased being in Finland 24% of subclinical and 18% of clinical cases the host responses in CNS mastitis are not well defined. Pro-inflammatory cytokines are central signalling molecules in infections initiating a cascade of innate immunity events. Their kinetics reflects the disease severity and host's innate immune response. Our aim was to measure kinetics of pro-inflammatory cytokines IL-1 $\beta$ , TNF- $\alpha$  and IL-8 in experimental CNS mastitis.

**Methods:** 8 cows were infected with *S. simulans* and *S. epidermidis*. Milk samples from control and infected quarters, bacteriological and clinical data were collected between day -7 and +14. The concentrations of IL-1 $\beta$ , TNF- $\alpha$  and IL-8 were determined by ELISA. The animal experiments were approved by the local animal welfare authorities.

**Results:** Viable bacteria were recovered from all infected quarters. Our preliminary results indicate that cytokine levels (IL-1 $\beta$ , TNF- $\alpha$  and IL-8) in milk were typically slightly increased in infected quarters after 12 hours and returned to the background levels by 45 hours while the cytokine levels of control quarters remained unsubstantial.

**Conclusions:** Milk IL-8 concentration was significantly lower than reported in coliform, *S. aureus* or streptococcal mastitis whereas IL-1 $\beta$  and TNF- $\alpha$  concentrations were at the same level. These elevated cytokine levels indicate an active innate response. The differences in cytokine profiles reflect the differential host responses to the various microbes and could explain some variations in the clinical signs of the respective infections.

#### PC24/5 CYTOKINE EXPRESSION AND THE CELLULAR STRESS RESPONSE AFTER ACUPUNCTURE TREATMENT IN COWS WITH LEFT ABOMASAL DISPLACEMENT

C. N. Weber<sup>1</sup>, K. Freudenberg<sup>2</sup>, K. Doll<sup>2</sup>, K. E. Mueller<sup>1</sup>

<sup>1</sup>Freie Universität Berlin, Clinic for Ruminants and Pigs, Berlin, Germany, <sup>2</sup>Justus-Liebig-Universität Giessen, Clinic for Ruminants and Pigs, Giessen, Germany

**Objectives of study:** The effects of acupuncture on the immune response are described extensively in rats and human beings. Accordingly, acupuncture modulates the secretion and reduces the mRNA expression of pro-inflammatory cytokines as well as promotes the activity of natural killer cells by increased level of Interferon- $\gamma$  (IFN- $\gamma$ ), Interleukin-2 (IL-2) and Interleukin-12 (IL-12) in the blood serum. At the same time acupuncture is often connected with a decrease of the body temperature in patients with fever. In chronic allergies acupuncture can reduce the IL-10 level in the plasma. A continuous treatment with acupuncture is supposed to improve an existing immunosuppression after trauma or surgery, significantly. An effect of acupuncture on the cellular stress system, however, has been rarely examined so far, but is believed to increase the expression of inducible Heat Shock proteins and to prolong their presence in the cell. In the presented study expressions of selected cytokines and the cellular stress response as represented by the inducible Heat Shock protein Hsp70 in cows with left abomasal displacement after corrective surgery with and without acupuncture are compared.

**Materials and methods:** 28 cows with left abomasal displacement underwent corrective surgery at the Clinic for Ruminants in Giessen. Of these 14 cows were subjected to acupuncture stimulation of bowel motility after surgery twice. Samples of venous blood were drawn from each cow before and after the acupuncture treatment using PaxGene Blood RNA tubes (Becton Dickinson). Total RNA was isolated from the blood samples (PaxGene Blood RNA kit, Qiagen) and transcribed into cDNA (StrataScript First-Strand Synthesis System, Stratagene). Different expression of Hsp70 and IL-1 $\beta$ , IL-6, IL-10, INF- $\gamma$  and TNF- $\alpha$  were calculated against house-keeping genes using Real Time-PCR (Brilliant SYBR Green QPCR Master Mix, Stratagene).

**Results:** Acupuncture significantly reduced the mRNA expression of pro-inflammatory cytokines Interleukin-6 (IL-6) and Interleukin-1 $\beta$  (IL-1 $\beta$ ). We also found increased levels of Interferon- $\gamma$  (IFN- $\gamma$ ) and Interleukin-2 (IL-2) mRNA. Comparison between acupuncture-treated and non-treated cows also showed a slight up-regulation of Hsp70 in acupuncture-treated cows.

**Conclusions:** Use of acupuncture supporting surgery could therefore improve the recovery of the cows and thus prevent the occurrence of post-surgical infections.

#### PC24/6 MAMMARY GLAND INFLAMMATORY RESPONSE IN MICE FED WITH MILK FROM *STREPTOCOCCUS AGALACTIAE*-INDUCED MASTITIS DAMS

G. Trigo<sup>1</sup>, R. Gil da Costa<sup>1</sup>, M. Dinis<sup>1</sup>, A. França<sup>1</sup>, P. Ferreira<sup>1</sup>, D. Tavares<sup>1</sup>

<sup>1</sup>Instituto de Ciências Biomédicas de Abel Salazar, Porto, Portugal

*Streptococcus agalactiae* is a well known major contagious pathogen causing bovine subclinical. In this study, we characterized the effects of maternal *S. agalactiae* mastitis on their offspring's inflammatory response upon mastitis induction with the bacteria in the adult age. As control, the inflammatory response was evaluated in mice breastfed with milk from non-infected dams. The results of this study demonstrate that animals fed with infected milk developed a much more severe mastitis with an inflammatory vascular congestion and interstitial oedema than those fed with non-infected milk, which showed only moderate and transient congestion and oedema. In addition, animals fed with infected milk also presented much larger necrotic, purulent areas that affected whole lobes of the gland and extended to the adjacent dermis, hence risking of cutaneous ulceration. Moreover, these animals showed a reduction on the number of *Streptococcus agalactiae* in mammary glands which was accompanied by a more severe mastitis when compared with control animals. The severity of the inflammatory response and the reduction of bacterial colonization observed in mice fed with infected milk can, at least partly, be explained by increased tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ) and IL-6 levels in mammary glands, which most likely triggered the imposing vascular changes and mammary necrosis observed in these animals.

This results suggest that feeding of animals with infected milk has a significant impact on their immune response to *S. agalactiae* infection on adult age.

#### PC24/7 MOLECULAR IDENTIFICATION OF B HAPLOTYPE IN IRANIAN ENDOGENOUS CHICKEN

N. Barjesteh<sup>1</sup>, G. Nikbakht Brujeni<sup>1</sup>, A. Esmailnezhad<sup>1</sup>

<sup>1</sup>University of Tehran, Faculty of Veterinary Medicine, Microbiology & Immunology, Tehran, Iran, Islamic Republic of

There has been growing interest in genetic markers suitable for drawing population genetic interferences about immune response. Microsatellite is one of these genetic markers. The use of microsatellites is a standard technique for molecular genetics evaluation and mapping of chickens. The Microsatellite marker LEI0258 maps to chromosome 16 where MHC genes were located. In addition, marker MCW0371 is located in this chromosome 10,560 bp downstream of LEI0258 marker. It is investigated that these markers are closed to the MHC genes, they are genetic indicator for the MHC haplotype.

In this study 141 Blood samples belong to Iranian endogenous in Iran were analyzed (85 parents and 56 F1). After the extraction of DNA, Polymerase chain reaction (PCR) for LEI0258 marker was carried out. PCR products were separated on a 4% agarose gel. Because of similarity between some sizes in the first marker, second marker was used.

Finally, 10 blood group in this population were detected (B74, B1, B6, B5, B14, B72, B13, B18, B12, B4). B74 has highest frequency (21.63%) and B4 has lowest frequency (0.35%).

From this study it was concluded that polymorphism exists within Iranian endogenous chicken that might be useful for selecting desirable immunological traits.

#### PC24/8 MYCOBACTERIUM BOVIS INFECTION IN CATTLE INDUCE DIFFERENTIAL EXPRESSION OF PRLR ISOFORMS

A. L. Pereira<sup>1</sup>, G. López-Rincón<sup>2</sup>, F. Díaz<sup>3</sup>, C. Arriaga<sup>3</sup>, J. Gutiérrez Pabello<sup>4</sup>, A. Corbacho<sup>5</sup>, A. Daneri<sup>6</sup>, J. Ramírez<sup>2</sup>, C. Estrada<sup>2</sup>

<sup>1</sup>Universidad de Guadalajara, Fisiología, Guadalajara, Mexico, <sup>2</sup>Ciatej, Guadalajara, Mexico, <sup>3</sup>CENID-Microbiología INIFAP, Mexico, Mexico, <sup>4</sup>UNAM, Medicina Veterinaria, Mexico, Mexico, <sup>5</sup>Univeidad de California, Davis, United States, <sup>6</sup>Universidad de Guadalajara, Fisiología, Guadalajara, Mexico

Bovine tuberculosis (bTB) caused by the intracellular pathogen *Mycobacterium bovis* is characterized to produce chronic inflammation as shown by granulomatous lesions. In order to gain information of prolactin/ prolactin receptor (PRL/PRLR) system during chronic inflammation, the expression patterns of PRLR were surveyed in tissues samples (spleen, thymus, mediastinal, tracheobronchial and retropharyngeal lymph nodes, lung, liver); in bronchioalveolar lavage cells (BAL); and in peripheral blood mononuclear cells (PBMC)-derived macrophages (MΦ). Samples were taken from both, experimentally and naturally M. bovis infected cattle, as well as, from healthy non-tuberculous controls. It was demonstrated a high expression of PRLR in M. bovis infected tissues, mRNA and protein, using RT-PCR and Western blot, respectively. Different patterns with multiple PRLR forms were observed regarding tissues: 120, 90, 75 and 55 kDa in thymus and spleen; 55 kDa in liver; 100 and 55 kDa in lung. Forms of 75, 55 and 40 kDa were the most ubiquitous found, being the 75 kDa the most common detected, except in liver or lung. Interestingly, expression of PRLR appears also be affected by the grade of infection since all lymph nodes surveyed expressed 75 kDa form, but only if they contain visible gross lesion, forms of 40 and 65 kDa were detected. The induction of PRLR expression by M. bovis in PBMC-derived MΦ was assessed in vitro. In conclusion, M. bovis infection promotes the expression of PRLR and different isoforms pattern regarding tissues in bovine, particularly in MΦ. Results suggest that heterogeneity in isoforms expression during active tuberculosis is a consequence of the pathological state in cows, since in healthy animals it was not observed. Short PRLR isoforms could modulate the chronic inflammatory responses during tuberculosis in different tissues. We propose the activation of the PRL/PRLR signaling by M. bovis could play important role for pathogenesis of bTB.

#### PC24/9 IMMUNOCORRECTION IN NEWBORN PIGLETS

N. Salyha<sup>1</sup>

<sup>1</sup>Institute of Animal Biology, Lviv, Ukraine

It is well known that the resistance of animal organism to infections depends mainly on development and functioning of immune system. The immunomodulators in dependence on dose can stimulate or inhibit the immune response system. Thus, the aim of our investigation was to select the optimal scheme and doses of thymalin for the formation of full value immune response in piglets during the early period of ontogenesis.

The study was carried out on 3 groups of piglets (n=5) of 10- to 30-days old age. At the 10- and 20-days old age the immunomodulator Thymalin was administered during 3 days with the administration exposition of 7 days correspondingly in doses: to animals of experimental group 1 (E 1) – 1 and 2 mg, experimental group 2 (E 2) – 2 and 4 mg. To control group (C) the correspondent volume of 0.9% NaCl was administered.

The obtained results show that the use of different doses of thymalin is associated with the changes of separate populations of T- and B-system of immunity of piglets. So the administration of thymalin in dose 1 and 2 mg/day during 3 days promotes the reliable (p< 0.5) in experimental group 1 and (p< 0.001) in experimental group 2 increase of antigen-binding B-lymphocytes under simultaneous decrease of total T-lymphocytes and T-helpers at the day 20 of life. The analysis of obtained results showed that the administration of thymalin to piglets in various doses and schemes influence differently on the activation of T- and B-cell compartment immunity. These changes may be associated with thymalin characteristics in different doses to stimulate or inhibit the processes of cell activation, proliferation and differentiation.

The data obtained suggest that after the first administration of thymalin B-lymphocytes increase in blood of investigated piglets at the day 20.

Thus, the discovered changes of interrelation of immunocompetent cells in piglets blood after the use of thymalin in different doses suggest the reasonability of usage of thymalin on the day 20 of life in dose 3 mg during 3 days for the stimulation of T-cell immunity in piglets.

#### PC24/10 INTRACELLULAR TRAFFICKING OF BRUCELLA MELITENSIS IN J774A.1 MACROPHAGES CELLS

C. García-Cabrera<sup>1</sup>, E. González-González<sup>1</sup>, M. C. Moreno-Lafont<sup>1</sup>

<sup>1</sup>Instituto Politécnico Nacional – ENCB, Inmunología, México D.F., Mexico

**Introduction:** Brucellosis is one of the major zoonosis caused by infection with *Brucella* species. In humans, *Brucella* is mainly located in tissues and organs of the reticuloendothelial system. After *Brucella* spp. infection, innate immunity is activated and to kill the bacterium, it must be internalized into phagosomes, which fuse with lysosomes. Evidence suggests that some *Brucella* species are internalized through different pathways to classic phagocytosis, whose efficiency and pathological role are unknown. It has been shown that vesicles containing an attenuated *Brucella* strain, such as *B. abortus* RB51 fuse with lysosomes, whereas vesicles containing the virulent *B. abortus* 2308 strain migrate to a niche closely related to the endoplasmic reticulum of the host cell.

**Objective:** The aim of the present work was to analyze the intracellular trafficking of *B. melitensis*, highly pathogenic for humans, within professional phagocytic cells, comparing the behavior of the *B. melitensis* 16M virulent strain with that of the attenuated rough mutant *B. melitensis* VTRM1.

**Methods:** J774A.1 murine macrophages were infected with *B. melitensis* 16M or VTRM1, previously stained with PKH26 dye. At different post infection (p.i.) time points cells were stained with specific antibodies against Rab-5, Rab-7, LAMP-2 and cathepsin D, and a secondary antibody coupled to FITC. Samples were observed under a confocal microscope.

**Results:** A higher number of macrophages were infected with the VTRM1 strain as compared with those infected with the 16M strain; both strains were internalized at least 5 min p.i. into Rab5<sup>+</sup> vesicles; thereafter bacteria were located within Rab7<sup>+</sup> vesicles. Surprisingly, live *B. melitensis* 16M-containing vesicles acquired the markers LAMP2 and cathepsin D, suggesting their fusion with lysosomes. Heat-killed *B. melitensis* 16M was internalized into Rab5<sup>+</sup> vesicles by a much larger number of macrophages than those infected with the live strain. In both infections, vesicles were fused with lysosomes.

**Conclusion:** Our results suggest that *B. melitensis* 16M do not avoid phagolysosomal fusion, as virulent *B. abortus* 2308 does, but probably it may display mechanisms of resistance to or inhibition of intracellular killing. The lesser infection caused by the virulent 16M strain seems to be a phenomenon dependent on the bacterial viability.

#### PC24/11 SUPEROXIDE DISMUTASE (SOD Cu<sup>2+</sup>/Zn<sup>2+</sup>) OF BRUCELLA ABORTUS RB51 INDUCES SPECIFIC MURINE CYTOTOXIC T CELLS

R. Flores-Mejía<sup>1</sup>, E. Galicia-Silva<sup>1</sup>, M. E. Cancino-Díaz<sup>1</sup>, R. López-Santiago<sup>1</sup>

<sup>1</sup>Instituto Politécnico Nacional – ENCB, Inmunología, México D.F., Mexico

**Introduction:** Brucellosis is an infectious disease that affects animal and humans and is caused by *Brucella* spp. mainly *B. abortus*. In order to assess immunization efficiency against *B. abortus*, several important immunogenic molecules had been analyzed in the infection model using Cu<sup>2+</sup> and Zn<sup>2+</sup> dependent superoxide dismutase (Cu<sup>2+</sup>/Zn<sup>2+</sup> SOD) from *B. abortus* RB51 vaccine strain.

**Objective:** The aim was to evaluate the cytolytic effect of cytotoxic T cells from SOD immunized mice on *B. abortus* infected macrophages.

**Methods:** Recombinant SOD (rSOD) was purified from recombinant pBAD-SOD *E. coli*, previously induced with L-arabinose. Biomass was separate by centrifugation before the chemical and mechanical lysis. A pre-purification affinity column was performed before visualization of the rSOD in SDS PAGE. rSOD purification was done by FPLC using mass molecular exclusion columns. Two groups of female six weeks age BALB/c mice were immunized with PBS as control and 75 µg rSOD (three immunization on days 0, 7 and 14 with 25 µg each). Two days after the last injection, spleen cells were obtained and T cells purified by magnetic beads were co-cultured with *B. abortus* RB51-infected J774A.1 macrophages for 5 days. Effector T cytotoxic cells were recovered and used in a lactate dehydrogenase release cytotoxic assay against J774A.1 infected macrophages with the virulent strain (2308) and RB51 vaccinated one.

**Results:** Cytotoxicity of T cells was specific against J774A.1 macrophages infected with *B. abortus* 2308 and RB51. T cells from PBS mice did not show any cytotoxicity.

**Conclusions:** Cu<sup>2+</sup>/Zn<sup>2+</sup> rSOD was capable of eliciting specific cytotoxic T cell which exerted cytolytic effect on both infected macrophages with vaccine RB51 and virulent 2308 *B. abortus* strains, in vitro.

#### PC24/12 IMMUNOLOGICAL AND GENETIC ANALYSIS OF RHD (RABBIT HAEMORRHAGIC DISEASE) VIRUS STRAINS (THE PAPER WAS SPONSORED FROM GRANT NO. N308 03832/3662)

P. Niedźwiedzka-Rystwej<sup>1</sup>, M. Pawlikowska<sup>1</sup>, B. Hukowska-Szematowicz<sup>1</sup>, B. Tokarz-Deptuła<sup>1</sup>, W. Deptuła<sup>1</sup>

<sup>1</sup>University of Szczecin, Faculty of Natural Sciences, Department of Microbiology and Immunology, Szczecin, Poland

The aim of the study is immunological analysis of 4 strains of RHD virus (BS89, Rainham, Asturias, Frankfurt) in the scope of immunological response after infection of rabbits with them and their genetic analysis on the basis of genomes fragments comparison. The immunological studies were conducted on 60 rabbits, divided into 4 infected groups (10 rabbits each) and 4 control groups (5 rabbits each). Animals from infected groups were intramuscularly injected with one of 4 strains (Italian-BS89, English-Rainham, Spanish-Asturias, German-Frankfurt) of RHD virus suspended in 1 ml of glycerol and in the same way glycerol to control animals was injected. Blood was sampled before the application of antigen – “0” hour, and then in 4,8,12,24 hour after infection and the following parameters were

estimated: non-specific cell immunity (NOK) (adherence, ingesting and cidal ability of PMN cells), non-specific humoral immunity (NOH) (activity of myeloperoxidase, concentration and activity of serum lysozyme), specific cell immunity (SOK) (percentage of T lymphocytes and subpopulations – Th, Tc/s), specific humoral immunity (SOH) (percentage of B lymphocytes and the total amount of immunoglobulins). In genetic studies, 4 sequences of examined strains of RHD virus were compared on the basis of gene coding N-terminal part of structural capsid protein VP60. The most intensive changes were registered in NOK parameters respectively in Frankfurt, Asturias, BS89, Rainham strain of RHD virus. In terms of NOH parameters, those changes were 2,5-times less intensive respectively in Frankfurt, BS89, Rainham, Asturias and 6-times less intensive in SOK indices only in strains Asturias i Rainham of RHD virus and almost 30-times less intensive in SOH parameters only in strains Frankfurt and Rainham. This causes the division of 4 strains into two immunogroups: with high immunogenicity – Asturias and Frankfurt and low immunogenicity – strain Rainham and BS89. Genetic analysis divided those strains also into two genetic groups – first: Asturias and BS89; second: Frankfurt and Rainham. Summing up, no matter the fact that examined strains form 2 immunogroups and 2 genogroups, those groups bare a different content, what suggest that genetic resemblance of those strains do not correlate with their immunogenicity.

#### PC24/13 IMMUNE RESPONSE TO THE NEMATODE *LAGOCHILASCARIS MINOR* IN BALB/C AND C57BL/6 INFECTED MICE

M. Spadafora-Ferreira<sup>1</sup>, M. F. D. S. Prudente<sup>2</sup>, P. G. Lara<sup>1</sup>, F. M. Diniz<sup>1</sup>, P. H. Papotto<sup>1</sup>, D. V. Tambourgi<sup>1</sup>, M. S. Carvalhaes<sup>2</sup>

<sup>1</sup>Butantan Institute, Laboratory of Immunohistochemistry, São Paulo, Brazil, <sup>2</sup>Institute of Tropical Pathology and Public Health, Federal University of Goiás, Department of Microbiology, Immunology, Parasitology and Pathology, Goiânia, Brazil

**Objectives.** *Lagochilascaris minor* nematode usually affects the neck region with exudative abscesses. Mice are considered intermediate hosts of the parasite. We have shown that C57BL/6 are more susceptible to *L. minor* than BALB/c mice. The aim of this study was to investigate the immune response of both strains infected with *L. minor*. We analyzed the splenocytes proliferation and production of IL-10, IFN $\gamma$ , TNF $\alpha$ , TGF $\beta$ , IL-4 and IL-5 after re-stimulation with parasite antigens.

**Methods.** Mice were orally infected with 10<sup>3</sup> eggs of *L. minor*. After 7, 35 and 250 days of infection, groups of 5 mice were sacrificed. Non-infected mice were used as control. 5  $\times$  10<sup>5</sup> splenocytes were stimulated with 5  $\mu$ g/ml of crude extract (CE) or excretory/secretory (ES) products of L3 larvae of *L. minor*. Splenocytes proliferation was measured by [<sup>3</sup>H]-thymidine incorporation after 72h of culture. Cytokine production was determined by ELISA or FACS in cell supernatants collected 48h after antigen stimulation.

**Results.** SE induced proliferation at 7 days of infection in infected and control of both mice and in C57BL/6 at 35 days. In contrast, CE antigen inhibited the proliferative response at 7 and 250 days in infected and controls of both strains. SE induced IL-10 and TNF $\alpha$  in BALB/c at 7 days of infection compared to control group. SE also induced TNF $\alpha$  in C57 mice at 250 days of infection and IL-10 in the controls. CE induced IL-10 in Balb/c infected mice at 35 days and IFN $\gamma$  in BALB/c at 250 days of infection. C57BL/6 infected mice produced TGF $\beta$  at 7 days of infection. The two antigens did not induce IL-5 or IL-4 in both mouse strains during the infection.

**Conclusion.** Our results suggest that different T cell populations seem to be associated with the response to CE and SE antigens along the infection with *L. minor*. The two strains of mice display a different profile of cytokines. The greater production of IL-10 in the initial phase of the infection in BALB/c mice may be associated to its greater resistance to the infection compared to C57BL/6.

**Financial support.** FAPESP and FAPEG, Brasil.

#### PC24/14 DIFFERENTIAL CYTOKINE EXPRESSION IN *CHLAMYDOPHILA PSITTACI* GENOTYPE A-, B- OR D- INFECTED CHICKEN MACROPHAGES AFTER EXPOSURE TO *ESCHERICHIA COLI* O2:K1 LPS

D. Vanrompay<sup>1</sup>, D. Beeckman<sup>1</sup>, L. Rothwell<sup>2</sup>, P. Kaiser<sup>2</sup>

<sup>1</sup>Ghent University, Molecular Biotechnology, Ghent, Belgium, <sup>2</sup>Institute for Animal Health, Compton, United Kingdom

**Objectives:** Both *Chlamydomphila psittaci* and avian pathogenic *Escherichia coli* infections contribute to the respiratory disease complex observed in turkeys. Secondary infection with *E. coli* exacerbates *Cp. psittaci* pathogenicity and gives higher *E. coli* excretion. Little is known, however, about the innate immune response initiated by both pathogens in their avian host. The objective of this study was to determine the cytokine responses induced following a *Cp. psittaci* infection and *E. coli* superinfection of avian monocytes/macrophages by examining gene transcripts of IL-1 $\beta$ , IL-6, CXCLi2 (IL-8), CXCLi1 (K60), IL-10, IL-12 $\alpha$ , IL-12 $\beta$ , IL-18, TGF- $\beta$ 4 and CCLi2.

**Methods:** Corresponding mRNA levels were detected using qRT-PCR at 4 h post-inoculation (p.i.) with different *Cp. psittaci* strains or 4 h post-treatment with avian *E. coli* LPS of *Cp. psittaci* pre-infected HD11 cells. *Cp. psittaci* strains used were 84/55 and 92/1293 (both highly virulent), CP3 (low virulent) and 84/2334 (phylogenetic intermediate strain between *Cp. psittaci* and *Cp. abortus*).

**Results and conclusion:** At 4 h post *Cp. psittaci* infection, an increased expression of the pro-inflammatory cytokines IL-1 $\beta$  and IL-6 as well as the inflammatory chemokines IL-8, K60 and CCLi2 was observed compared to levels in uninfected HD11 controls. This effect was less pronounced for the milder CP3 strain. The pro-inflammatory response of *Cp. psittaci* infected cells to *E. coli* LPS was significantly lowered compared to the mock-infected controls, especially when the cells were pre-infected with highly virulent *Cp. psittaci* strains. In both experiments, exceptionally high levels of IL-10 and no TGF- $\beta$ 4 response were observed, and we propose that this could induce deactivation of macrophages and suppress the NF- $\kappa$ B pathway. As a result, pro-inflammatory and Th1-promoting responses to both the primary *Cp. psittaci* infection and *E. coli* would be inhibited, thus explaining the observed aggravated *in vivo* pathology.

#### PC24/15 EXPRESSION PROFILE OF ANGIOGENETIC FACTORS IN SPONTANEOUS UVEITIS CASES IN HORSES

J. K. Zippel<sup>1</sup>, S. M. Hauck<sup>2</sup>, B. Amann<sup>3</sup>, C. H. van der Meijden<sup>4</sup>, M. Stangassinger<sup>1</sup>, M. Ueffing<sup>5</sup>, C. A. Deeg<sup>1</sup>

<sup>1</sup>Institute of Animal Physiology, LMU Munich, Department of Veterinary Sciences, Munich, Germany, <sup>2</sup>Institute of Protein Sciences, Helmholtz Center, Neuherberg, Germany, <sup>3</sup>Institute of Animal Physiology, LMU Munich, Department of Veterinary Sciences, Munich, Germany, <sup>4</sup>IT Operation Team, Faculty of Veterinary Medicine, Munich, Germany, <sup>5</sup>Institute of Protein Sciences, Helmholtz Center, Neuherberg, Germany

**Purpose:** Equine recurrent uveitis (ERU) is an incurable autoimmune disease affecting the inner eye that leads to blindness, through activated T-cells that pass the blood-retinal barrier and destroy the retina. Serum markers are a desirable choice to monitor development of disease as sera are easy accessible and markers could serve to predict begin of disease or an imminent relapse.

**Methods:** In this study, serum proteomes of horses with ERU and healthy controls were compared with the 2D DIGE technique to identify differentially expressed proteins. A total of ten different proteins could be identified.

**Results:** Five proteins, namely IgM, IgG4 hc, Serotransferrin,  $\alpha$ -2HS-Glycoprotein and Complementfactor B were upregulated in uveitic state, whereas the five proteins Albumin, Apolipoprotein A-IV and H, IgG5 hc and high molecular weight Kininogen (HK) showed a significantly lower expression in sera of uveitis cases. Interestingly, HK was significantly upregulated in target tissues vitreous and retina. HK is a plasma protein with multiple physiological functions, with an important role in inflammation and promoting neovascularization. Most interesting is the as of yet unaddressed association of HK with uveitis. With immunohistochemistry, we could show co-expression of Kininogen and VEGF in inflamed eyes.

**Conclusions:** Since neovascularization plays a major role in the pathogenesis of uveitis, the identification of a proangiogenic factor in the retina presents an important finding and may contribute to elucidate the pathogenesis of uveitis.

**Support:** SFB 571 A5 Deeg

#### PC24/16 THE ROLE OF MUELLER GLIA CELLS IN EQUINE RECURRENT UVEITIS

C. Eberhardt<sup>1</sup>, B. Amann<sup>1</sup>, S. M. Hauck<sup>2</sup>, M. Stangassinger<sup>3</sup>, C. A. Deeg<sup>1</sup>

<sup>1</sup>Institute of Animal Physiology, LMU Munich, Department of Veterinary Sciences, Munich, Germany, <sup>2</sup>Institute of Protein Sciences, Helmholtz Center, Neuherberg, Germany, <sup>3</sup>Institute of Animal Physiology, Department of Veterinary Sciences, LMU Munich, Munich, Germany

**Purpose:** Mueller glia cells serve the functions of normal glial cells. However, following inflammation of the retina, we have shown that Mueller glia cells change their morphology significantly. Therefore, further investigations on the role of Mueller glia cells are needed to understand their role in ERU pathogenesis.

**Methods:** Expression of Mueller glia specific proteins was tested on Western blots, comparing the expression patterns in normal control retinas and retinas from ERU diseased horses. Monoclonal antibodies specific for glial fibrillary acidic protein (GFAP), glutamine synthetase (GS), vimentin and several ion channels were used. Activation of equine Mueller glia cells was further evaluated with immunohistochemistry on paraffin embedded sections.

**Results:** Mueller glia cells of ERU cases showed an activated phenotype with upregulation of GFAP and/or Vimentin, depending on the stage of inflammation (acute or chronic). Additionally, GS expression was significantly downregulated. Further, a change in the expression of K<sup>+</sup> and Na<sup>+</sup> channels indicates Mueller glia cell damage and edema as a result. Evaluation of the Mueller glia phenotype with immunohistochemistry confirmed the changed expression of the Mueller glia proteins and a stable expression of Cellular retinaldehyde-binding protein (CRALBP) in all stages of ERU.

**Conclusion:** Our data underscore the importance of Mueller glia cells in the pathogenesis of ERU. Further studies will use Mueller glia cell lines to investigate the differentiation of this cell type under various inflammatory stimuli.

**Support:** SFB 571 A5 Deeg



## PC25 – PATHOGENESIS OF VIRAL DISEASES

PC25/1 EXPANSION OF CD56<sup>+</sup> CD16<sup>+</sup> NK CELLS IN HIV INFECTION IS ASSOCIATED WITH DIFFERENTIAL EXPRESSION OF CD57 AND CCR7H.S. Hong<sup>1,2</sup>, J. Eberhard<sup>1</sup>, P. Keudel<sup>1</sup>, N. Bhatnagar<sup>1</sup>, M. Ballmaier<sup>3</sup>, R.E. Schmidt<sup>1</sup>, D. Meyer-Olson<sup>1</sup><sup>1</sup>Medizinische Hochschule Hannover, Klinik für Immunologie und Rheumatologie, Hannover, Germany, <sup>2</sup>Medizinische Hochschule Hannover, MD PhD program, Hanover Biomedical Research School, Hannover, Germany, <sup>3</sup>Medizinische Hochschule Hannover, Pädiatrische Hämatologie und Onkologie, Hannover, Germany**Objectives:** HIV infection is linked with loss of CD56<sup>+</sup> NK cells and expansion of dysfunctional CD56<sup>+</sup> CD16<sup>+</sup> NK cells. We hypothesized that disorders of NK cell homeostasis are caused by differential proliferative activity and distribution of NK cell subsets. We furthermore sought to phenotypically and functionally characterize CD56<sup>+</sup> CD16<sup>+</sup> NK cells in HIV infection.**Methods:** Cryopreserved peripheral blood mononuclear cells (PBMCs) from 34 untreated HIV-infected individuals and 14 healthy subjects were analyzed. We investigated expression of senescence marker CD57 and lymphnode homing receptor CCR7 and NK cell related markers by flow cytometry. Unpaired t test was performed when comparing two groups and One-way Anova followed by Tukey's Multiple Comparison test when more than two groups were analyzed.**Results:** We provide evidence that CD56<sup>+</sup> CD16<sup>+</sup> NK cells consist of three distinct subsets, namely, CCR7<sup>+</sup> CD57<sup>+</sup> cells, which are decreased in HIV-infected individuals (56.3% ± 4.7 SEM versus 36.5% ± 3.5 SEM,  $P = 0.004$ ), CCR7<sup>+</sup> CD57<sup>+</sup> double-negative cells and the CCR7<sup>+</sup> CD57<sup>+</sup> subset, which is robustly expanded in HIV-seropositive subjects (9.7% ± 1.3 SEM versus 30.2% ± 3.0 SEM,  $P = 0.0002$ ).Loss of CCR7 expression and expansion of CD57<sup>+</sup> cells within the CD56<sup>+</sup> CD16<sup>+</sup> NK cell subset strongly correlated with absolute numbers of CD56<sup>+</sup> CD16<sup>+</sup> NK cells. The CCR7<sup>+</sup> CD57<sup>+</sup> subset differed substantially from the CCR7<sup>+</sup> CD57<sup>+</sup> subpopulation as they were characterized by lower expression of HLA-DR ( $P < 0.001$ ) and CD45RO ( $P < 0.001$ ) but higher expression of CD38 ( $P < 0.001$ ), CD122 ( $P < 0.001$ ), Granzyme B ( $P < 0.001$ ) and Perforin ( $P < 0.001$ ).CCR7<sup>+</sup>/CD57<sup>+</sup>-double-negative cells resembled CD57<sup>+</sup> CD56<sup>+</sup> CD16<sup>+</sup> NK cells, yet they displayed significantly higher expression of HLA-DR ( $P < 0.01$ ) and significantly lower expression of Granzyme B ( $P < 0.001$ ). This population underwent vigorous proliferation as shown by intracellular Ki-67 staining.**Conclusion:** We demonstrate that HIV-induced expansion of CD56<sup>+</sup> CD16<sup>+</sup> NK cells is driven by different subpopulations, which can be further delineated by distinct phenotypic and functional properties. Our data suggest that HIV disturbs distribution and proliferative behavior of NK cell subpopulations.

## PC25/2 A TRANSITIONAL SUBSET OF EFFECTOR-MEMORY AND CENTRAL MEMORY CD4 T CELLS ARE THE MAJOR HIV RESERVOIR IN LONG TERM NON PROGRESSORS

B. Descours<sup>1</sup>, V.A. Fenollet<sup>2</sup>, C. Blanc<sup>3</sup>, A. Samri<sup>1</sup>, O. Pellé<sup>1</sup>, A. Mélard<sup>2</sup>, T. Prazuck<sup>4</sup>, L. Hocqueloux<sup>4</sup>, G. Carcelain<sup>1</sup>, C. Rouzioux<sup>2</sup>, B. Autran<sup>1</sup>, ALT ANRS CO15<sup>1</sup>INSERM/UPMC/UMRS945, Paris, France, <sup>2</sup>Université Paris 5 /EA3620/APHP, Paris, France, <sup>3</sup>Flow Cytometry Platform, Inter IFR-UPMC, Paris, France, <sup>4</sup>CHU la Source, Orléans, France**Objectives:** Long Term non Progressors (LTNP) are characterized by host-virus steady state with low HIV-DNA levels suggesting that a low HIV reservoir is controlled by peculiar host genetic traits and strong specific immune responses. To further understand this equilibrium, we investigated in those patients how cell differentiation and genetic background influence the distribution of HIV reservoir among CD4 T cells.**Methods:** We explored the distribution of cell-associated HIV-DNA (caHIV-DNA) of 2 groups of LTNP: either Controllers or Low Vireemics and in Slow Progressors. We sorted live resting cells from each CD4 subsets as defined by combination of CD45RA/CD27/CCR7, quantified the cell-associated HIV-DNA in each fraction and tested the in vitro inducibility of this HIV.**Results:** Cell sorting revealed a highly reproducible distribution hierarchy of caHIV-DNA, stable over up to 10 years: TM cells followed by CM contained the majority of caHIV-DNA (medians: 3.12 and 2.87 log cps/million cells) and it was significantly higher in TM ( $p < 0.01$ ) than in EM, E27- and N cells (medians: 2.29, 2.42 and 1.95). This distribution is not systematically correlated with CCR5 expression and not influenced by genetic background (HLA or CCR5Δ32 deletion). The in vitro activation induced strong HIV replication in Memory subsets independently of their infection levels and proliferation ability. In contrast HIV replication is poorly inducible in the highly proliferative Naïve subset though they are as infected as EM. Preliminary results indicate that IL-7 rescue HIV production from LTNP-C Naïve cells.**Conclusions:** In LTNP, HIV-DNA is concentrated in CD4-T cell populations with intermediate in vivo turn-over and survival: mainly the TM followed by CM cells while it remains low in cells with high turn-over but low survival such as EM or E27- cells or with low turn-over and hi survival such as the N cells. This distribution is highly stable overtime and independent of the host genetic background. Inducibility of HIV production does not strictly parallel this distribution. Altogether our results demonstrate that distinct mechanisms of HIV control dictated by or associated with T cell maturation and dynamics influence the stable low level of cell-associated HIV-DNA of LTNP.

## PC25/3 THE ROLES OF NEUTROPHILS, PLASMACYTOID DENDRITIC CELLS AND NATURAL KILLER CELLS IN RECOVERY FROM SECONDARY POXVIRUS INFECTION

V. Tahiliani<sup>1</sup>, V. Panchanathan<sup>1</sup>, G. Chaudhri<sup>1</sup>, G. Karupiah<sup>1</sup><sup>1</sup>Australian National University, The John Curtin School of Medical Research, Program in Immunology, Canberra, Australia

The potential threat of intentional or unintentional release of variola virus has generated renewed interest in smallpox. Since smallpox has been eradicated, surrogate animal models of the closely related orthopoxviruses are utilized to understand protective immunity to smallpox. The best small animal model for smallpox is mousepox, a disease caused by ectromelia virus (ECTV) infection of mice. Neutrophils, plasmacytoid dendritic cells (pDC), natural killer (NK) cells, T cell subsets and antibody are critically important for recovery of mice from a primary ECTV infection. In contrast, recovery of mice from secondary ECTV infection is strictly dependent on antibody responses that does not require CD4 T cell help or effector functions of CD8 cytotoxic T lymphocytes. We reasoned that neutrophils, pDC and NK cells could contribute to virus control during a secondary infection through antibody-dependent cell-mediated cytotoxicity involving Fc receptors. Our data show that in contrast to a primary infection, the ability of mice depleted of any one of these leukocyte subsets with specific monoclonal antibody to overcome a secondary ECTV challenge was not diminished. Importantly, a strong neutralizing antibody response was generated even in the absence of neutrophils, pDCs or NK cells. We conclude that antibody is essential, but the roles of neutrophils, pDCs and NK cells are not required for recovery from secondary poxvirus infection. This work lends further support to our previous work, which has underscored the role of antibody in recovery from secondary poxvirus infection.

## PC25/4 CELLULAR IMMUNE REACTION OF YOUNG RABBITS TO CALICIVIRUS INFECTION: COMPARISON OF LIVER AND SYSTEMIC RESPONSES

R.M. Marques<sup>1</sup>, A. Costa-e-Silva<sup>1</sup>, A.P. Águas<sup>1</sup>, P.G. Ferreira<sup>1</sup><sup>1</sup>Abel Salazar Institute for Biomedical Science, University of Porto, Anatomy, Porto, PortugalRabbit haemorrhagic disease (RHD) is a lethal infection caused by a calicivirus that kills more than 90% of the infected adult animals within 1 a 3 days. The virus replicates in the liver and causes a fulminant hepatitis. A mystery of the calicivirus infection is that young rabbits (less than 8-weeks old) are naturally resistant to the infection, expressing only a mild and transient hepatitis after inoculation. To investigate the immunological rationale of this natural resistance to RHD, we have studied by flow cytometry the kinetics of the major subpopulations of leukocytes in the liver and in the spleen of young rabbits after infecting the animals with calicivirus. In the liver, 48 hours after viral inoculation, the infection caused significant increase in all leukocyte subpopulations (macrophages, B and T cells). This contrasted with the splenic response that at this timing only showed increase in B lymphocytes. After 7 days of infection, only T lymphocytes (both CD4<sup>+</sup> and CD8<sup>+</sup> cells) were seen in enhanced numbers in the liver and spleen of the rabbits. Thymic cells remained unchanged along the first week of calicivirus infection of young rabbits. We conclude that the natural resistance to calicivirus infection that is expressed by young rabbits is associated with a rapid and strong local inflammatory response in the liver (the main organ of viral replication), and also with a secondary elevation of local and systemic T cells.PC25/5 DIFFERENT EVOLUTION OF THE FUNCTIONAL PROFILE OF HIV-SPECIFIC CD8<sup>+</sup> T CELLS IN HIV PATIENTS WITH DISTINCT HIV DISEASE PROGRESSION OVER 4 YEARSA. Peris Pertusa<sup>1</sup>, M. López<sup>1</sup>, N.I. Rallón<sup>1</sup>, C. Restrepo<sup>1</sup>, M. Salgado<sup>1</sup>, B. Rodés<sup>1</sup>, J. González-Lahoz<sup>1</sup>, V. Soriano<sup>1</sup>, J.M. Benito<sup>1</sup><sup>1</sup>Hospital Carlos III, Infectious Diseases, Madrid, Spain**Background:** Transversal studies suggest that a polyfunctional HIV-specific immune response directed against Gag might be involved in the control of HIV replication. There is scarce information about the stability of this response over time in different groups of HIV patients.**Methods:** 10 ARV-naïve typical progressors (TP), 10 progressors on HAART with undetectable viremia (H), 9 viremic controllers (VC, >10 years of infection with >500 CD4<sup>+</sup> cells without ARV and < 2000 HIV-RNA copies/ml), and 8 elite controllers (EC, >10 years of infection with undetectable viremia) were followed for 4 years. Three functions of CD8<sup>+</sup> T cells (production of MIP1β, IL2 and TNFα) were simultaneously examined in response to HIV Gag- and Nef-peptide pools using flow cytometry.**Results:** In TP patients, the functional profiles of Gag and Nef did not change over 4 years. However, the contribution of subsets with 2 functions tended to decrease whereas those with 1 function tended to increase although the differences did not reach statistical significance. In VC patients, the functional profiles of Gag- and Nef-specific CD8<sup>+</sup> responses evolved differently. The contribution of the MIP1β-TNFα+IL2- subset significantly declined and was replaced by other subsets with 1 function (MIP-TNF-IL2+, for Gag and MIP1β+TNFα-IL2- for Nef). In H patients, the profile of Nef-specific CD8<sup>+</sup> responses evolved to an increase of

the MIP1 $\beta$ +TNF $\alpha$ -IL2- subset ( $p=0.04$ ) while the Gag profile did not significantly change. In EC patients, the profile of Gag-specific CD8+ responses evolved to an increase in polyfunctionality. The Gag-specific CD8+ subsets significantly increased were MIP1 $\beta$ +TNF $\alpha$ +IL2- ( $p=0.04$ ) and the subsets with 2 functions ( $p=0.04$ ). No significant changes were found for Nef-specific responses.

**Conclusion:** The functional profile of HIV-specific CD8+ cells may evolve in a different manner depending of the targeted HIV protein and the ability to control virus replication. In patients with uncontrolled HIV replication, the functionality of Gag-specific CD8+ responses tends to diminish over time, whereas in EC it becomes polyfunctional, highlighting its importance in controlling HIV replication.

#### PC25/6 PROTECTIVE HLA CLASS I ALLELES ARE ASSOCIATED WITH A HIGHER EXPANSION ABILITY OF POLYFUNCTIONAL SUBSETS OF HIV-SPECIFIC CD8+ T CELLS IN LONG TERM NON-PROGRESSORS

L. Mariola<sup>1</sup>, V. Soriano<sup>1</sup>, S. Lozano<sup>1</sup>, J.L. Vicario<sup>2</sup>, A. Peris<sup>1</sup>, M. Salgado<sup>1</sup>, B. Rod  s<sup>1</sup>, N.I. Rall  n<sup>1</sup>, C. Restrepo<sup>1</sup>, J. Gonz  lez-Lahoz<sup>1</sup>, J.M. Benito<sup>1</sup>

<sup>1</sup>Hospital Carlos III, Laboratorio de Biolog  a Molecular, Madrid, Spain, <sup>2</sup>Centro de Transfusiones de la Comunidad Aut  noma de Madrid, Madrid, Spain

**Background:** Studies in long-term non-progressors (LTNP) have suggested that the quality of the CD8+ response is associated with non-progression and with protective HLA class-I alleles. However, there are no studies analyzing the ability of expansion of different functional subsets of CD8 cells and their association with HLA class-I alleles.

**Methods:** 25 LTNP were examined. HLA class I typing was performed using a sequence specific primer assay. Different functional subsets of Gag and Nef-specific CD8+ cells were analysed based on the production of MIP1 $\beta$ , TNF $\alpha$  and IL2. The expansion ability of these subsets was evaluated after 10-day culture in the presence of Gag and Nef-HIV peptides. Differences between groups were evaluated using non-parametric tests.

**Results:** According to the presence/absence of the protective-HLA alleles (B\*58 and B\*27 supertypes), patients with protective HLA presented a higher expansion ability of MIP+TNF+IL2+CD8+ subsets for Gag ( $p=0.04$ ) and MIP+TNF+IL2+ ( $p=0.02$ ), MIP+ TNF-IL2+ ( $p=0.02$ ) and MIP+TNF-IL2- ( $p=0.03$ ) CD8+ subsets for Nef.

According to presence/absence of HLA-B\*5701, patients with this allele showed a higher expansion ability in MIP+TNF-IL2+CD8+ cells ( $p=0.03$ ) for Gag, and MIP+TNF-IL2+ ( $p=0.03$ ) and MIP+TNF-IL2- ( $p=0.02$ ) CD8+ subsets for Nef.

Finally, according to the presence/absence of HLA-B\*2705 no differences were observed in the expansion ability of different subsets of Gag-specific CD8+ cells. Interestingly, the expansion ability of MIP+TNF+IL2+ in HLA-B\*2705 patients for Nef was lower ( $p=0.016$ ).

**Conclusions:** The expansion ability of polyfunctional CD8+ T-cell subsets is influenced by the HLA class-I allele and the targeted protein. LTNP with HLA class I protective alleles (specially HLA-B\*5701) show a better expansion ability of polyfunctional HIV-specific CD8+ T-cells than the rest, suggesting that other factors might contribute to viral replication control in LTNP without HLA-B\*5701.

#### PC25/7 INFLUENCE OF GENOTYPES FOR CCR5DELTA32 ON THE RESPONSE TO THERAPY IN ASYMPTOMATIC HIV-1 CHRONICALLY INFECTED PATIENTS

D. Hern  ndez Fl  rez<sup>1</sup>, L. Valor<sup>1</sup>, J. Modrego<sup>1</sup>, D. Alecsandru<sup>1</sup>, C. Rodr  guez-S  niz<sup>1</sup>, E. Fern  ndez-Cruz<sup>1</sup>

<sup>1</sup>Hospital General Universitario Gregorio Mar    n, Immunology, Madrid, Spain

**Objective:** CCR5delta32/CCR5wt heterozygous genotype has been associated to patterns of slower HIV-1 disease progression in untreated individuals. In this retrospective study we have assessed the influence of the genotypes for CCR5delta32 on the immunovirological outcome in 187 antiretroviral-naive individuals initiating treatment with antiretrovirals plus a therapeutic vaccine (STIR-2102).

**Methods:** In 187 individuals participating in the clinical trial STIR-2102 were have retrospectively analysed CCR5delta32 genotypes by polymerase chain reaction and sequencing. Virological (viral load > 5.000 copies HIV-1 RNA/ml) and immunological (CD4 cell counts below 350 cells/ $\mu$ L) endpoints were analyzed by Kaplan-Meier analysis (Vaccine. 2008 May 23;26(22):2738-45). Aminoacid sequence of the HIV-1 loop V3 for the genetic evaluation of the viral tropism was determined in a group of 16 patients at the baseline and at the end of the study (13 individuals CCR5wt/CCR5wt, 2 individuals CCR5delta32/CCR5wt and 1 individual CCR5delta32/CCR5delta32).

**Results:** Twenty five (13.4%) individuals were heterozygous and one (0.5%) individual was homozygous for CCR5delta32 genotype. In Kaplan-Meier analysis CCR5Delta32 heterozygous genotype did not impact significantly on the virological outcome (Log rank,  $p \geq 0.187$ ). Patient CCR5delta32/CCR5delta32 showed an HIV V3 region with genotypic features attributable to X4 viruses and did not reach an endpoint. In three out of 16 patients we determined the presence of V3 loop sequences characteristic of X4 viruses (CCR5delta32/CCR5delta32, CCR5delta32/CCR5wt and CCR5wt/CCR5wt). The patient homozygous for CCR5wt with X4 virus at the end of the study achieved a rapid decline of T CD4+ cells while patients with CCR5delta32 and X4 viruses remained without immunological endpoint.

**Conclusion:** CCR5Delta32 did not influence immunovirological outcome in patients undergoing antiretroviral therapy. The genotypic background for CCR5delta32 could be associated with the sequence of the HIV-1 V3 loop and this might influence the immunovirological outcome of X4 viruses. Immunovirological outcome might depend on the interaction between CCR5 genotype and R5 or X4 viral variants.

#### PC25/8 A DISTINCT DIFFERENTIATION PROFILE BETWEEN HIV-GAG AND NEF SPECIFIC CD8+ T CELLS WITH PRESERVATION OF CD27+ CENTRAL MEMORY T CELLS IS DICTATED BY HLA-B57/B5801 AND ASSOCIATED WITH VIRUS CONTROL

J. Xie<sup>1</sup>, W. Lu<sup>1</sup>, A. Samr  <sup>1</sup>, D. Costagliola<sup>2</sup>, A. Schnuriger<sup>1</sup>, B.C. Maciel da Silva<sup>1</sup>, C. Blanc<sup>1,3</sup>, M. Almeida<sup>1</sup>, O. Pell  <sup>1</sup>, I. Theodorou<sup>1,4</sup>, C. Rouzioux<sup>5</sup>, B. Autran<sup>1,4</sup>, the ALT study group

<sup>1</sup>INSERM, UMR945, Laboratoire d'Immunologie Cellulaire et Tissulaire, UPMC Univ Paris 06, Paris, France, <sup>2</sup>INSERM U943,   pid  miologie, strat  gies th  rapeutiques et virologie cliniques dans l'infection    VIH, H  pital Piti  -Salp  tri  re, UPMC Univ Paris 06, Paris, France, <sup>3</sup>Flow Cytometry Platform, Inter IFR-UPMC, Paris, France, <sup>4</sup>AP-HP, H  pital Piti  -Salp  tri  re, Laboratoire d'Immunologie Cellulaire et Tissulaire, Paris, France, <sup>5</sup>Laboratoire de Virologie, H  pital Necker, Universit   Ren   Descartes, Paris, France

**Objectives:** Multifunction and memory T cell characteristics or HLA-B57 and the closely related B5801 (HLA-B57/B5801) alleles appear to play a key role in controlling HIV, although this is not yet fully understood. To gain novel insight into the nature of the protective effect mediated by specific CD8+ T cells in HIV-infected HLA-B57/B5801+ individuals, we compared the frequency, cytokine production, differentiation, and functional avidity of HIV-specific-CD8+ T cells, in B57/B5801+ and B57/B5801- nonprogressors.

**Methods:** This study investigated in a cohort of 53 untreated nonprogressors whether CD8+ T cells specific for three classes of HIV gene products, i.e., the dominant structural Gag and early non structural Nef antigens, and the highly conserved RT protein, differed in their relations to two virus parameters – plasma HIV-RNA and cell-associated HIV-DNA loads. Twenty-two subjects, 11 HLA-B57/B5801+ and 11 B57/B5801-, with simultaneous positive responses to Gag and Nef detected by ELISpot assays, were selected for analyzing whether HIV-Gag and Nef trigger the same CD8+ T cell functions and differentiation stages and their correlation with viral burden.

**Results:** The frequency of Gag-specific CD8+ T cells negatively correlated with HIV-DNA loads ( $n=50$ ,  $r=-0.395$ ,  $p=0.004$ ), while that of Nef- and RT-specific cells did not. None of these frequencies correlated with plasma HIV-RNA levels. The HIV-Gag and Nef-specific CD8+ T cells did not differ for IL-2 production both in two HLA groups. In the B57/B5801+ group, the IFN- $\gamma$ -producing Gag-specific central memory (CD45RA CCR7+) CD8+ T cells showed a significantly higher proportion of CD27+ cells than their Nef-specific counterparts ( $p=0.007$ ). This differentiation pattern was not observed in B57/B5801- individuals. The percentage of CD27 expression on Gag-specific IFN- $\gamma$ +TCM-like CD8+ T cells negatively correlated with HIV-DNA in the B57/B5801+ group ( $r=-0.683$ ,  $p=0.042$ ) but not in the B57/B5801- group. The same subset specific for Nef was not correlated either with the HIV-RNA or the HIV-DNA loads, whatever the HLA group considered.

**Conclusion:** Our findings indicate that in HLA-B57/B5801+ individuals HIV-Gag induces a preferential CD27+ central-memory differentiation profile distinct from that caused by Nef and that this profile may contribute to the protective effect of Gag-specific CD8+ T cells in HLA-B57/B5801+ nonprogressors.

#### PC25/9 INCREASED ARGINASE ACTIVITY CORRELATES WITH DISEASE SEVERITY IN HIV SEROPOSITIVE PATIENTS

T.E. Cloke<sup>1</sup>, L.J. Garvey<sup>2</sup>, B.-S. Choi<sup>1</sup>, T. Abebe<sup>3</sup>, A. Hailu<sup>3</sup>, M.R. Hancock<sup>4</sup>, U. Kadolsky<sup>1</sup>, C.R.M. Bangham<sup>1</sup>, M. Munder<sup>5</sup>, I. Muller<sup>1</sup>, G.P. Taylor<sup>2</sup>, P. Kropf<sup>1</sup>

<sup>1</sup>Imperial College, Department of Immunology, St Mary's Campus, London, United Kingdom, <sup>2</sup>Imperial College London, St Mary's Campus, Departments of Infectious Diseases and Genito-Urinary Medicine, London, United Kingdom, <sup>3</sup>University of Addis Ababa, Department of Microbiology, Parasitology and Immunology, Addis Ababa, Ethiopia, <sup>4</sup>Imperial College Healthcare NHS Trust, Department of Clinical Biochemistry, Charing Cross Hospital, London, United Kingdom, <sup>5</sup>Max-Planck-Institute for Immunobiology, Department of Cellular Immunology, Freiburg, Germany

Infection with human immunodeficiency virus (HIV) results in a chronic infection that progressively impairs the immune system. Although depletion of CD4+ T cells is frequently used to explain immunosuppression, chronicity of infection and progressive loss of CD4+ T cells are not sufficient to fully account for immune dysregulation and the precise mechanisms leading to immunodeficiency and progression from HIV infection to AIDS remain unexplained.

Arginase-induced L-arginine deprivation is emerging as a key mechanism for the downregulation of immune responses.

**Objectives:** The objective of the study was to test the hypothesis that arginase activity increases with disease severity in HIV seropositive patients and impairs T cell responses, thereby contributing to disease progression.

**Methods:** To test this hypothesis, we isolated peripheral blood mononuclear cells (PBMCs) and sera from 38 HIV positive patients and 11 uninfected controls. The levels of arginase activity in PBMCs were determined using an enzymatic assay.

The levels of L-arginine in the sera of HIV positive patients were quantified using postcolumn ninhydrin derivatisation after separation by ion-exchange chromatography.

The expression of CD3   was determined by western blot.

**Results:** Our results show that PBMCs from HIV seropositive patients with low CD4<sup>+</sup> T cell counts expressed significantly more arginase activity as compared to patients with high CD4<sup>+</sup> T cell counts or uninfected controls. Furthermore, we found a significant correlation between high arginase activity in HIV seropositive patients and low CD4 count and high viral load. Finally, higher arginase expression in PBMCs from HIV seropositive patients was associated with decreased levels of CD3 $\zeta$  expression, a marker of T cell dysregulation.

**Conclusion:** Our results suggest that arginase-induced L-arginine metabolism might be a novel mechanism in patients infected with HIV that contributes to impaired T cell functions.

#### PC25/10 EXPANSION OF TRANSITIONAL B CELLS IN PATIENTS WITH CHRONIC HUMAN HERPESVIRUS-8 INFECTION AND KAPOSI'S SARCOMA

A. Taddeo<sup>1</sup>, P. Romeo<sup>1</sup>, M. Bellinva<sup>2</sup>, E. Colombo<sup>1</sup>, B. Scoppio<sup>2</sup>, A. Toulaki<sup>2</sup>, L. Brambilla<sup>2</sup>, M. L. Villa<sup>1</sup>, S. Della Bella<sup>1</sup>

<sup>1</sup>Università degli Studi di Milano, Dipartimento di Scienze e Tecnologie Biomediche, Segrate, Italy, <sup>2</sup>Fondazione IRCCS Ospedale Maggiore Policlinico, Mangiagalli e Regina Elena, Institute of Dermatological Sciences, Milan, Italy

**Objectives:** Human Herpesvirus 8 (HHV-8) is the etiological agent of Kaposi's Sarcoma (KS) and it is also associated with two B cell lymphoproliferative diseases: primary effusion lymphoma (PEL), and the plasmablastic form of multicentric Castelman's disease (MCD). HHV-8 establishes persistent infection in the host with tropism for multiple cell types. In KS patients, the virus is found in tumor-spindle cells, peripheral blood monocytes, T and B lymphocytes. Peripheral B cells represent the major virus reservoir in most patients but the consequences of HHV-8 infection of these cells have been poorly characterized. Therefore, in this study we analysed the frequency and the immunophenotypic profile of different peripheral B cell subsets in patients with KS to identify potential alterations of these cells.

**Methods:** Whole-blood samples from patients with the classical form of KS (cKS) (n=62) and healthy age and sex-matched seronegative controls (HSN) (n=43) were analyzed by multiparametric flow-cytometry to determine the frequency of B cells and their subpopulations, as well as their surface expression of immunoglobulins and activation markers.

**Results:** The frequency of circulating B cells was significantly higher in cKS patients than controls. The analysis of the B cell subsets revealed that the frequency of naïve (CD19<sup>+</sup>CD27<sup>+</sup>), memory (CD19<sup>+</sup>CD27<sup>+</sup>) and B1 (CD19<sup>+</sup>CD5<sup>+</sup>) B cells did not differ between the two study groups. Further analysis of the peripheral B cell phenotype demonstrated an expansion of transitional CD19<sup>+</sup>CD38<sup>high</sup>CD5<sup>+</sup> B cells in cKS patients. The characterization of membrane surface immunoglobulins on B lymphocytes showed a significant higher proportion of IgM<sup>+</sup>IgD<sup>+</sup> B cells in cKS patients than in controls. Moreover, B cells from cKS patients expressed higher levels of CD20 but lower levels of the activation markers CD80 and CD86.

**Conclusion:** Taken together, these results clearly indicate that circulating B cells are altered in patients with cKS. These B cell alterations in turn may contribute to the pathogenesis of KS possibly by interfering with the capacity of the immune system to control HHV-8 infection.

#### PC25/11 FREQUENCY OF CYCLING REGULATORY T CELLS IN TWO AIDS-ASSOCIATED DISEASES WITH DISTINCT CLINICAL OUTCOMES: HIV-1 AND HIV-2 INFECTIONS

R. B. Foxall<sup>1</sup>, A. A. Albuquerque<sup>1</sup>, A. P. Baptista<sup>1</sup>, R. S. Soares<sup>1</sup>, R. Tendeiro<sup>1</sup>, P. Gomes<sup>2</sup>, R. M. M. Victorino<sup>1,3</sup>, A. E. Sousa<sup>1</sup>

<sup>1</sup>Unidade de Imunologia Clínica, Instituto de Medicina Molecular, Faculdade de Medicina de Lisboa da Universidade de Lisboa, Lisboa, Portugal, <sup>2</sup>Laboratório de Virologia, Hospital Egas Moniz, Lisboa, Portugal, <sup>3</sup>Clínica Universitária de Medicina 2, Hospital de Santa Maria, Lisboa, Portugal

**Objectives:** Naturally-occurring regulatory T cells (Treg) make up 2-3% of circulating CD4<sup>+</sup> T cells in humans. They are enriched within CD4<sup>+</sup>CD25<sup>bright</sup> T cells, and Foxp3, a transcription factor absolutely required for their function, is currently their most widely accepted marker.

Treg play an important role in many aspects of immunity. As with most infections, their role in HIV immunopathogenesis may be beneficial (limiting activation-induced damage) or deleterious (blunting HIV-specific responses).

HIV-1 infection induces a generalized immune-activation which is thought to be a major factor in HIV-associated immunodeficiency. This phenomenon is also observed in HIV-2+ adults with comparable degrees of CD4 depletion, despite a much slower rate of CD4 loss and limited impact on survival in these individuals. Here we compare both the frequency of Treg, and the proportion that are expressing Ki67, in HIV-1 and HIV-2 infection, to assess whether quantitative or qualitative differences in these populations may relate to the different disease courses.

**Methods:** A cross-sectional study of HIV-2+, HIV-1+ and seronegative individuals was conducted. Freshly isolated PBMC were analyzed using multiparameter Flow Cytometry with an antibody panel designed to characterize CD25<sup>bright</sup>CD4<sup>+</sup> T cells in parallel with Foxp3 expression and cell-cycling, measured using Ki67. Spearman correlations and Mann-Whitney tests were used for statistical analysis.

**Results:** The proportion of Treg expressing Ki67 was similarly increased in HIV-1 and HIV-2 infected individuals. However, this increase was not correlated with the degree of CD4 T-cell depletion and immune activation in either HIV cohort, despite such relationships being reported for effector T-cell subsets in both HIV-1+ and HIV-2+ individuals. Moreover, we observed a progressive increase in the proportion of Treg within CD4 T cells with disease progression in both infections in parallel with a relative expansion of the memory pool.

**Conclusion:** In spite of their distinct natural history, the mechanisms that regulate the circulating Treg pool seem to be operating similarly in both HIV-1 and HIV-2 infections. Moreover, these mechanisms do not seem to be directly affected by the hyper-immune activation and the associated CD4 depletion that are the hallmarks of HIV/AIDS pathogenesis.

#### PC25/12 ACTIVATION LEVELS OF CENTRAL MEMORY CD8 T CELLS DIFFERENTIATE HIV PATIENTS WITH SPONTANEOUS VERSUS HAART-INDUCED CONTROL OF HIV REPLICATION

L. Mariola<sup>1</sup>, A. Peris<sup>1</sup>, V. Soriano<sup>1</sup>, S. Lozano<sup>1</sup>, M. Salgado<sup>1</sup>, B. Rodés<sup>1</sup>, N. I. Rallón<sup>1</sup>, C. Restrepo<sup>1</sup>, J. González-Lahoz<sup>1</sup>, J. M. Benito<sup>1</sup>

<sup>1</sup>Hospital Carlos III, Laboratorio de Biología Molecular, Madrid, Spain

**Background:** T cell-activation is a major factor involved in CD4 depletion in HIV patients. Information on the level of activation of different T cell subsets in patients with spontaneous versus antiretroviral-induced control of viral replication is scarce.

**Methods:** 120 HIV-1 subjects were examined: 11 elite controllers (EC), 14 viremic controllers (VC), 61 antiretroviral-naïve typical progressors (TP), 34 subjects on undetectable viral load on HAART for at least one year (HP), and 9 non-infected subjects were examined.

The activation level of naïve (TN), central-memory (TCM), effector-memory (TEM) and terminally differentiated effector-memory (TEMRA) subsets of CD4 and CD8 T-cells was analysed by flow cytometry using a quantitative assay for CD38 expression. Differences between groups were evaluated using non-parametric tests.

**Results:** EC showed normal activation levels in CD4 T-cells whereas H showed higher activation levels in TNCD4 cells than uninfected individuals (p=0.012). However, EC, VC and TP showed higher levels of activation in all CD8 subsets than uninfected subjects (p< 0.05 for all comparisons). Interestingly, HP had normal levels of activation in TN and TCM CD8 cell subsets.

EC had lower activation levels than VC, especially on TCM cells (p=0.001 and p=0.04 for CD4 and CD8 cells, respectively). Moreover, EC had significantly higher activation levels than HP in total (p=0.03), TN (p=0.04), and TCM (p=0.002) CD8 cells. In TP, the activation level in TCMCD8 was significantly correlated with viral load, regardless CD4 counts.

**Conclusions:** Elite controllers show abnormal and higher activation levels of central memory CD8 cells than patients successfully treated with HAART, suggesting that residual viral load may be higher in patients spontaneously controlling viral replication than in those successfully treated with HAART.

#### PC25/13 CIITA INTERACTS WITH HTLV-I (HUMAN T CELL LEUKEMIA VIRUS TYPE 1) TAX-1 PROTEIN AND INHIBITS BOTH ITS TRANSCRIPTIONAL FUNCTION ON THE VIRAL LTR PROMOTER AND ITS CAPACITY TO CONSTITUTIVELY ACTIVATE NFkB

G. Tosi<sup>1</sup>, L. Badarou<sup>1</sup>, V. Andresen<sup>2</sup>, G. Franchini<sup>2</sup>, R. S. Accolla<sup>1</sup>

<sup>1</sup>University of Insubria, Dept. of Clinical and Biological Sciences, Varese, Italy, <sup>2</sup>National Cancer Institute, NIH, Animal Models and Retroviral Vaccines Section, Bethesda, United States

**Objectives:** To assess whether CIITA, the activator of MHC class II gene transcription, inhibits the replication of HTLV-I oncogenic retrovirus, as it inhibits HIV-1 and HTLV-II, by targeting the viral transactivator (Tax-1). Possible molecular mechanisms responsible for this inhibitory effect were studied. We also assessed whether CIITA can inhibit Tax-1-mediated activation of NFkB, a major mechanism through which the viral transactivator deregulates the expression of cellular genes controlling cell proliferation and survival.

**Methods:** The effect of CIITA on Tax-1-mediated transactivation of the viral LTR promoter and of a NFkB-responsive promoter was assessed by classical luciferase gene reporter assays. CIITA-mediated inhibition of HTLV-I replication was evaluated in 293T cells co-transfected with CIITA and a plasmid containing the entire HTLV-I genome, by measuring Tax-1 activity and the secretion of p19 viral protein. Co-immunoprecipitation experiments were performed to analyze the interaction between CIITA and Tax-1.

**Results:** CIITA inhibits Tax-1-dependent LTR activation and this inhibitory effect maps to a short stretch of 60 aminoacids at the N-term of CIITA. Importantly, the functional inhibition of Tax-1 by CIITA results in an impaired HTLV-I replication. Moreover, Tax-1 interacts with CIITA in vivo and the over-expression of PCAF, a cellular co-factor commonly used by both CIITA and Tax-1 to activate transcription of their target promoters (MHC-II and LTR), can rescue Tax-1-directed LTR transactivation inhibited by CIITA. Finally, CIITA suppresses the constitutive activation of NFkB by Tax-1.

**Conclusions:** Our data suggest that CIITA may inhibit Tax-1 activity because of P/CAF sequestration or because CIITA binding to Tax-1 abrogates the crucial Tax-1-PCAF interaction. Moreover, CIITA counteracts the activation of NFkB pathway by Tax-1. All together these results indicate that CIITA exerts multiple effects against HTLV-I infection: it increases presentation of pathogen antigens; it inhibits viral replication and it may decrease, at least in part, the oncogenic potential of HTLV-I virus. This investigation is supported by Fondazione Cariplo, Milan, Italy, 2008: Biomedical Research "Cellular and Molecular Basis of Human Retroviral-Dependent Pathology" to R. S. A.



**PC25/14 CYTOMEGALOVIRUS INDUCES CHRONIC INFLAMMATION**P.J. van de Berg<sup>1,2</sup>, R.R. Raabe<sup>2</sup>, K.M. Heutink<sup>1,2</sup>, S.L. Yong<sup>1</sup>, R.A. van Lier<sup>1</sup>, I.J. ten Berge<sup>2</sup><sup>1</sup>Amsterdam Medical Center / University of Amsterdam, Department of Experimental Immunology, Amsterdam, Netherlands, <sup>2</sup>Amsterdam Medical Center / University of Amsterdam, Renal Transplant Unit, Amsterdam, Netherlands

Chronic inflammation plays an important role in cardiovascular disease but the exact mechanisms leading to this inflammatory state are not fully known. Cytomegalovirus (CMV) is a persistent virus that generates a permanent pool of highly differentiated CMV specific effector CD4<sup>+</sup> and CD8<sup>+</sup> T cells through chronic antigenic stimulation. We examined if CMV infection can also lead to a permanent increase of inflammatory mediators in blood.

Therefore, we longitudinally measured cytokines, chemokines, soluble endothelial adhesion molecules and acute phase proteins using multiplex bead assay. Based on the availability of serum samples, we selected 14 renal transplant patients developing a primary CMV infection after transplantation and 9 renal transplant patients that remained CMV seronegative. Serum samples of the primary CMV infected patients were chosen before transplantation when all patients were CMV seronegative, during the peak of the primary CMV infection and 1 year after the transplantation, resembling the latency phase of the CMV infection. The serum samples of the patients that remained CMV seronegative were from comparable time points relative to time of transplantation.

We found that during the peak of the primary CMV infection cytokines inducing Th1 cells such as IL-18 (p=0.003) and cytokines produced by Th1 cells such as IL-10 (p=0.03), IP-10 (p=0.0001) and MCP-2 (p=0.04) were elevated. Furthermore, the acute phase proteins SAA-1 (p=0.02) and C-reactive protein (CRP) (p=0.0005) and the soluble form of the endothelial adhesion molecules VCAM-1 were increased (p=0.004). Importantly, we found that IP-10 (p=0.002) and CRP (p=0.004) remained elevated during latency.

These data show that during the peak of the primary CMV infection a vigorous Th1 response is generated, acute phase proteins are released and endothelial cells are activated. Moreover, CMV causes chronic inflammation as indicated by the permanent increase in CRP and IP-10. In conclusion, CMV induces a state of systemic chronic inflammation.

**PC25/15 CYTOKINES GENE POLYMORPHISM ASSOCIATED TO SEVERE DENGUE ILLNESS**A.B. Perez<sup>1</sup>, B. Sierra<sup>1</sup>, G. Garcia<sup>1</sup>, E. Aguirre<sup>1</sup>, N. Babel<sup>2</sup>, L. Valdes<sup>3</sup>, L. Sanchez<sup>1</sup>, H.D. Volk<sup>2</sup>, M.G. Guzman<sup>1</sup><sup>1</sup>Institute for Tropical Medicine 'Pedro Kouri', Havana, Cuba, <sup>2</sup>Institute for Medical Immunology, Charité-Universitätsmedizin Berlin, Berlin, Germany, <sup>3</sup>Center of Hygiene and Epidemiology, Santiago de Cuba, Cuba

The susceptibility to develop the severe disease during a dengue infection has been associated to host genetic factors. Taking into account the involvement of T cell response and several cytokines in the immune-pathogenesis of the disease and the exceptional epidemiological situation of dengue in Cuba, valuable for genetic studies, we investigate the TNF-alpha and TGF-beta gene single nucleotide polymorphisms (SNP) in individuals who have suffered Dengue Haemorrhagic Fever (DHF) and controls.

The TNF-alpha (-308) and TGF-beta (codon 10 and codon 25) gene SNP were studied by polymerase chain reaction-sequence specific primer (PCR-SSP).

Significant differences in the TNF-alpha (-308) allele frequencies were found when comparing controls and DHF cases, showing an association between a high expression allele (A) with severe disease. On the other hand, allele C in the TGF-beta gene, codon 25 position, was significantly associated to DHF when compared with controls, relating the TGF-beta low expression genotype with the severe disease.

Our results suggest that individuals carrying high TNF- $\alpha$  and low TGF- $\beta$  producer alleles are at greater risk of developing DHF. This study gives insights about the predictive value of cytokine genotype for the development of clinical output of dengue infection, and confirms the role of pro-inflammatory pattern in the pathogenesis of the severe disease.

**PC25/16 LONGITUDINAL FOLLOW UP OF AN HIV-1 INFECTED SUBJECT WITH PARTIAL CONTROL OF VIRAL REPLICATION**C.J. Dembek<sup>1</sup>, S. Kutscher<sup>1</sup>, S. Allgayer<sup>2,3</sup>, J.R. Bogner<sup>1</sup>, F.D. Goebel<sup>1</sup>, V. Erflé<sup>1,2</sup>, A. Cosma<sup>1,3</sup><sup>1</sup>Helmholtz Zentrum München, Institute of Virology, Munich, Germany, <sup>2</sup>Technische Universität München, Institute of Virology, Munich, Germany, <sup>3</sup>Helmholtz Zentrum München, Clinical Cooperation Group 'Immune Monitoring', Munich, Germany, <sup>4</sup>Ludwig-Maximilians-Universität, Medizinische Poliklinik, Munich, Germany

**Objectives:** Immunological correlates associated with the control of viremia in HIV infection are still poorly understood. To understand the dynamic of HIV disease, we monitored the interplay between the host and the virus in a long term follow up.

**Methods:** We performed a seven years follow-up of one HIV infected subject (V4) by intensive immunological characterization using multiparameter flow cytometry combined with sequence analysis.

**Results:** V4 participated to a phase I clinical trial to assess safety of a HIV-1 Nef based vaccine delivered by a modified vaccinia virus Ankara (MVA-nef). Following interruption of anti-retroviral therapy, we observed a first phase in which the viral load increased to reach a viral set point (median: 31,000 copies/ml; mean: 34,285.39 copies/ml). During this phase HIV-specific CD4 and CD8 T-cell responses strongly increased. The phase of stable viral load lasted 3.1 years. During this long period of partial viral control, we observed a gradual modification of the quality of the immune response. Specific CD4 T-cells lost the capacity to express the CD154 activation marker and specific CD8 T-cells progressively increased their activation status (HLA-DR and CD137). Subject V4 finally lost the capacity to control viral replication and the viral load rebounded to >100,000 copies/ml while CD4 counts dropped below 500 cells/ml. During this phase, the total amount of specific CD4 T-cells decreased while the amount of specific CD8 T-cells stayed unchanged.

**Conclusion:** These data suggest that in a situation of partial viral control there is a progressive modification of the HIV-specific immune response, which eventually leads to the incapacity of the immune system to control viral replication. The modifications of the quality of the immune response observed in the present study constitute the basis for a more comprehensive longitudinal follow-up.

**PC25/17 HERPES VIRUS AND MIGRAINE: NEW THEORY OF PATHOGENESIS. IS MIGRAINE ATTACK ASSOCIATED WITH HERPES-VIRUS SWITCH FROM LATENCY TO RE-ACTIVATION?**V. Garib<sup>1</sup>, U. Ashonov<sup>1</sup>, S. Shamansurov<sup>1</sup><sup>1</sup>Tashkent Institute for Post-Graduate Medical Education, Medical Immunology, Tashkent, Uzbekistan

Until now migraine is an idiopathic headache of differential pathogenesis, much evidence let us think about the involving of cytokines system modification. Here we discuss the hypothesis that HSV, a neurotropic virus, can be a trigger of migraine attack. Following initial infection of cells, the virus gains access to the distal axonal terminate of sensory nerves and travels by retrograde axonal transport to neuronal cell bodies in trigeminal ganglia, where further replication or latency can occur. In latently infected neurons, the viral genome is active under the control of "Latency-Associated Transcript". Recurrences can be triggered by numerous environmental stimuli, also important for migraine attack. HSV recurrence could mediate both the inflammatory process and pain. Trigeminal ganglia can be stimulated locally through a herpes virus reactivation and releases vasoactive substances into the area surrounding the vasculature or other biochemical mediators, such as nitric oxide, and then to cause inflammation. At day 1<sup>st</sup> of HSV reactivation high concentrations of IFN-Gamma, IL-1 Beta, IL-6 and active levels of IL-12, MIP-1alpha, MIP-1-Beta, RANTES had been determined previously. 30 patients with migraine had been investigated by PCR-analysis, and HSV-1 had been found in 100% cases. Pain syndrome causes to release of pain hormone – substance P. Substance P predominantly binds to neurokinin-1 receptor and inhibits migration activity of natural killer cells. All mechanisms of migraine pathogenesis had been previously discovered and discussed, can be combined via herpes virus theory. This work had been partly performed by author in Institute of Immunology, Witten/Herdecke University, Germany.

**PC25/18 SEROTYPING OF HBSAG-MUTANTS OF HEPATITIS B VIRUS (HBV) BY THE MUTANT-SPECIFIC DEFECTS OF HBSAG RECOGNITION BY MONOCLONAL ANTI-HBSAG ANTIBODIES**A.P. Suslov<sup>1</sup><sup>1</sup>State Gamaleya Research Institute of Epidemiology and Microbiology, Russian Academy of Medical Sciences, Moscow, Russian Federation

The HBSAg of HBV is the most important target for diagnostics and immunoprophylaxis of HBV infection. HBSAg-escape mutations, predominantly accumulated within the "a"-determinant, could result in breakthrough infection in vaccinated subjects and hinder detection of HBSAg by the monoclonal antibody (mAb)-based assay. In this study we analyzed defects in the recognition of HBV mutants by mAbs specific to wild-type HBSAg. In total, 37 mAbs were tested, and dramatic mutant-specific drops in mAb's sensitivity were found. These drops ranged from 10 to 10000 and were highly reproducible. Based on the pattern of defects in HBSAg mutant' recognition, all mAbs were divided into the six groups, defective for their ability to recognize the following mutants:

- (i) G145R mutant only (10 out of 37 mAbs, 27.0%);
- (ii) mutants G145R and S143L (8 mAbs, 21.6%);
- (iii) mutant G145R and different HBV subtype variants (11 mAbs, 29.7%);
- (iv) mutants G145R, S143L and ad subtype (5 mAbs, 13.5%); ay subtype (2 mAbs, 5.4%); mAbs without significant defect (1 mAb, 2.7%).

Of note, more than 90% of mAbs tested almost totally lost their reactivity with the mutant G145R, indicating that this mutation had a dramatic effect on the HBSAg antigenic structure. Another mutation that had a significant impact on HBSAg recognition was mutation S143L: 37% of wild-type specific mAbs lost their reactivity with this mutant. In summary, using a panel of mAbs specific to wild-typeHBSAg it is possible to create HBSAg mutant-specific serological "portraits" and develop defect-based mutant-specific ELISA. Serotyping of HBSAg by mAb mutant-specific defects represents a valuable and informative test to be used for HBV infection diagnosis and analysis of precise mechanisms of HBV escape from immune surveillance.

**PC25/19 THE ROLE OF TUMOR MICROENVIRONMENT AND EPIGENETIC CHANGES IN IMMUNE ESCAPE AND TUMOR PROGRESSION TRIGGERED BY HHV8-VGPCR**N. Mallem<sup>1</sup>, N. Thirunarayanan<sup>1</sup>, C. Krause<sup>1</sup>, G. Mueller<sup>1</sup>, M. Lipp<sup>1</sup><sup>1</sup>Max-Delbrueck Center for Molecular Medicine, Molecular Tumor Genetics and Immunogenetics, Berlin, Germany

Human herpesvirus 8 (HHV-8), or Kaposi sarcoma-associated herpesvirus is an oncogenic herpesvirus that has been implicated in the development of all epidemiological forms of Kaposi's sarcoma (KS), the HHV-8 plays also a critical role in other lymphoproliferative disorders like "body cavity-based lymphoma" or "primary effusion lymphoma" (BCBL or PEL) and multicentric Castelman's disease (MDC). The HHV-8 encodes a constitutively active chemokine receptor homolog (vGPCR), which may play an important role in virus mediated tumorigenesis. A recent work in our laboratory had shown that Balb/c3T3 expressing vGPCR induce tumors in nude mice, but as expected fail to induce tumors in their immunocompetent counterparts. However, tumor fragments obtained from nude mice grow progressively in immunocompetent Balb/c mice. Unexpectedly, vGPCR expressing cells established from grafted tumor fragments gave rise to tumors in immunocompetent mice. These tumors exhibit a striking histological resemblance to KS. This model is currently used to understand the molecular and the cellular mechanisms underlying immune escape and progressive tumorigenesis triggered by the human herpesvirus 8 chemokine receptor vGPCR. To attain this objective a large scale gene expression mapping was done using affymetrix chips. And as the methylation of eukaryotic DNA and histone modification are the major mechanisms by which epigenetic changes regulate gene expression, we are currently performing a large-scale DNA methylation profiling using DNA methylation immunoprecipitation (MeDIP) and chromatin immunoprecipitation on affymetrix promoter chips. The result of the MeDIP on chip and ChIP will be compared to the gene expression profiling in order to identify potential target genes responsible for immune escape and tumor progression.

**PC25/20 COMPLEMENT MEDIATED ENHANCEMENT OF FRIEND VIRUS SPECIFIC CTL RESPONSE BY B CELLS IN VITRO**C. Bila<sup>1</sup>, A. Ejaz<sup>1</sup>, V. Oberhauser<sup>1</sup>, M. P. Dierich<sup>1</sup>, H. Stoiber<sup>1</sup>, Z. Banki<sup>1</sup><sup>1</sup>Innsbruck Medical University, Department of Hygiene, Microbiology and Social Medicine, Innsbruck, Austria

Friend Virus (FV) represents a mouse model for retroviral infections. FV infects various cells of the lymphohematopoietic systems, including B cells. B cells are professional antigen presenting cells and express complement receptors (CR1 and CR2) on their surface. In this study, we investigated the role of complement on FV infection of B cells and on the induction of FV-specific cytotoxic T-cell (CTL) response by B cells. We found that complement opsonization significantly enhance the infection of B cells, compared to non-opsonized virus. Furthermore, B cells loaded with complement opsonized FV showed significant higher capacity to induce FV-specific CTLs as determined by the upregulation of activation markers CD69 and CD25 as well as by the induction of cell proliferation.

**PC25/21 EFFECT OF DENGUE VIRUS SEROTYPE-2 IN HUMAN T CELLS PROLIFERATION**C. J. Fuentes-Miranda<sup>1</sup>, F. J. Sánchez-García<sup>1</sup>, O. Rojas-Espinosa<sup>1</sup>, R. Salinas-Tobón<sup>1</sup>, M. B. Moreno-Altamirano<sup>1</sup><sup>1</sup>ENCB – IPN, Immunology, Mexico, D.F., Mexico

**Aims:** To study if dengue virus serotype-2 (DEN-2) has any effect in the proliferation of human T lymphocytes in response to PMA/Ionomycin or anti-CD3/anti CD28 and if so, to analyze whether intracellular calcium mobilization is involved.

**Methods:** Dengue-2 was propagated on C6/36 cells. Virus activity and titer were assessed by non-structural 1 protein (NS1) synthesis by ELISA (Platelia) and Plaque Forming Units. Human peripheral blood mononuclear cells (PBMC) were separated from Buffy coats by Ficoll-Hypaque gradients. PBMC were cultured and passed through nylon wool columns to enrich the T cell fraction. Cell viability was determined by trypan blue exclusion. Proliferation assays were performed as follows: 2x10<sup>5</sup> CFSE labeled cells/well were cultured in medium alone or stimulated with PMA (50ng/ml), Ionomycin (500ng/ml) or PMA plus Ionomycin, in the presence or in the absence of DEN-2, in which case DEN-2 was added to the cultures 30 min before the mitogenic stimulus. Cells were cultured for 72 h and proliferation assessed by CFSE labeling. Intracellular calcium mobilization was assessed by calcium indicator Fluo-4 (Invitrogen) and flow cytometry. Fluo-4 pulsed cells in a 2mM CaCl<sub>2</sub> medium were assayed for base intracellular calcium concentration and then stimulated with PMA/Ionomycin with or without DEN-2, as indicated. Intracellular calcium mobilization was followed for about 7 min post-stimulus.

**Results:** Cells cultured in the presence of DEN-2 did not show any significant difference in proliferation as compared to cells cultured in medium alone. DEN-2 inhibited PMA/Ionomycin-induced T cell proliferation by about 80%. Pre-incubation of T cells with DEN-2 did not have any effect on PMA/Ionomycin-induced intracellular calcium mobilization.

**Conclusions:** Dengue virus inhibits T lymphocyte proliferation no matter whether T cells are in contact with DEN-2 before or several hours after the mitogenic stimulus and apparently, calcium influx is not modified by DEN-2. Although several reports suggest that T cells are not infected by dengue virus, their ability to inhibit T cell proliferation is a matter of investigation. Here we suggest that mechanisms other than alterations in calcium influx are involved in the ability of dengue virus to inhibit T cell proliferation.

Supported by CONAYT46767,COFAA and SIP-IPN.

**PC25/22 LOCALIZATION AND FINE MAPPING OF AN ANTIGENIC SITE ON THE NUCLEOCAPSID PROTEIN OF HUMAN PARAINFLUENZA VIRUS TYPE 3**A. Zvirbliene<sup>1</sup>, I. Sezaite<sup>1</sup>, M. Pleckaityte<sup>1</sup>, I. Kucinskiute-Kodze<sup>1</sup>, M. Juozapaitis<sup>1</sup>, K. Sasnauskas<sup>1</sup><sup>1</sup>Institute of Biotechnology, Vilnius, Lithuania

**Objectives:** Human parainfluenza virus type 3 (hPIV3) is a respiratory tract pathogen. The current study was aimed at investigating immunodominant regions of hPIV3 nucleocapsid (N) protein by using monoclonal antibodies (MAbs) raised against recombinant N protein and human serum specimens from hPIV3-infected individuals.

**Methods and Results:** A panel of murine MAbs was generated following immunization with yeast-expressed hPIV3 N protein self-assembled to nucleocapsid-like particles. All MAbs recognized native viral nucleocapsids in hPIV3-infected cells as confirmed by an indirect immunofluorescence analysis. Antigenic sites recognized by the MAbs were mapped using recombinant overlapping N protein fragments. One major immunodominant site was identified in the carboxy-terminal region (amino acids [aa] 397 to 486) of hPIV3 N protein. Further analysis with smaller N protein fragments and a synthetic peptide revealed one linear epitope representing aa 437 to 446 of the N protein located within this antigenic site. This epitope was reactive with about 50% of hPIV3 IgG-positive human sera. These results suggest that the above antigenic site on the N protein is important in eliciting humoral immune response against hPIV3.

**Conclusion:** The current study enhances the knowledge of the antigenic structure of hPIV3 N protein and may facilitate the development of better diagnostic methods for hPIV3 infection.

**PC25/23 MXA GENE POLYMORPHISMS IN SUBACUTE SCLEROSING PANENCEPHALITIS**N. B. Hasbal<sup>1</sup>, S. P. Yentür<sup>1</sup>, C. Gürses<sup>1</sup>, V. Demirebilek<sup>2</sup>, H. Cetin<sup>1</sup>, S. Uysal<sup>3</sup>, Z. Yapiçci<sup>4</sup>, G. Yilmaz<sup>5</sup>, O. Cokar<sup>6</sup>, E. Onal<sup>7</sup>, C. Yalcinkaya<sup>2</sup>, Ü. Kuru<sup>8</sup>, A. Gökyigit<sup>4</sup>, G. Saruhan-Direskeneli<sup>1</sup>

<sup>1</sup>I. U. Istanbul Medical Faculty, Physiology, Istanbul, Turkey, <sup>2</sup>I. U. Cerrahpasa Medical Faculty, Neurology, Istanbul, Turkey, <sup>3</sup>I. U. Cerrahpasa Medical Faculty, Pediatrics, Istanbul, Turkey, <sup>4</sup>I. U. Istanbul Medical Faculty, Neurology, Istanbul, Turkey, <sup>5</sup>I. U. Istanbul Medical Faculty, Microbiology, Istanbul, Turkey, <sup>6</sup>Haseki Hospital, Neurology, Istanbul, Turkey, <sup>7</sup>I. U. Istanbul Medical Faculty, Public Health, Istanbul, Turkey, <sup>8</sup>Bayrampasa Public Hospital, Pediatrics, Istanbul, Turkey

**Objective:** Subacute Sclerosing Panencephalitis (SSPE) is a fatal degenerative and slowly progressive neuronal disease of childhood caused by persistent infection of measles virus (MV). In addition to viral factors, host factors seem to contribute to the development of SSPE. Myxovirus A (MxA) protein is induced by interferon  $\alpha/\beta$  and inhibits the replication of single-stranded RNA viruses including MV. This protein may play a role in the persistence of MV in human neurons. Two single nucleotide polymorphisms (SNP) at the promoter of MxA gene have been screened previously in SSPE. MxA -88 G/T polymorphism was associated with the development of SSPE, whereas -123 A/C was not. We have tried to replicate this association in the Turkish patients.

**Methods:** In this study, exon 14890 G/A (rs469390), promoter -88 G/T and -123A/C SNPs of MxA gene were investigated with the DNA samples of 119 SSPE patients and compared with age matched 116 children. The mean age of SSPE patients was 9.0 and 67.2% of them were boys. In the control group, the mean age was 8.7 and 47.4% of them were boys. PCR-RFLP method was used and the results were compared by using  $\chi$ -square test.

**Results:** The distribution of MxA exon SNP revealed genotype frequencies of 53.4 vs. 57.5% of GA, 23.3 vs. 25.7% of GG and 23.3 vs. 16.8% of AA. The distribution of MxA -88 SNP revealed genotype frequencies of GT as 48.6 vs. 56.4%, GG as 43.9 vs. 37.6% and TT as 7.5 vs. 5.9%. MxA -123 CA, CC and AA genotypes were present in 46.8, 44.0 and 9.2% of patients and 53.4, 36.9 and 10.7% of controls. The differences between SSPE patients and controls were not significant.

**Conclusion:** These three MxA polymorphisms in the exon (14890 G/A) and promoter of MxA gene (-88 G/T and -123C/A) were not associated with SSPE in Turkey.

This work was supported by a grant from Istanbul University Research Fund.

**PC25/24 DETECTION OF BOVINE LEUKAEMIA VIRUS INFECTION IN HUMAN AND CATTLE SAMPLES**G. Nikbakht Bruni<sup>1</sup>, M. Emam<sup>2</sup>, M. Rabbani<sup>3</sup>, E. Tofighi<sup>4</sup>

<sup>1</sup>University of Tehran, Faculty of Veterinary Medicine, Microbiology and Immunology, Tehran, Iran, Islamic Republic of, <sup>2</sup>University of Tehran, Tehran, Iran, Islamic Republic of, <sup>3</sup>University of Isfahan, Isfahan, Iran, Islamic Republic of, <sup>4</sup>University of Chamran, Ahvaz, Iran, Islamic Republic of

Bovine leukaemia virus (BLV) is a retrovirus responsible for lymphoproliferative disorders in ruminants. Marketed beef and dairy products from infected animals might be an important source for human infection. Although BLV infection in animals is well known, little is known about its possible occurrence in humans. This

study investigated for the presence of anti-BLV antibodies and BLV provirus in human and cattle samples. For detection of antibodies to BLV, indirect enzyme-linked immunosorbent assay (ELISA) was used and for detection of BLV provirus, nested PCR was used. The overall prevalence of anti-BLV antibodies in the human and cattle samples were 12.50% and 16.73%, respectively. When using ELISA as a reference test, sensitivity and specificity for nested PCR were 0.625 and 0.970, respectively. Predictive value of a positive test was 0.862, and predictive value of a negative test was 0.897. The percentage of cows correctly classified by nested PCR assay was 89.1%. Nested PCR and Southern blot analysis, using primers specific for BLV gag sequences revealed that BLV provirus was detectable in cattle and humans. Our results suggest the risk of human exposure to BLV and the need for further investigations to determine whether BLV infection is a health hazard for humans.

#### PC25/25 REGULATORY MECHANISMS OF CHRONIC INFLAMMATION DURING HERPESVIRUS INFECTION IN CHILDHOOD

H.M. Stamenkovic<sup>1</sup>, B.A. Kamenov<sup>1</sup>, L. Saranac<sup>2</sup>

<sup>1</sup>Clinic for Paediatric Medicine, Immunology and Allergology, Nis, Serbia, <sup>2</sup>Clinic for Paediatric Medicine, Endocrinology, Nis, Serbia

**Introduction:** An important characteristic of the herpes group viruses (HSV, CMV, EBV) is their ability to persist in the tissues of their hosts for many years after initial infection as intracellular viruses. Characteristic life of virus (chronic persistent and cyclic replication) in organisms is often followed by immune dysregulation (Th1 or Th2 tip immune response). Chronic stimulation of immune system and immunodeficiency are development by virus persisting in organisms.

**Materials and Methods:** Clinically manifestations in patients with herpesvirus infections were examined. We analysed: white blood cell count, hemoglobin level, serum immunoglobulins level, enzymes of cell destruction (LDH, CPK, AST and ALT), oxidative metabolism of the peripheral blood phagocytes as ability of NBT reduction, ELISA test of antibody for one of the viruses: HSV, CMV and EBV. Serum level of IFN- $\gamma$ , IL-4 and DHEAS, cortisol were measured by ELISA test.

**Results:** Our patients had and all of them had positive ELISA test on one of viruses (CMV, HSV or EBV). This were initial parameters for separate our patients in our analysis. Our parameters approved low level of hemoglobin, monocytosis, lymphocytosis, virocytosis and leukopenia. Our patients had high level LDH, CPK, low ability of NBT reduction and hypergammaglobulinemia. High levels of IFN $\gamma$  (70%) followed high levels of LDH, CPK, GOT and GPT. Decrease levels of DHEAS and cortisol opposite control group were evident.

**Conclusion:** Chronic activation of immune system is background of pathogenetic mechanisms during herpes virus infection. Different level of DHEAS and cortisol are part of regulatory mechanisms of immune response across endocrine system. Increase levels of DHEAS in our patients can display chronic inflammation. Absence of increase level of cortisol may suggestion that our patients had a little "acute" phase of infection opposite a lot of chronic disorders. Increase level of IFN- $\gamma$  can suggestion on dominant Th1 response in our patients. Analyse of immunoregulatory mechanisms is essential to order level and place of damage cells, tissue and organs. It is important for therapy and prognosis of disease.

#### PC25/26 IMMUNE RESPONSE EVALUATION IN FULMINANTLY DEAD PATIENTS OF CRIMEAN-CONGO HEMORRHAGIC FEVER IN IRAN

S. Chinikar<sup>1</sup>, A. Ghalyanchi Langeroudi<sup>1</sup>, S.M. Ghiasi<sup>1</sup>, R. Mirahmadi<sup>1</sup>, M. Moradi<sup>1</sup>, T. Jalali<sup>1</sup>, M. Rahpeyma<sup>1</sup>, M. Ohani Markarian<sup>1</sup>, N. Afzali<sup>1</sup>, H.S. Taghavi Larijani<sup>1</sup>

<sup>1</sup>Pasteur Institute of Iran, Tehran, Iran, Islamic Republic of

**Objectives:** Crimean-Congo Hemorrhagic Fever (CCHF) is a zoonotic disease that can develop into a severe hemorrhagic fever in humans. CCHF virus is a member of the genus *Nairovirus*, family *Bunyaviridae*. This tick-borne pathogen is transmitted to humans primarily by the bite of an infected Ixodid ticks, or by direct contact with blood or tissues of infected livestock. CCHF is a potentially fatal disease affecting multiple organ system. It has a case-fatality rate of 10-80%. To checking immune response in fulminantly CCHF dead patients we did this experiment.

**Methods:** In this study from 1999 to 2009 (February), we analyzed 76 sera of CCHF dead patients for anti-CCHF virus IgM antibody through IgM specific ELISA. Similarly, the sera were investigated for CCHF virus genome through real-time and gel-based RT-PCR.

**Results:** Among 76 CCHF dead patients, 40 patients had no antibody response. Notably, all these 40 patients (fulminantly dead patients) were RT-PCR positive.

**Discussion:** The results show that no antibody response was initiated in fulminant dead cases. Molecular assay could only detect the virus genome in these fulminantly dead patients; so, this assay is taken into more account for laboratory diagnosis of CCHF. Prospectively, for precise study in difficult aspects of CCHF virus pathogenesis, evaluation of inflammatory mediators of immune system is required.

#### PC25/27 ANALYSIS OF MIP 1 ALFA , MCP1 Y RANTES EXPRESSION IN A MODEL THAT RESEMBLE EARLY IMMUNE RESPONSE IN THE CUBAN DENGUE EXCEPTIONAL CONDITIONS

B. Sierra<sup>1</sup>, K. Vogt<sup>2</sup>, A. Pérez<sup>1</sup>, G. García<sup>3</sup>, K. Schmolke<sup>2</sup>, E. Aguirre<sup>1</sup>, M. Alvarez<sup>1</sup>, H.-D. Volk<sup>2</sup>, M.G. Guzman<sup>1</sup>

<sup>1</sup>Institute for Tropical Medicine 'Pedro Kouri', Havana, Cuba, <sup>2</sup>Institute for Medical Immunology, Charité-Universitätsmedizin Berlin, Berlin, Germany, <sup>3</sup>Institute for Tropical Medicine 'Pedro Kouri', Havana, Cuba

Dengue fever/dengue hemorrhagic fever (DF/DHF) has emerged as the most important mosquito-borne viral diseases in tropical areas. The dengue virus (DV) has become endemic in most tropical urban centers throughout the world, and DHF has appeared concomitantly with this expansion. Given the fact that intensity of DV replication during the early times of infection could determine clinical outcomes, which ranges from febrile illness (DF) to life-threatening disease (DHF), it is important to understand the impact of DV infection on innate immunity, the earliest defence against microbial infection, that also profoundly regulates the adaptive T-B immune responses. In this study we demonstrated the strong inflammatory cell activation induced in peripheral blood mononuclear cell after 24 culture with dengue virus through the analysis of the gene expression and quantification in supernatant of IL8, MIP-1  $\alpha$ , CCR1, MCP-1 and Rantes.

### PD09 – IMMUNOTHERAPY OF TUMORS

#### PD09/1 CROSSTALK BETWEEN CD4+ T CELLS AND LEUKEMIC CELLS IN VIVO LEADS TO LEUKEMIC-APC FORMATION AND AN EFFICIENT ANTI-TUMOR EFFECT BY CD4+ DONOR LYMPHOCYTE INFUSION IN A NOD/SCID MOUSE MODEL FOR HUMAN ACUTE LEUKEMIA

S. Stevanovic<sup>1</sup>, M. Griffioen<sup>1</sup>, B.A. Nijmeijer<sup>1</sup>, M.L.J. Van Schie<sup>1</sup>, R. Willemze<sup>1</sup>, J.H.F. Falkenburg<sup>1</sup>

<sup>1</sup>Leiden University Medical Center, Experimental Hematology, Leiden, Netherlands

Donor T-cells administered by donor lymphocyte infusion (DLI) after allogeneic stem cell transplantation may not only mediate Graft-versus-Leukemia (GvL) reactivity, but also induce Graft-versus-Host Disease (GvHD). Since HLA-class II molecules are predominantly expressed on hematopoietic cells, CD4+ donor T-cells may selectively mediate GvL reactivity without GvHD. Clinical efficacy of CD8+ T-cell depleted DLI has been shown, but it remains unknown whether GvL reactivity and/or GvHD observed in these patients are mediated by CD4+ T-cells or by contaminating CD8+ T-cells in the DLI or residual CD8+ T-cells in the patient. We assessed the capacity of human CD4+ donor T-cells to mediate GvL reactivity in a NOD/scid mouse model for human acute leukemia. Mice inoculated with primary acute lymphoblastic leukemic cells from different patients were treated with purified CD4+ DLI (consisting of CD4+ T-cells) or CD3+ DLI (consisting of CD4+ and CD8+ T-cells) from various donors. Highly purified CD4+ DLI was demonstrated to efficiently eradicate leukemic cells, with similar kinetics as CD3+ DLI. In mice treated with DLI, the leukemic cells acquired in vivo an antigen presenting cell (APC) phenotype, characterized by upregulation of HLA-class II, adhesion and costimulatory molecules, whereas leukemic cells from untreated mice remained unchanged. Clonal isolation of CD4+ T-cells after CD4+ DLI showed that 97% of isolated CD4+ T-cell clones (n=118) recognized primary leukemic cells that were in vitro modified into APC (leukemic-APC). Further characterization of 30 CD4+ T-cell clones with strong reactivity against leukemic-APC revealed that 35% of the clones also showed strong reactivity against primary leukemic cells, whereas 65% of the clones were not reactive against primary leukemic cells both in IFN- $\gamma$  ELISA as well as <sup>51</sup>Cr-release cytotoxicity assays. Finally, in vitro experiments demonstrated that primary leukemic cells acquired an APC phenotype when cocultured with leukemia-specific, but not non-specific, CD4+ T-cell clones. In conclusion, our data show that CD4+ T-cells can be potent effector cells and sole mediators of anti-tumor responses, and provide evidence that crosstalk between leukemic cells and specific CD4+ T-cells in vivo is the fundamental basis for induction of leukemic cells with APC phenotype and efficient anti-tumor immunity.

#### PD09/2 EFFECTIVE TUMOR IMMUNOTHERAPY WITH ENGINEERED HUMAN NEOVESSELS SECRETING RECOMBINANT BISPECIFIC ANTIBODIES

M. Compte<sup>1</sup>, V. Alonso-Camino<sup>1</sup>, P. Santos Valle<sup>1</sup>, Á.M. Cuesta<sup>1</sup>, D. Sánchez-Martín<sup>1</sup>, M. Rodríguez López<sup>1</sup>, J.L. Vicario<sup>2</sup>, L. Sanz<sup>1</sup>, L. Álvarez-Vallina<sup>1</sup>

<sup>1</sup>Hospital Universitario Puerta de Hierro – Majadahonda, Molecular Immunology Unit, Majadahonda, Spain, <sup>2</sup>Centro de Transfusiones de la Comunidad Autónoma de Madrid, Madrid, Spain

Recent studies showed that a network of human mature blood vessels can be formed by co-implantation of primary human endothelial cells (EC) and human mesenchymal stem cells (MSC) in immunodeficient mice. These findings have generated considerable interest in the potential application of engineered blood vessels in regenerative medicine and the opportunity for therapeutic intervention. In this context, the genetic modification of ECs would ensure the secretion of a therapeutic protein into the systemic circulation for a prolonged period of time.

Previously, we have reported that vasculature generated from lentivirally transduced human-umbilical vein endothelial cells (HUEVC) expressing firefly luciferase (HUEVC<sup>Luc</sup>) and co-implanted with MSC can be assessed quantitatively by *in vivo* bioluminescence imaging (BLI). The stability of vascular implants, the permissive-ness of HUEVC to be transduced by lentiviral vectors, encouraged us to use engineered blood vessels as therapeutic protein factories.



We employed a recombinant bispecific diabody with specificity for both the human tumor-associated carcinoembryonic antigen (CEA) and the CD3 $\epsilon$  chain of human TCR/CD3 complex (aCEA/aCD3). Mixtures of genetically modified HUVEC (HUVEC<sup>Δab</sup> or HUVEC<sup>Δlac</sup>) and MSC were embedded in Matrigel and inoculated contralaterally in the ventral area of nude mice. Vascular implants containing HUVEC<sup>Δlac</sup> exhibited stable luciferase activity for more than 30 days, indicating a good connection to the mouse circulatory system. Vascular implants containing HUVEC<sup>Δab</sup> (factory neovessels) supported diabody release into the bloodstream at detectable levels. Moreover, when factory neovessels were established into CEA-positive human colon cancer xenograft-bearing mice and human T lymphocytes were administered, secreted aCEA/aCD3 diabody activated T cells and promoted tumor cell lysis. Reduction of tumor growth in HUVEC<sup>Δab</sup>-treated mice was statistically significant when compared with animals that only received HUVEC<sup>Δlac</sup> implants. In summary, we demonstrate for the first time the therapeutic effect of a protein locally secreted by engineered human neovessels.

#### PD09/3 A HLA-A2 RESTRICTED T CELL RECEPTOR RECOGNIZING A SHARED TUMOR-ASSOCIATED ANTIGEN OF RENAL CELL CARCINOMA

A. Turqueti-Neves<sup>1</sup>, M. Leisegang<sup>2</sup>, B. Engels<sup>3</sup>, T. Blankenstein<sup>2,4</sup>, D. Schendel<sup>1</sup>, W. Uckert<sup>2,5</sup>, E. Noessner<sup>1</sup>

<sup>1</sup>Helmholtz Zentrum München, Institute of Molecular Immunology, Munich, Germany, <sup>2</sup>Max-Delbrück-Center for Molecular Medicine, Berlin, Germany, <sup>3</sup>University of Chicago, Department of Pathology and Committee on Immunology, Chicago, United States, <sup>4</sup>Free University of Berlin, Institute of Immunology, Berlin, Germany, <sup>5</sup>Humboldt-University of Berlin, Institute of Biology, Berlin, Germany

Renal cell carcinoma (RCC) is one of the few human solid tumors considered immunogenic. In fact, immune therapies for RCC are being intensively explored, as tumour resistance to radiation or chemotherapy leaves patients with limited treatment options. In particular, adoptive therapy is a promising strategy for the treatment of RCC and expectations are raised by the significant clinical success achieved in melanoma utilizing this approach. RCC has a long history of adoptive cell therapy, including the transfer of lymphokine activated killer cells or tumor infiltrating lymphocytes (TIL) alone or in combination with IL-2 and most recently allogeneic hematopoietic stem cell transplantation. A broad implementation of the adoptive T cell therapy for RCC and the augmentation of efficacy are hampered by the poor availability of tumor-specific T cells for patient infusion. We identified among TIL, isolated from RCC tumors, those with MHC-class I restriction. One TIL population (TIL-53) showed reactivity against several HLA-A2 positive tumor lines without recognizing normal kidney cells. The T cell receptor (TCR) specificity was rescued through T cell cloning, TCR sequence identification and retroviral engineering. Murinization and codon optimization achieved high TCR expression and optimal function of the TCR sequences after transduction into peripheral blood lymphocytes. Donor lymphocytes transduced with the optimized TCR53 recapitulated the specificity of the parental TIL-53 population, including HLA-A2 restriction, shared recognition of RCC lines and no recognition of normal tissue. 60% of RCC lines expressing HLA-A2 endogenously or after transfection were recognized by TCR53 (N=30). No recognition was observed for 17 HLA-A2 positive normal kidney cultures and other non-tumor cells (N=6). The TCR53-pMHC ligand seems also to be expressed in some tumors of other histologies. TCR53-engineered T cells were cytotoxic toward tumor lines expressing the TCR53-pMHC ligand, secreted IFN- $\gamma$ , IL-2, TNF- $\alpha$  and MIP-1 $\beta$ . Broad tumor recognition, complex effector function and restriction by the prevalent HLA-A2 class I molecule provide TCR53-engineered T lymphocytes with the required hallmarks for beneficial application in adoptive T cell therapy.

#### PD09/4 REDUCTION OF ADENOSINE PRODUCTION AND BLOCKADE OF ITS A<sub>2A</sub> RECEPTOR REDUCE TUMORIGENICITY

H. Eini<sup>1</sup>, E. C. Lewis<sup>1</sup>, C. Chaimovitz<sup>2</sup>, A. Douvdevani<sup>1,3</sup>

<sup>1</sup>Ben-Gurion University of the Negev, Department of Clinical Biochemistry, Beer-Sheva, Israel, <sup>2</sup>Ben-Gurion University of the Negev, Beer-Sheva, Israel, <sup>3</sup>Soroka Medical Center, Beer-Sheva, Israel

**Objectives:** Adenosine is one of the factors contributing to tumor growth and survival by a variety of mechanisms. It accumulates in solid tumors at high concentrations, and has been shown to stimulate angiogenesis and to inhibit the activity of immune cells. These functions are mainly mediated by the ligation of adenosine to the A<sub>2A</sub> receptor (A<sub>2A</sub>R).

There are several independent sources of adenosine, including cell death and nucleotide degradation, however the constant release of adenosine could be counteracted by its deamination which catalyzed by adenosine deaminase (ADA). Here, we examine the cellular mechanisms underlying the ability of adenosine to mediate tumor escape from immune surveillance.

**Methods:** Tumors were induced in wild-type mice by 3-methylcholanthrene (3-MCA) intramuscular injection (200 mg/mouse). Several cell lines were developed by isolating cells from excised tumors. Established cell lines were injected into mouse footpad, and tumor growth and cytokine profile were monitored. To examine the role of adenosine in tumor progression, cell lines were injected to A<sub>2A</sub>R antagonist-treated mice and A<sub>2A</sub>R<sup>-/-</sup> mice. In addition, wild-type mice were injected with fibrosarcoma tumor cells that were genetically modified to express secretable form of ADA.

**Results:** Our preliminary results indicate that A<sub>2A</sub>R<sup>-/-</sup> mice have reduced rate and delayed tumor development after tumor induction by the complete carcinogen 3-MCA. Accordingly, treatment of mice with A<sub>2A</sub>R antagonist, caffeine (0.1% in drinking water), significantly decreased the rate of tumor-bearing mice after 3-MCA inoculation and induced spontaneously resolved autoimmunity. Injection of a weakly tumorigenic cell line which was found to be rejected by WT mice showed elevated IL-15, IL-15R $\alpha$  and IFN $\gamma$  mRNA levels compared to the intact tissues. These cytokine levels were further increased following caffeine treatment. Interestingly, mice injected with high ADA-expressing cells showed significantly improved inhibition of tumor growth compared to low ADA-expressing cells.

**Conclusion:** Our data shows that adenosine may suppress immune response towards developing tumor through A<sub>2A</sub> receptor. Furthermore, the data suggests that a shift from adenosine-producing to adenosine-catabolizing phenotype may serve as a potential target to cancer therapy.

#### PD09/5 IDENTIFICATION OF FOUR NEW HLA-CLASS II RESTRICTED MINOR HISTOCOMPATIBILITY ANTIGENS IN ANTI-TUMOR IMMUNITY

A. N. Stumpf<sup>1</sup>, E. D. van der Meijden<sup>1</sup>, C. A. M. van Bergen<sup>1</sup>, R. Willemze<sup>1</sup>, J. H. F. Falkenburg<sup>1</sup>, M. Griffioen<sup>1</sup>

<sup>1</sup>Leiden University Medical Center, Department of Hematology, Leiden, Netherlands

**Objectives:** Donor lymphocyte infusion (DLI) can be an effective cellular immunotherapy for patients with hematological malignancies after HLA-matched allogeneic stem cell transplantation (alloSCT). The effect of DLI is mediated by donor-derived T-cells recognizing minor histocompatibility antigens (mHags) on malignant cells of the recipient. These T-cells may also induce Graft-versus-Host Disease (GVHD) when directed against mHags with broad expression on non-malignant tissues. HLA-class II is predominantly expressed on hematopoietic cells, and therefore, CD4+ T-cells may selectively confer graft versus leukemia (GvL) effect without GVHD. The aim of this study was to identify HLA-class II restricted mHags in anti-tumor immunity.

**Methods:** CD4+ T-cell clones were isolated from a patient successfully treated with DLI for relapsed chronic myeloid leukemia (CML) more than one year after HLA-matched alloSCT. GvL reactivity in this patient was accompanied with mild GVHD of the skin. Reactivity of the T-cell clones was analyzed and HLA-class II mHags were characterized by screening a recombinant bacteria cDNA library.

**Results:** CD4+ T-cells specific for the previously identified HLA-DQ restricted P14K2B-derived mHag as well as T-cells recognizing 5 unknown mHags were isolated. By screening a recombinant bacteria cDNA library, we identified four new HLA-DR mHags encoded by the genes for MTHFD1, LY-75, PTK2B and MR-1. These genes all show selective or predominant expression in cells of hematopoietic origin, and the immunogenic variants have balanced population frequencies of 25-70%. All T-cell clones recognized high HLA-class II expressing B-cells, mature dendritic cells and in vitro cultured leukemic cells with antigen-presenting cell phenotype. The T-cell clone for the MTHFD1-derived mHag also showed direct recognition of CD34+ CML precursor cells.

**Conclusions:** We identified four new HLA-DR restricted mHags and provide evidence for different roles of these mHags in the onset and execution of GvL reactivity. The data also illustrate the efficacy of the recombinant bacteria cDNA library to identify HLA-class II mHags. The method, which is based on exogenous antigen delivery to EBV-transformed B cells, may also have broad value for characterization of non-polymorphic tissue specific antigens as targets for CD4+ T-cells in anti-tumor or autoimmunity.

#### PD09/6 IMMUNOTHERAPY (IT) OF NEUROBLASTOMA (NB) BY AN INTERLEUKIN-21-SECRETING CELL VACCINE IS POTENTIATED BY TRANSIENT CD4+ T CELL DEPLETION

M. Croce<sup>1,2</sup>, A. M. Orengo<sup>1</sup>, M. Borghi<sup>1</sup>, A. Brizzolara<sup>1</sup>, V. Rigo<sup>1</sup>, R. Meazza<sup>3</sup>, B. Carlini<sup>2,4</sup>, V. Pistoi<sup>4</sup>, M. V. Corrias<sup>4</sup>, S. Ferrini<sup>1</sup>

<sup>1</sup>Lab of Immunological Therapy – Istituto nazionale per la ricerca sul cancro, Genoa, Italy, <sup>2</sup>Italian NB Foundation, Genoa, Italy, <sup>3</sup>Istituto Nazionale per la Ricerca sul Cancro, Genoa, Italy, <sup>4</sup>Lab of Oncology – Gaslini Institute, Genoa, Italy

**Objectives:** IL-21 is a new member of the IL-2 cytokine family. We studied, in a syngeneic metastatic NB (Neuroblastoma) model, the therapeutic efficacy of IL-21-gene-modified Neuro2a (Neuro2a/IL-21) NB cells as vaccine in combination with anti-CD25 or anti-CD4 mAbs.

**Methods:** Three doses of Neuro2a/IL-21 or empty-vector-Neuro2a (Neuro2a/mock) cells were injected sc in syngeneic A/J mice bearing Neuro2a parental cells (Neuro2a/pc) micrometastases (induced by i.v. challenge) and tumor-free survival was evaluated. Cytokine mRNA expression was studied by a quantitative cytokine PCR-Array.

**Results:** IT with Neuro2a/IL-21 cured 33% of mice bearing systemic NB, whereas Neuro2a/mock cells had no effect. The administration of anti-CD25 mAb (depleting Treg cells) slightly potentiated the effect of vaccine IT (50% cure rate), while anti-CD4 mAb treatment displayed a more potent effect, leading to 80% cure rate. These findings reflect a partial depletion of CD25+FoxP3+ Treg cells by anti-CD25 mAb and a more effective depletion by anti-CD4 mAb. A Winn assay showed that naïve CD4+ T cells coinjected with Neuro2a cells favoured NB growth in NOD-SCID mice. Syngeneic mice receiving vaccine+anti-CD4 mAbs recovered CD4+ T cell counts in about 90 days and developed immunity to Neuro2a cells. Depletion of CD8+ T cells abrogated the effect of the combined IT, indicating a predominant role of CTL. In spleen cells from mice receiving Neuro2a/IL-21 vaccination up-regulation of IL-17 gene cluster and of IFN $\alpha$ 2,  $\beta$ 1, and  $\gamma$  genes was observed. This may depend on the production of TGF $\beta$  and IL-6 Th17-polarizing cytokines by Neuro2a cells. CD4 mAb depletion reduced IL-17 gene cluster expression in vaccinated mice but IL-17 protein was still detectable in the sera.

**Conclusions:** Anti-CD4 mAb was more effective than anti-CD25 mAb in potentiating the effect of a IL-21-secreting vaccine by removing: 1) Treg cells and/or their precursors, 2) “cytokine sinks” that compete for IL-21, 3) a feeding effect of CD4<sup>+</sup> T cells on NB growth. Mice vaccinated with Neuro2a/IL-21 showed a Th17 skewing of the immune response but Th17 cells were not involved in IT, which was strictly dependent on CD8<sup>+</sup> T cells. *MC is the recipient of Italian Neuroblastoma Foundation fellowship*

#### PD09/7 TUMOR-SPECIFIC T HELPER 1 CELLS INFLUENCE THE DE-DIFFERENTIATION OF TUMOR CELLS IN AN ENDOGENOUS TUMOR MODEL

H. Braumüller<sup>1</sup>, T. Wieder<sup>1</sup>, N. Bauer<sup>1</sup>, M. Röcken<sup>1</sup>

<sup>1</sup>Eberhard-Karls-Universität Tübingen, Dermatology, Tübingen, Germany

In most cancer immuno-therapies CD8<sup>+</sup> T cells (CTL) are thought to be essential for anti-tumor responses by the adaptive immune system. However, in vitro obtained potent anti-tumor activity often fail to clear tumors in vivo. On the other hand, a few studies using adoptive transfer of CD4<sup>+</sup> T cells could show that T helper cells can eliminate tumors that are resistant to CTL-mediated rejection. To answer the question whether MHC class II restricted CD4<sup>+</sup> cells can control MHC class II-negative tumors, we used a model of an endogenous tumor, the RIP1-Tag2 mouse. The oncoprotein T antigen (Tag2) of the SV40 is expressed under the control of the rat insulin promoter (RIP1) in all insulin-producing beta cells of the pancreas. Tag2 expression successively induces the development of first islet cell hyperplasia, adenomas and then carcinomas. At 14-15 weeks, mice die of hypoglycemia. Adoptive transfer of interferon-gamma (IFN- $\gamma$ )-producing, Tag2-specific Th1 cells (Tag2-Th1) strongly inhibited tumor development. Tag-Th1-treated mice had small, barely vascularized tumors. Depletion of CTL by a monoclonal antibody in vivo had no effect on Tag2-Th1-mediated survival or angiogenesis. In TUNEL assays we could show that the reduced tumor growth was not due to enhanced apoptosis of the tumor cells. However, in BrdU assays we could show a significant reduction of proliferating tumor cells. Based on these results, we asked whether Tag2-Th1 cells may retard de-differentiation and analyzed two beta cell-specific markers. In normal islets all beta cells express insulin and the beta cell-specific glucose transporter 2 (Glut2). In tumors of 12 weeks old RIP1-Tag2 only about half of the beta cells still express insulin whereas Glut2 is completely lost. In sharp contrast, in RIP1-Tag2 mice treated with Tag-Th1 cells all tumor cells express both insulin and Glut2. As normal islet cells do not divide any more, the observed reduction of tumor growth may be the result of a reduced loss of differentiation, leading to the inhibition of cell proliferation.

#### PD09/8 CHARACTERIZATION OF THE SPONTANEOUSLY INDUCED TUMOR-REACTIVE MEMORY T CELL REPERTOIRE IN MALIGNANT MELANOMA PATIENTS

C. Pfirschke<sup>1</sup>, C. Gebhardt<sup>2</sup>, A. Enk<sup>2</sup>, P. Beckhove<sup>1</sup>

<sup>1</sup>German Cancer Research Center (DKFZ), Translational Immunology Unit, Heidelberg, Germany, <sup>2</sup>University of Heidelberg, Department of Dermatology, Heidelberg, Germany

**Objectives:** Malignant melanoma is the leading cause of skin diseases related deaths. Hints for spontaneous tumor-specific T cell responses in malignant melanoma patients have recently been described. The studies mainly used HLA-A2 restricted peptides to investigate CD8<sup>+</sup> T cell responses against only few selected antigens. We here determined the specificity of spontaneously induced memory T cells against a broad variety of different melanoma tumor-associated antigens (TAAs) in the peripheral blood (PB) of melanoma patients in a HLA-A2 unrestricted setting using long peptides (50aa) that cover MHC class I- and II-restricted epitopes. Moreover, we investigated the contribution of regulatory T cells (Treg) to the activation status of tumor reactive memory T cells via depletion of Tregs from the analyzed T cell fraction.

**Methods:** PB samples were taken from melanoma patients (stage I, II). Dendritic cells (DC) were generated *in vitro* from monocytes and pulsed with synthetic 50aa peptides derived from immunodominant regions of described melanoma TAAs MelanA/MART-1, tyrosinase, gp100/pmel17, NY-Eso-1, p53, MDM2, NA17-A, TRP2, MAGE-A1, MAGE-A4, MAGE-C2, GAGE-1, GAGE-3, MIF, RAB38/NY-Mel-1 or with a negative control antigen (human IgG). Treg were depleted using  $\alpha$ CD25-magnetic beads. Presence of antigen-specific T cells was analyzed *ex vivo* by a 40-hour IFN $\gamma$  ELISPOT assay. Statistical comparison was performed by student's t test.

**Results:** More than 60% T cell samples reacted against at least one tested polypeptide.

T cells of HLA-A2 positive, as well as negative patients recognized the polypeptides to a similar extent. On average 3 (1-4) different antigens were recognized per tested sample. Some peptides, such as MelanA/MART-1, were preferentially recognized (33%) while other melanoma-associated antigens, such as gp100/pmel17 elicited spontaneous T cell responses only rarely (6%). T cell reactivity against some (MelanA/MART-1, p53), but not all antigens (NA17-A, TRP-2) was increased after depletion of Treg.

**Conclusion:** Our data demonstrate that a polyvalent repertoire of tumor-reactive memory T cells exists in a majority of malignant melanoma patients. This repertoire is partially controlled by Tregs. Some antigens appear to be dominant targets of spontaneous T effector/Treg responses providing a potential new criterion for selection of vaccine antigens.

#### PD09/9 ORAL ADMINISTRATION OF TNF $\alpha$ -EXPRESSING SALMONELLA INHIBITS TUMOR GROWTH AND PROLONGS HOST SURVIVAL INDEPENDENT OF INTRA-TUMORAL BACTERIAL ACCUMULATION

B. Al-Ramadi<sup>1</sup>, H. El-Hasasna<sup>1</sup>, M.J. Fernandez-Cabezudo<sup>2</sup>, G. Bashir<sup>1</sup>, S. Chouaib<sup>3</sup>

<sup>1</sup>Faculty of Medicine, UAE University, Medical Microbiology & Immunology, Al Ain, United Arab Emirates, <sup>2</sup>Faculty of Medicine, UAE University, Biochemistry, Al Ain, United Arab Emirates, <sup>3</sup>National de la Sante et de la Recherche Medicale, Institut Gustave Roussy, Villejuif, France

**Objectives:** We previously reported that attenuated Salmonella strains, engineered to express eukaryotic cytokines, have the capacity to retard the growth of experimental melanomas in a syngeneic murine model. The systemic administration of a bacterial strain expressing IL-2 resulted in a superior degree of inhibition of tumor growth, concomitant with enhanced host survival, in comparison with the parental non cytokine-expressing strain (BRD509). The purpose of the current study was to evaluate the anti-tumor potential of a recombinant strain of *S. typhimurium* which has been engineered to express murine TNF- $\alpha$  (designated GIDTNF strain).

**Methods:** The GIDTNF strain was administered to B16 melanoma-bearing mice via the i.p. or oral routes. Tumor growth and animal survival were then followed for up to 4 weeks post treatment initiation. In a separate set of experiments, we also assessed bacterial burden in various organs and within tumor tissue at different time points post treatment. In all experiments, the effect of GIDTNF was evaluated in comparison with the parental, non cytokine-expressing, *Salmonella* strain (designated BRD509).

**Results:** Intraperitoneal inoculation of GIDTNF strain resulted in a preferential accumulation within tumor tissue as compared with other target organs and >80% inhibition in tumor growth at early time points but was also associated with substantial general toxicity that resulted in high mortality rates among treated mice. In contrast, oral administration of GIDTNF produced significantly higher rates of tumor retardation but this wasn't associated with increased mortality. This was particularly evident in the first 7-10 days after a single dose of treatment when GIDTNF resulted in 79% retardation of tumor growth compared to 21% in BRD509-treated mice. Animal survival was also improved significantly, with 80% of treated mice surviving beyond 16 days post treatment as supposed to 0% in non-treated or BRD509-treated groups. Surprisingly, despite this strong anti-tumor effect of oral GIDTNF organisms, no bacteria could be detected in tumor tissue.

**Conclusion:** The data strongly suggests that the anti-tumor properties of TNF $\alpha$ -expressing *Salmonella* bacteria are unrelated to their ability to home to and multiply inside tumors. The precise anti-tumor mechanism(s) of live *Salmonella* bacteria are under study.

#### PD09/10 CTLs SPECIFIC FOR ALLO-HLA-A2-CD20 KILL CHRONIC LYMPHOID LEUKEMIA CELLS AND DISPLAY COMPLEMENTARITY-DETERMINING REGION 3 HOMOLOGY

I. W. Abrahamsen<sup>1</sup>, E. Strömen<sup>1</sup>, J. Johansen<sup>1</sup>, S. Wälchli<sup>1</sup>, S. Kumar<sup>1</sup>, G. Gaudernack<sup>1</sup>, G. Tjønnfjord<sup>2</sup>, F. Lund-Johansen<sup>2</sup>, J. Olweus<sup>1</sup>

<sup>1</sup>Institute for Cancer Research, Radiumhospitalet, Oslo University Hospital, Department of Immunology, Oslo, Norway, <sup>2</sup>Department of Medicine, Rikshospitalet, Oslo University Hospital, Section for Hematology, Oslo, Norway

**Objectives:** The aim of the current study was to target allo-reactive T cells to defined cell types, which may have important implications for adoptive immunotherapy of cancer. Allo-recognition appears to involve the peptide. On the other hand, the high frequency of allo-reactive T cells may indicate that a given peptide recruits a wider range of T cell receptors (TCRs) when presented on a foreign HLA-molecule. Here, we show that an HLA-peptide complex found on B cells only (HLA-A\*0201/CD20), engages a highly restricted and conserved TCR repertoire in HLA-A2 negative donors.

**Methods and results:** Dendritic cells were transfected with a foreign HLA molecule (HLA-A2), pulsed with a peptide derived from CD20, and co-cultured with autologous T cells. Whereas T cells from HLA-A2 positive individuals are tolerant to this self-antigen, reactive T cells were obtained from 6/6 HLA-A2 negative donors after only 19 days of co-culture. T cell clones isolated from four donors showed a remarkable conservation in the lengths and sequences of the TCRbeta complementarity-determining region 3 (CDR3). Homology in CDR3 is commonly observed across individuals for T cell repertoires recognizing viral epitopes presented by a shared HLA-allele, and is associated with high avidity and specificity. In contrast, the four donors from which the HLA-A2 negative, CD20-specific T cells were isolated, shared no HLA-allele. The T cells selectively bound cognate pentamers, and killed primary chronic lymphocytic leukemia cells (CLL) from HLA-A2 positive donors, while sparing non-B cells and HLA-A2 negative CLL cells. The CD20-specific T cells furthermore responded vigorously to human embryonic kidney cells (HEK293) following co-transfection with HLA-A2 and CD20, but not when transfected with CD20 or HLA-A2 alone, indicating a high degree of specificity.

**Conclusion:** The results demonstrate that negative selection is not essential for TCR repertoire focusing, and suggest that HLA-peptide complexes found on defined cell types can be targets for immunotherapy of cancer using allo-reactive T cells or T cell receptors.

**PD09/11 HSP90 INHIBITORS INCREASE MHC CLASS I-RELATED CHAIN A AND B LIGANDS AND SENSITIVITY TO NK CELL-MEDIATED CYTOTOXICITY IN MULTIPLE MYELOMA CELLS**

C. Fionda<sup>1,2</sup>, A. Soriani<sup>1</sup>, G. Malgarini<sup>1,2</sup>, M.L. Iannitto<sup>1</sup>, A. Santoni<sup>1,2</sup>, M. Cippitelli<sup>1,2</sup>

<sup>1</sup>Sapienza – University of Rome, Department of Experimental Medicine, Rome, Italy, <sup>2</sup>Regina Elena Cancer Institute, C.R.S. - Immunology, Rome, Italy

Modulation of host immune system represents a promising therapeutic approach against cancer, including multiple myeloma (MM), an aggressive hematologic malignancy that remains incurable. Recent findings indicate that the NKG2D and DNAM-1 activating receptors play a prominent role in tumor recognition and elimination by cytotoxic effector lymphocytes, suggesting that the levels of NKG2D ligands and DNAM-1 ligands expression on target tumor cells may be one critical factor to improve the immune response against cancer. In this study, we tested the effect of 17-allylaminogeldanamycin (17AAG) and Radicol, drugs targeting the Hsp90 chaperone protein and displaying anti-myeloma activity, on the expression of NKG2D and DNAM-1 ligands in human myeloma cell lines. We demonstrate that Hsp90 inhibitors are able to up-regulate both MICA and MICB protein surface and mRNA expression in human myeloma cell lines. Although Hsp90 inhibition is often associated with the activation of the unfolded protein response (UPR), we observed only a partial activation of this pathway by 17AAG and Radicol. In addition, classical inducers of the endoplasmic reticulum stress did not enhance NKG2D/DNAM-1 ligand expression, suggesting that UPR activation is not sufficient to induce MICA/MICB expressions on myeloma cells, and may not be involved in their regulation by Hsp90 inhibitors. On the contrary, Hsp90 inhibitors-mediated activation of the transcription factor Heat Shock Factor-1 (HSF-1) is essential for the induced MICA/MICB expression. We found that knockdown of HSF-1 by shRNA interference blocks the up-regulation of NKG2D-ligands by 17AAG and Radicol. Indeed, *in vitro* and *in vivo* binding of HSF-1 to MICA and MICB promoters, in Hsp90 inhibitors-treated cells, indicates that it can enhance NKG2D-ligands expression at the transcriptional level. Finally, exposure to Hsp90 inhibitors renders myeloma cells more sensitive to natural killer (NK) mediated cytotoxicity, and a blocking Ab specific for NKG2D significantly reduces this effect, confirming the functional impact of higher MICA/MICB expression on NK recognition of target myeloma cells. Thus, our results provide evidence that targeting NKG2D-ligands expression may be an additional mechanism supporting the anti-myeloma activity of Hsp90 inhibitors and suggest their possible immunotherapeutic value.

**PD09/12 GLYCOLIPID ANTIGEN AS ADJUVANT FOR SKIN IMMUNIZATION AGAINST MELANOMA**

C.H. Tripp<sup>1</sup>, F. Sparber<sup>1</sup>, N. Romani<sup>1</sup>, I.F. Hermans<sup>2</sup>, P. Stoitzner<sup>1</sup>

<sup>1</sup>Innsbruck Medical University, Department of Dermatology, Innsbruck, Austria, <sup>2</sup>Malaghan Institute of Medical Research, Cancer Immunotherapy Group, Wellington, New Zealand

**Objectives:** Glycolipid antigens are currently being tested as adjuvant for immunotherapy as they are able to enhance T cell responses after being presented by DC to NKT cells. We were interested in testing the potential of glycolipid antigen as adjuvant for skin immunization against melanoma.

**Methods:** With flow cytometry analysis we examined the level of expression of CD1d and activation markers on murine skin DC and lymph node cells. The ability of skin DC to present the synthetic glycolipid  $\alpha$ -Galactosylceramide ( $\alpha$ -GalCer) to the NKT cell hybridoma cells (DN32.D3) was tested *in vitro*. T cell responses were investigated in mice after intradermal immunization with  $\alpha$ -GalCer plus ovalbumin protein (OVA) by pentamer stainings and *in vivo* killing assays. OVA as a tumor model antigen was used together with  $\alpha$ -GalCer for immunization against murine OVA-expressing B16-melanoma (B16.OVA). The involvement of skin DC in this process was tested by removal of the immunization site and with transgenic mice which allow the depletion of skin DC subsets.

**Results:** We observed that all skin DC subsets express CD1d upon migration to the lymph nodes and were able to present  $\alpha$ -GalCer to NKT cells *in vitro*. Intradermally injected  $\alpha$ -GalCer was presented by skin and lymph node DC to NKT cells *in vitro*. When we injected  $\alpha$ -GalCer intradermally into the skin, T, B and NKT cells upregulated activation marker. The application of  $\alpha$ -GalCer together with the tumor model antigen OVA induced expansion of cytotoxic CD8<sup>+</sup> T cells that could inhibit the growth of B16.OVA melanoma. However, mice that were depleted of skin DC subsets developed similar cytotoxic immune responses after intradermal immunization with  $\alpha$ -GalCer and OVA.

**Conclusion:** The synthetic glycolipid  $\alpha$ -GalCer is a useful adjuvant for intradermal immunization strategies through the skin, however, it works independently of migratory skin DC.

**PD09/13 REVERSAL OF IMMUNE TOLERANCE AND COMPLETE REGRESSION OF ADVANCED PRIMARY HGF-CDK4<sup>R24C</sup> MOUSE MELANOMAS FOLLOWING COMBINATION CHEMO-IMMUNOTHERAPY**

M. Renn<sup>1</sup>, M. Cron<sup>1</sup>, J. Kohlmeyer<sup>1</sup>, J. Landsberg<sup>1</sup>, E. Gaffal<sup>1</sup>, T. Tüting<sup>1</sup>

<sup>1</sup>University of Bonn, Department of Dermatology, Bonn, Germany

The development of therapeutic strategies to reverse immune tolerance in the tumor microenvironment *in vivo* represents a major goal of cancer immunology. Here we utilized the unique features of the genetically engineered Hgf-Cdk4<sup>R24C</sup> mouse model to identify a combination treatment protocol for melanoma. These mice develop primary cutaneous melanomas which grow progressively and metastasize in the absence of immunogenic foreign proteins as oncogene or antigen. As a consequence, primary and metastatic tumors evade innate and adaptive immune defense but naturally express lineage differentiation antigens which can be targeted with antigen-specific immunotherapy. However, primary tumors continue to grow despite infiltration with adoptively transferred, *in vivo* activated melanocyte-specific CD8<sup>+</sup> T cells. To overcome immune tolerance in the tumor microenvironment we developed a treatment protocol consisting of four complementary components:

- (1) chemotherapeutic preconditioning prior to
- (2) adoptive lymphocyte transfer and
- (3) viral vaccination followed by
- (4) adjuvant peritumoral injections of immunostimulatory nucleic acids.

Lymphocyte ablation and innate anti-viral immune stimulation cooperatively enhanced expansion and effector cell differentiation of adoptively transferred lymphocytes. The efficacy of the different treatment approaches converged in the tumor microenvironment and induced a strong cytotoxic inflammatory response enabling preferential recognition and destruction of melanoma cells. This combination chemo-immunotherapy caused complete regression and long-term cure of advanced, macroscopically visible primary melanomas in the skin with minimal autoimmune side effects. Our results in a clinically highly relevant experimental model provide a scientific rationale to evaluate similar strategies which unleash the power of innate and adaptive immune defense in future clinical trials.

**PD09/14 RHO GTPASES INHIBITORS AND IFN- $\gamma$  TREATMENT STIMULATES ANTI-MELANOMA IMMUNE RESPONSE**

C. Pich<sup>1,2</sup>, G. Sarabayrouse<sup>1</sup>, C. Cormary<sup>1</sup>, A.-F. Tilkin-Mariame<sup>1</sup>

<sup>1</sup>INSERM, U563, Toulouse, France, <sup>2</sup>Université Paul Sabatier, Toulouse, France

The lack of MHC class I and costimulatory molecules on tumour cells results in inefficient stimulation of tumour-reactive T lymphocytes. The costimulatory signal can be provided by members of the TNF/TNFR super family, including CD27, CD70, CD40, CD40L which are expressed on the T-cell membrane. Stably transfected murine melanoma cells, expressing CD40L, CD70 and MHC class I molecules enhanced the immune anti-tumour response and favour a long-lasting immune memory.

These observations suggest that new immunotherapeutic protocols, using melanoma cells expressing costimulatory molecules can be conceivable. Consequently, we tried to find drugs, which could enhance expression of costimulatory and MHC class I complexes on melanoma cells. We have shown that Rho GTPases inhibitors could be good candidates. Indeed, *in vitro* treatments with IFN- $\gamma$  and Rho GTPases inhibitors, such as statins, isoprenyl transferase inhibitors, C3 exotoxin and small interfering RNA against RhoA, are able to stimulate membrane expression of costimulatory and MHC class I molecules on murine and human melanoma cell lines. Furthermore, these treated melanoma cells favour *in vitro* the stimulation of specific CD8 T lymphocytes and *in vivo* the proliferation of these effector cells and the reduction of the tumor growth.

**PD09/15 TOLL-LIKE RECEPTOR-8 ENGAGEMENT ON ARTIFICIAL ANTIGEN-PRESENTING CELL-EXPANDED CD8<sup>+</sup> T LYMPHOCYTES**

J.-F. Chatillon<sup>1</sup>, C. Abasq<sup>1</sup>, E. Fauquemberg<sup>2</sup>, F. Bayeux<sup>1</sup>, A. Drouet<sup>2</sup>, J.-B. Latouche<sup>2</sup>, P. Musette<sup>1</sup>

<sup>1</sup>Inserm U905, Rouen, France, <sup>2</sup>Inserm U614, IFRMP 23, Institute for Biomedical Research, University of Rouen, Rouen, France

Adoptive transfer of *in vitro* activated and expanded tumor antigen-specific cytotoxic T lymphocytes (CTLs) is a promising approach to cure cancer but numerous difficulties are encountered. The main problem is to obtain highly cytotoxic cells that could home to the tumor.

Our team studied the impact of Toll-Like Receptor-8 (TLR8) engagement on peripheral CD8<sup>+</sup> T lymphocytes expanded by co-culture with artificial antigen-presenting cells (AAPCs, Latouche and Sadelain, Nat biotechnol, 2000).

These AAPCs have been transduced to recreate a HLA-A2.1 immunological synapse which presents MART-1, an auto-antigen which is overexpressed in melanoma, to CD8<sup>+</sup> T cells. We defined the optimal conditions for obtaining MART-1-specific CTLs from 6 A2.1+ healthy donors, using AAPCs and anti-MART-1 phycoerythrin (PE)-coupled pentamer.

After a 2 to 3 week co-culture, we obtained, in a donor-dependent manner, 3 to 25% of specific CD8<sup>+</sup> T cells. We found that CD8<sup>+</sup> T cells expressed TLR8 at the cell surface and in intracellular compartments. MART-1 specific T cells activated by TLR8 agonist (CL075) were able to induce an increase of cytotoxic activity against MART-1-pulsed target cells, between 10 and 20% at each tested ratio for two donors, and at the highest ratio for two other donors. However, TLR8 engagement did not change significantly the production of cytokines implicated in cytotoxicity (TNF- $\alpha$ , IFN- $\gamma$  and Granzyme B).

Here we confirmed the observation of Zarembler *et al* (2002) that TLR8 mRNA is present in CD8<sup>+</sup> T cell population. Furthermore, we found that TLR8 expression levels as well as the increase of cytotoxicity observed after TLR8 engagement were highly dependent on the donor. We could not correlate these effects with an increase of cytokine production, and further analyses are needed to understand the mechanisms underlying the role of TLR8 engagement on CD8<sup>+</sup> T cells.



**PD09/16 CD8<sup>+</sup> EFFECTOR T CELLS WITH A SINGLE SPECIFICITY REJECT LARGE GENETICALLY UNSTABLE TUMORS**K. Anders<sup>1</sup>, C. Buschow<sup>1</sup>, J. Charo<sup>1</sup>, T. Blankenstein<sup>1</sup><sup>1</sup>Max-Delbrück Center for Molecular Medicine, Berlin, Germany

Cancer cells are genetically unstable and, therefore, single therapies frequently select resistant clones, as shown for chemotherapy or drugs interfering with onco-gene activity. A priori, one would expect that adoptive T cell therapy similarly selects escape variants. We established a model that allowed direct comparison of therapeutic efficacy by targeting an oncogene via drug-mediated inactivation or CD8<sup>+</sup> effector T (T<sub>E</sub>) cells with a single peptide-specificity. Tumor cells were generated by expression in fibroblasts of a doxycycline (dox)-regulatable SV40 large T-firefly luciferase (TagLuc) fusion protein, which could be switched off *in vivo* and allowed visualization of tumor growth and regression by bioluminescence imaging. Switching off TagLuc expression by dox in large tumors always resulted in rapid regression, followed by growth of dox-unresponsive variants. Each variant revealed a unique point mutation in the dox-binding domain of the transactivator, demonstrating a seemingly unlimited reservoir of genetic variants in the tumor. In contrast, targeting TagLuc in large tumors (>500 mm<sup>3</sup>) by Tag peptide I-specific T<sub>E</sub> cells resulted in complete rejection and no escape variant ever emerged. These results demonstrate that T cells with a single specificity can reject large tumors containing many genetic variants. Furthermore, T cells but not “chemotherapy”-like treatment allows “bystander” elimination of genetic variants.

**PD09/17 A KEY REGULATORY ROLE OF THE TRANSCRIPTION FACTOR NFATC2 IN BRONCHIAL ADENOCARCINOMA VIA CD8<sup>+</sup> T LYMPHOCYTES**J.H. Maxeiner<sup>1</sup>, R. Karwot<sup>1</sup>, K.A. Sauer<sup>1</sup>, P. Schuster<sup>1</sup>, I. Boross<sup>1</sup>, M. Koslowski<sup>2</sup>, Ö. Türeci<sup>3</sup>, R. Wiewrodt<sup>3</sup>, M.F. Neurath<sup>4</sup>, S. Finotto<sup>1</sup>

<sup>1</sup>Laboratory of Cellular and Molecular Immunology of the Lung, I. Medical Clinic, Mainz, Germany, <sup>2</sup>Johannes Gutenberg-University, 2 Department of Internal Medicine III, Mainz, Germany, <sup>3</sup>Johannes Gutenberg-University, Department of Internal Medicine III, Mainz, Germany, <sup>4</sup>Institute of Molecular Medicine, University Mainz, Mainz, Germany

There is substantial evidence that cancer patients harbor tumor-reactive T cells, although their number and/or activity are apparently insufficient to eradicate tumors. How such tumor-reactive T cells can be expanded and activated to turn against established tumors is a promising scientific endeavor in effective immunotherapy for cancer. Naturally occurring CD25<sup>+</sup>CD4<sup>+</sup> Foxp-3<sup>+</sup> regulatory T (T<sub>reg</sub>) cells maintain immunologic self-tolerance in the periphery but also inhibit immune-surveillance against autologous tumor cells. NFAT (Nuclear Factor of Activated T cells) transcription factors were originally described as nuclear proteins that bind to and thus control the activity of the interleukin 2 (IL-2) promoter in T lymphocytes. IL-2 and IL-7 have been used to activate T cell responses in patients with tumors. We thus asked whether NFATc2 expression would be defective in the lung of patients with lung adenocarcinomas.

In this study, we describe for the first time a profound deficiency of the T cell transcription factor NFATc2 in carcinoma-bearing lungs as compared to lungs of control patients. Consistently, NFATc2 deficient mice showed more aggressive tumors in a murine model of bronchial adenocarcinoma compared to wild-type littermates. Using both *in vitro* and *in vivo* systems we found a tolerogenic effect of lung CD8<sup>+</sup> T cells in NFATc2<sup>-/-</sup> mice in this disease model due to a defect in IL-2 and TNF-α production. Finally, we could overcome this tumor promoting effect by activating GITR signaling, leading to enhanced long lived memory cells CD4<sup>+</sup>CD127<sup>+</sup> (IL-7Rα<sup>+</sup>) in the lungs of NFATc2 deficient mice. As GITR mRNA expression is increased in the lungs of some patients with bronchial adenocarcinoma, we propose that ligation of GITR, in the absence of NFATc2, emerges as a novel possible strategy for treatment of selective human bronchial adenocarcinoma by enhancing IL-2Rα<sup>+</sup> effector and IL-7Rα<sup>+</sup> long lived memory cells.

**PD09/18 4-1BB LIGAND ALLOWS THE LONG TERM EXPANSION OF NATURAL KILLER CELLS**A.C. Dowell<sup>1</sup>, P.F. Searle<sup>1</sup>, S.P. Lee<sup>1</sup><sup>1</sup>University of Birmingham, School of Cancer Science, Birmingham, United Kingdom

**Background:** Immunotherapy is a promising area for the development of novel treatments for cancer. A major emphasis has been placed on T cell based interventions, one area of which is the enhancement of pre-existing immune responses. Natural killer cells (NK) were initially defined by their ability to spontaneously lyse tumour but are now understood to play a central role in the coordination of the immune response. During T cell activation the interaction of TNF super-family member 4-1BB (CD137) with its ligand 4-1BBL (CD137L) provides a powerful co-stimulatory signal, with an established role in the enhancement of the T cell immune response. However, its function in the NK immune response is less well defined.

**Method:** Adenoviral (Ad) vectors have been widely used for cancer gene therapy. Replication defective Ad vectors encoding either 4-1BBL or IL-12, enabled us to engineer chosen cells (including OVCAR-3 or autologous cells) to produce artificial APC, which we used to stimulate PBMC from healthy lab donors and renal cell carcinoma patients.

**Results:** Cells transduced with 4-1BBL or IL-12 alone induced NK proliferation and a significant additive effect was observed with dual transductants. Within 1 day post stimulation NK cells are shown to increase CD56 expression while losing CD16 expression and begin to express CD25 and 4-1BB. NK subsequently began to proliferate between days 3-5 post stimulation. The CD56<sup>bright</sup> NK subset (CD56<sup>bright</sup>CD16<sup>-</sup>) appears to predominate following proliferation. Stimulated cells also produced IFN γ and GM-CSF and had cytolytic function. Cells transduced with 4-1BBL supported the long term proliferation of NK cells *in vitro*. Dual transductants supported ~1000 fold expansion within three week culture.

**Conclusion:** Our data suggest that resting CD56<sup>dim</sup> NK cells are able to transition to an activated CD56<sup>bright</sup> state upon stimulation with 4-1BBL and IL-12 and undergo substantial proliferation. The use of Ad vectors to deliver 4-1BBL and IL-12 in combination for the immunotherapy of solid tumours may promote innate and adaptive anti-tumour immune responses allowing a comprehensive targeted activation of the immune system. The use of 4-1BB stimulation may also be a useful tool for the *ex vivo* expansion of NK.

**PD09/19 ISOLATION, CHARACTERIZATION AND CLINICAL RELEVANCE OF TUMOR STEM CELLS EXPRESSING MEMBRANE GRP78 IN COLON CANCER**B. Hardy<sup>1</sup>, A. Raiter<sup>1</sup><sup>1</sup>Tel-Aviv University School of Medicine, Felsenstein Medical Research Center, Petach-Tikva, Israel

Colorectal cancer is a common malignancy that in its metastatic stages is rarely responding to therapy. Evidence has accumulated that cancers are generated and maintained by tumor stem cells. Tumor stem cells have been experimentally isolated from a number of solid cancers, including colon cancer. CD133 was described as a useful marker to detect stem cells and tumor stem cells. An additional membrane protein GRP78, a heat shock protein, has been identified on surface of tumor cells linked to a greater degree of malignancy.

**Our objectives** were to isolate the subpopulation expressing CD133/GRP78 from colon tumor cells in order to determine their tumorigenic and metastatic potential.

**Methods:** Isolation of stem cells from human blood and tumor cells was done using antibody bound magnetic beads. The percent of CD133/GRP78 positive cells was determined by FACS analysis. Isolated sub-populations were studied for migration in Boyden chambers. The potential of colon tumor cells to metastasize was studied using a metastatic liver nude mouse model. Histological sections of the liver specimens were stained and examined for the presence of tumor metastases.

**Results:** In human peripheral blood from healthy donors we have determined that 50.6 ± 12.3 percent of CD133 cells expressed membrane GRP78. The isolated subpopulation was found to migrate 3 fold more than CD133/GRP78 negative cells. Also human colon carcinoma cells contain CD133/GRP78 positive cells. However, in colon tumor cell lines the percent of cells expressing GRP78 was variable; 25% of HM7 and LIM6 expressed GRP78 while only 4.4, 3.3 and 1.5 percent respectively in HT, HCT116 and RKO cells. HM7 colon carcinoma demonstrated a higher metastatic potential compared to the tumor cell lines with low percent of GRP78 as seen in liver histology. Anti GRP78 antibody inhibited *in vitro* proliferation of HM7 cells.

**Conclusions:** Our results suggest a correlation between the percent of GRP78 positive tumor stem cells and their metastatic potential. It is possible, that targeting CD133/GRP78 tumor stem cells will lead to treatment of the disease.

**PD09/20 MYELOID-DERIVED SUPPRESSOR CELLS: A NEW IMMUNE ESCAPE MECHANISM IN MELANOMA**A. Tsianakas<sup>1,2,3</sup>, J.M. Ehrchen<sup>1,2,3</sup>, S. Seeliger<sup>4</sup>, A. Rattenholl<sup>1</sup>, T.A. Luger<sup>1</sup>, M. Steinhoff<sup>1,2</sup>, J. Roth<sup>1</sup>, G. Varga<sup>1,3</sup>, C. Sunderkötter<sup>1,2,3</sup>

<sup>1</sup>University of Münster, Dpt. of Dermatology, Münster, Germany, <sup>2</sup>University of Münster, Interdisciplinary Centre of Clinical Research, Münster, Germany, <sup>3</sup>University of Münster, Institute of Immunology, Münster, Germany, <sup>4</sup>University of Göttingen, Dpt. of Pediatrics, Göttingen, Germany

**Introduction:** Recent studies have described the so-called myeloid-derived suppressor cells (MDSC) in various tumors. They suppress anti-tumor CD8 T cell responses and represent a newly detected important immune escape mechanism of tumors. In mice they can be phenotypically identified by cell surface expression of CD11b, Gr-1 and IL-4 receptor alpha (CD124).

**Question:** We wanted to know if MDSC could also be detected in (murine) melanoma, define their phenotype and analyse if they are able to down-regulate immune responses.

**Methods:** After injection of 5x10<sup>5</sup> B16 melanoma cells into C57/BL/6 mice, the presence of MDSC was analysed in blood, bone marrow and spleen.

**Results:** We were able to detect an increased fraction of CD11b<sup>+</sup>Gr-1<sup>+</sup>CD124<sup>+</sup> cells in several organs of tumor-bearing mice. MDSC from melanoma-bearing mice down-regulated T cell proliferation not only of CD8<sup>+</sup> but also of CD4<sup>+</sup> T cells. Blocking of known immunosuppressive mechanisms of MDSC such as arginase, iNOS (inducible NO synthase), and ROS (reactive oxygen species) that have been described in other tumor entities, led to reversion of the immunosuppressive function of MDSC.

**Conclusion:**

1) Our data show that the generation of MDSC represents a new immunosuppressive mechanism in melanoma.

2) Pharmacological inhibition of arginase, iNOS and ROS could enlarge therapeutic perspectives.

**PD09/21 ROLE OF ENDOGENOUS NK CELLS IN TUMOUR IMMUNOTHERAPY**A. Bouwer<sup>1</sup>, P. Stoitner<sup>2</sup>, A. McLellan<sup>1</sup><sup>1</sup>University of Otago, Department of Microbiology and Immunology, Dunedin, New Zealand, <sup>2</sup>Innsbruck Medical University, Department of Dermatology, Innsbruck, Austria**Objectives:** To investigate the roles of endogenous natural killer (NK) cells in tumour immunotherapy.**Methods:** ELISA, blocking antibodies, proliferation and cytotoxicity assays, immunodepletion and transwell cultures were used to optimise the activation of NK cells. We utilised the B16.OVA melanoma model with therapeutic immunisation using antigen-pulsed DC activated with various microbial components three days following tumour inoculation to determine if the activation of endogenous NK cells would be of benefit in tumour immunotherapy.**Results:** Compared to Gram negative cell components, Gram-positive bacteria provided optimal dendritic cell activation of NK cells, stimulating a rapid release (< 24 hours) of Th1 cytokines. NK activation was predominantly exerted on the CD27<sup>+</sup> NK cell subset and was dependent on membrane-contact with DC and IL-12/IL-18 expression. In contrast, NK activation was independent of LFA-1 or NKG2D interactions or NK 'licensing', as defined by Ly49A expression. We observed a significant delay ( $P < 0.0001$ ) in tumour growth in mice immunised with heat-killed whole bacteria/OVA-pulsed DC. *In vivo* depletion of NK cells with anti-asialo GM antibodies inhibited Th1 polarisation and completely abolished the benefit of DC immunotherapy. In contrast, NK cell depletion did not affect the tumour progression in mice that did not receive DC therapy, demonstrating that NK cells are essential for the priming of antigen specific T cells.**Conclusions:** Our results indicate that Gram positive bacterial components are able to optimally induce the recruitment and activation of NK cells thereby driving Th1 polarisation. Furthermore results from our tumour experiments clearly demonstrate a role for NK cells in directing the T cell mediated immune response against melanoma.**PD09/22 DEVELOPMENT OF A NEW IMMUNOTHERAPY STRATEGY AGAINST MELANOMA BASED ON THE USE OF ARTIFICIAL ANTIGEN-PRESENTING CELLS**J.-F. Chatillon<sup>1</sup>, E. Fauquemberg<sup>2</sup>, F. Bayeux<sup>1</sup>, A. Drouet<sup>2</sup>, P. Musette<sup>1</sup>, J.-B. Latouche<sup>2</sup><sup>1</sup>Inserm U905, Rouen, France, <sup>2</sup>Inserm U614, IFRMP 23, Institute for Biomedical Research, University of Rouen, Rouen, FranceAdoptive immunotherapy based on *in vitro* activation and expansion of tumor antigen-specific cytotoxic T lymphocytes (CTLs) is a very promising approach against cancer. Numerous techniques have been proposed to activate such CTLs, including the culture of peripheral T lymphocytes with allogeneic tumor cells (Labarriere *et al*, Cancer Immunol Immunother, 2008).

Our team has developed a strategy based on the use of artificial antigen-presenting cells (AAPCs) co-cultured with peripheral T lymphocytes (Latouche and Sadelain, Nat biotechnol, 2000).

These AAPCs have been transduced to express molecules involved in the immunological synapse, restricted to the HLA-A2.1 molecule, and essential to activate CTLs against MART-1, auto-antigen which is overexpressed in melanoma. We defined, starting from peripheral blood lymphocytes of 6 A2.1 + healthy donors, the optimal conditions for proliferation and purification of MART-1-specific CTLs, using AAPCs and anti-MART-1 phycoerythrin (PE)-coupled pentamer. After one round of a 2 to 3 week co-culture, we obtained 3 to 25% of specific CD8<sup>+</sup> T cells, depending on the donor. Cytotoxic assays showed the capacity of these cells to specifically lyse MART-1-pulsed T2 cells and A2.1+ melanoma cells. Flow cytometry analysis revealed a high potency to produce mainly TNF- $\alpha$ , IFN- $\gamma$  and Granzyme B. By sorting anti-MART-1 PE-coupled pentamer stained cells with anti-PE magnetic beads, we highly purified specific CD8<sup>+</sup> T lymphocytes which were then co-cultured with AAPCs to expand them.We will now apply this strategy to obtain MART-1-specific CTLs from stage II, III or IV melanoma patients, and precisely study expansion, phenotype (IFN- $\gamma$ , TNF- $\alpha$ , IL-2 production and V $\beta$  T Cell Receptor repertoire) and function (cytotoxic assays against MART-1-pulsed or melanoma-derived cell lines) of the expanded cells.**PD09/23 VIRAL VECTOR-BASED PRIME-BOOST IMMUNIZATION REGIMENS: A POSSIBLE INVOLVEMENT OF T-CELL COMPETITION**A. de Mare<sup>1</sup>, A. Lambeck<sup>1</sup>, G. van Dam<sup>2</sup>, H. W. Nijman<sup>2</sup>, H. Snippe<sup>3</sup>, J. Wilschut<sup>1</sup>, T. Daemen<sup>1</sup><sup>1</sup>University Medical Center Groningen, Medical Microbiology, Section Molecular Virology, Groningen, Netherlands, <sup>2</sup>University Medical Center Groningen, Groningen, Netherlands, <sup>3</sup>University Medical Centre Utrecht, Utrecht, NetherlandsRecombinant viral vectors are being developed for immunotherapeutic applications, including immunotherapy of cervical neoplasia. Although these approaches are promising, it is generally recognized that viral vectors *in vivo* may induce immunity against the (non-)structural proteins of the vector, which may impede the efficacy of booster immunizations with the same vector. In general, neutralizing antibodies are thought to be the main effector mechanism of vector-specific immunity, while cellular immunity would appear to play a secondary role.

We previously demonstrated that immunizations with recombinant Semliki Forest virus (rSFV) is very effective in inducing transgene-specific CTLs (Riezebos-Brilman A, Gene Ther.2007;14:1695). We also demonstrated that to achieve optimal responses and specifically to induce strong memory CTL responses, at least two immunizations with rSFV are required.

Now we investigated the effect of vector-specific immunity, induced by a priming immunization with rSFV, on transgene expression and CTL activation by a subsequent injection of SFV expressing E6E7 from human papillomavirus (HPV) (SFVE6,7). We furthermore determined which immune mechanisms may be involved in SFV vector-specific immunity.

Secondary immune responses against E6E7 were neither affected by vector-specific antibodies nor by CTL-mediated killing of infected cells. Instead, the presence of the antigen during the prime immunization is the main determinant for the boosting efficacy. After priming with rSFVE6,7, a homologous booster stimulated the primed E6E7-specific CTL response and induced long-lasting memory. Conversely, in mice primed with irrelevant rSFV, induction of E6E7-specific CTLs was inhibited presumably due to vector-specific responses induced by the priming immunization. When during the priming with irrelevant rSFV, E7-protein was co-administered, the inhibitory effect was abolished.

These observations indicate that T cell competition may determine the outcome of secondary immunizations in viral vector immunization strategies. For rSFV this immune mechanism however does not hamper homologous prime-boost immunization.

Study supported by Dutch Cancer Society grant RUG-2001-2361 (de Mare).

**PD09/24 COOPERATION BETWEEN TUMOR- AND MINOR HISTOCOMPATIBILITY ANTIGEN-SPECIFIC T CELLS INITIATE REJECTION AND MAINTAIN REMISSION FROM ESTABLISHED SPONTANEOUS SOLID TUMORS**R. Hess Michelini<sup>1,2</sup>, M. Freschi<sup>3</sup>, T. Manzo<sup>1,2</sup>, E. Jachetti<sup>1,2</sup>, E. Degl'Innocenti<sup>1</sup>, M. Grioni<sup>1</sup>, V. Basso<sup>1</sup>, C. Bonini<sup>1</sup>, E. Simpson<sup>4</sup>, A. Mondino<sup>1</sup>, M. Bellone<sup>1</sup><sup>1</sup>Istituto Scientifico San Raffaele, Department of Immunology, Milan, Italy, <sup>2</sup>Università Vita-Salute San Raffaele, Milan, Italy, <sup>3</sup>Istituto Scientifico San Raffaele, Unità Operativa Anatomia Patologica, Milan, Italy, <sup>4</sup>Hammersmith Hospital, Imperial College London, Department of Immunology, London, United Kingdom

Peripheral tolerance induction and/or suboptimal priming of tumor-specific T lymphocytes may hinder the success of hematopoietic cell transplantation against solid tumors. Here we report that in mice developing spontaneous prostate cancer, non-myeloablative minor histocompatibility mismatched hematopoietic stem cell transplantation and donor lymphocyte infusion of unmanipulated donor-derived lymphocytes combined with tumor-specific vaccination support tumor remission and favor the establishment of protective immunosurveillance. Minor histocompatibility antigen (H-Y) specific T cells readily accumulate in transplanted tumor-bearing mice, but are insufficient for tumor regression. Nevertheless, we found that tumor rejection is supported by the concomitant and optimal generation of both minor histocompatibility antigen- and high affinity tumor-specific effector T lymphocytes, best provided by the combination of allotransplantation and post-transplant tumor-specific vaccination. Together our data indicate that while individually inefficient, tumor and minor-histocompatibility effector lymphocytes circumvent functional peripheral T cell tolerance, allowing tumor rejection and disease-free survival. Thus non-myeloablative allo-transplantation combined with tumor-specific vaccination might prove suitable for the cure of advanced solid tumors.

**PD09/25 CHARACTERIZATION OF A HABL-SPECIFIC CD4+ T CELL RESPONSE AND THE APPLICATION OF ADETOH AS CATALYST OF MHC CLASS II PEPTIDE LOADING**S. Höpner<sup>1,2</sup>, K. Dickhaut<sup>2</sup>, S. Günther<sup>2</sup>, S. Gupta<sup>2</sup>, B. Rupp<sup>3</sup>, C. Freund<sup>3</sup>, K. Falk<sup>2</sup>, O. Röttschke<sup>4</sup><sup>1</sup>Geneva University Hospital, Oncology Division, Geneva, Switzerland, <sup>2</sup>MDC, Berlin, Germany, <sup>3</sup>FMP, Berlin, Germany, <sup>4</sup>Singapore Immunology Network (SigN), Singapore, SingaporeThe expression of the BCR-ABL fusion protein is associated with the development of various leukemias. Since several leukemia-associated antigens are known, which could be targets for cellular immunotherapy, peptide-vaccination comes into focus. These peptides are mainly CD8<sup>+</sup> T cell antigens. However clinical benefit of vaccination trials often fails.In addition to MHC class I antigens recent studies give evidence for the crucial role of CD4<sup>+</sup> T cells in tumor regression. In this study we focused on the characterization of a CD4<sup>+</sup> T cell specific immune response against hABL908-922. By using the SYFPEITHI prediction program and *in vitro* analysis of binding capacity to HLA-DR1 soluble molecule we identified hABL908-922 as a promising target for CD4<sup>+</sup> T cell response. In order to analyze the hABL-specific CD4<sup>+</sup> T cell responses regarding to tumor development we used a HLA-DRtg mouse model. We could demonstrate the injection of BCR-ABL tumor cells leads to the generation of hABL908-922 specific CD4<sup>+</sup> T cells *in vivo*. Furthermore we could show that vaccination of the CD4<sup>+</sup> T cell antigen in combination with Treg depletion leads to a significant tumor delay. In addition to depletion of regulatory T cells the enhancement of peptide loading onto MHC-class II molecules catalyzed by small molecular compounds (MHC-loading enhancer, MLE) could be also useful in order to modulate specific immune responses. By the identification of the small organic compound AdEtOH we could show, that MHC class II peptide loading can be catalyzed in an allele-specific manner. The catalytic activity of AdEtOH correlates with the presence of Glycin at the position  $\beta$ 86 in the P1 pocket of the MHC class II molecule. In our study we could further demonstrate that MLEs enhance specific immune responses *in vitro* and *in vivo*. Regarding to our results MLEs could be useful tools in diagnostic or therapeutic applications.

**PD09/26 SALMONELLA-INDUCED CONNEXIN 43 IS REQUIRED FOR AN EFFECTIVE IMMUNOTHERAPY AGAINST THE MURINE B16 MELANOMA**F. Saccheri<sup>1</sup>, C. Pozzi<sup>2</sup>, D. Mittal<sup>1</sup>, F. Avogadri<sup>3</sup>, S. Barozzi<sup>1</sup>, M. Faretta<sup>1</sup>, P. Fusi<sup>2</sup>, M. Rescigno<sup>1</sup><sup>1</sup>European Institute of Oncology, Experimental Oncology, Milan, Italy, <sup>2</sup>University of Milano-Bicocca, Department of Biotechnology and Biosciences, Milan, Italy,<sup>3</sup>Memorial Sloan-Kettering Cancer Center, Department of Immunology, New York, United States

The general objective of this project is to investigate the potential of the bacterium *Salmonella Typhimurium* as a melanoma cancer treatment aid, studying its mechanism of action in the immunotherapeutic protocol previously developed in our lab.

Intratumoral injection of *Salmonella Typhimurium* induces the slowing down the growth of a distal untreated lesion, through the cross-presentation of the tumor antigens by dendritic cells to CD8 T cells. Thus, we investigated deeply the mechanism that leads to the antitumor systemic response.

The results show that *Salmonella* or their products upregulate the expression of connexin43 in melanoma cells and therefore facilitate the formation of small molecular pores called gap junctions (GJs) between tumor cells and dendritic cells. Hence, tumor-associated peptides generated within the tumor cells can be efficiently transferred from the tumor cell to DCs for T cell activation via the newly formed GJs.

This approach allows the generation of a potent anti-tumor immunity that controls the growth of distant untreated tumors via a CD8-dependent mechanism. Negative controls also show that tumor cells infected *in vivo* with bacteria when stably silenced for the expression of connexin 43 fail to elicit the antitumor response. DCs loaded *in vitro* with bacteria-treated tumor cells but not with tumor cells initiate a potent antitumor response via a connexin 43-dependent mechanism that restrains the growth of the tumor in a therapeutic setting.

Therefore, we anticipate that this approach will become a powerful tool to allow DCs to present a repertoire of tumor peptides that reflects those presented by tumor cells. In turns, this allows the activation of T cells that will find their targets on tumor cells with a huge therapeutic implication.

**PD09/27 THE GD2-SPECIFIC 14G2A MONOCLONAL ANTIBODY INDUCES APOPTOSIS AND ENHANCES CYTOTOXICITY OF CHEMOTHERAPEUTIC DRUGS IN IMR-32 HUMAN NEUROBLASTOMA CELLS**A. Kowalczyk<sup>1</sup>, M. Gil<sup>1</sup>, L. Horwacik<sup>1</sup>, Z. Odrowaz<sup>1</sup>, D. Kozbor<sup>2</sup>, H. Rokita<sup>1</sup><sup>1</sup>The Jagiellonian University, Faculty of Biochemistry, Biophysics and Biotechnology, Krakow, Poland, <sup>2</sup>Roswell Park Cancer Institute, Department of Immunology, Buffalo, United States

Neuroblastoma (NB) is the most common extracranial solid tumor of childhood. The majority of children suffers from high risk neuroblastoma and has disseminated disease at the time of diagnosis. Despite recent advances in chemotherapy, the prognoses for children with high risk NB remain poor. Therefore, new treatment modalities are urgently needed. GD2 ganglioside is an antigen that is highly expressed on NB cells with only limited distribution on healthy tissues. Consequently, it appears to be an ideal target for both active and passive immunotherapy. The immunological effector mechanisms mediated by anti-GD2 monoclonal antibodies (mAbs) have been already well characterized. However, a growing number of reports suggest that GD2-specific antibodies may exhibit anti-proliferative effects without the immune system involvement. Here, we have shown for the first time that the anti-GD2 14G2a mAb is capable of decreasing survival of IMR-32 human neuroblastoma cells in a dose-dependent manner. Death induced by this antibody exhibited several characteristics typical for apoptosis such as increased number of Annexin V- and propidium iodide-positive cells, cleavage of caspase 3 and prominent rise in caspase activity. The use of a pan caspase inhibitor Z-VAD-fmk suggested that the killing potential of this mAb is partially caspase-dependent. The 14G2a mAb is rapidly endocytosed upon antigen binding; however, ceramide, the product of ganglioside degradation and a known mediator of apoptosis, was not responsible for the observed cell death. Most importantly, our studies showed that at particular drug concentrations the 14G2a mAb exerts a synergistic effect with doxorubicin and topotecan, as well as an additive effect with carboplatin in killing IMR-32 cells *in vitro*. Our results provide guidance regarding how to best combine the GD2-specific 14G2a antibody with existing cancer therapeutic agents to improve available treatment modalities for neuroblastoma.

This work was supported by grant number N301 158635 from the Polish Ministry of Science and Higher Education and by the statute financing number WBBB 8/2007 (HR).

**PD09/28 HIGHER FUNCTIONAL AVIDITY OF T CELL RECEPTOR (TCR)-GENE-MODIFIED T CELLS BY EXCHANGING FEW AMINO ACIDS IN THE HUMAN TCR CONSTANT REGIONS**D. Sommermeyer<sup>1</sup>, W. Uckert<sup>1</sup><sup>1</sup>MDC Berlin, Berlin, Germany

The *in vitro* generation of T cells with defined antigen specificity by T cell receptor (TCR) gene transfer is an established method to create cells for immunotherapy. One prerequisite for TCR gene therapy is the sufficient expression of the transferred TCR. To reach this goal several strategies were developed. One of those – the “murinization” – is based on replacing the human TCR $\alpha$  and TCR $\beta$  constant regions by murine counterparts to enhance preferential pairing and the stability of the transferred TCR chains within the TCR complex. Using a series of hybrid constructs, which included different domains of the murine constant regions, we identified those amino acids responsible for the improved expression of murinized TCR. Strikingly, in the TCR $\beta$  constant region one amino acid exchange from an acidic glutamic acid (human) to a basic lysine (mouse) was found to be most important. Four additional amino acids further improved the TCR expression. Within the TCR $\alpha$  constant region, a domain of four amino acids was found to be sufficient for the enhanced expression. For the identification, we used a NY-ESO-1-specific TCR in a TCR replacement model. In this model the NY-ESO-1-specific TCR had to compete with a strong second TCR to be expressed on the T cell line Jurkat 76. Therefore only the murinized variant but not the unmodified NY-ESO-1-TCR was expressed on those cells. Using the minimal murinized TCR variant (9 amino acids from the mouse sequence) the surface expression could be retained. To show a broader applicability, minimal murinized TCR variants of different TCR were tested in primary human T cells. For example, cells transduced with the minimal murinized variant of a renal cell carcinoma-reactive TCR expressed significant more interferon-gamma after cocultivation with antigen-expressing tumor cells compared to cells transduced with the unmodified TCR.

For TCR gene therapy the utilization of minimal instead of completely murinized constant regions will reduce the amount of foreign sequences and thus the risk of immunogenicity of the therapeutic TCR. Therefore, we suggest the use of minimal murinized constant regions instead of completely murinized ones for applications in TCR gene therapy.

**PD09/29 MECHANISM OF ACTION OF IMIQUIMOD IN ANTI-TUMOR IMMUNE RESPONSES**B. Drobits<sup>1</sup>, M. Holcman<sup>1</sup>, M. Sibilni<sup>1</sup><sup>1</sup>Medical University of Vienna, Department of Medicine I/ Institute for Cancer Research, Vienna, Austria

The immune response modifier Imiquimod (Imi), a ligand for TLR7, has been shown to exert anti-viral and anti-tumor activities. Treatment of melanomas with Imi leads to tumor regression, accompanied by increased tumor infiltration of plasmacytoid dendritic cells (pDCs), a DC subpopulation expressing TLR7. So far, the mechanism how Imi mediates anti-tumor activity is poorly understood.

In this study, we investigated if pDCs or other cell types e.g. skin cells, play an active role in anti-tumor immune responses mediated by Imi. Although TLR7/8 expression was not detectable either in epidermis or dermis by RT-PCR analysis, *in vitro* stimulation of primary keratinocytes from wt, TLR7 and MyD88 knock-out mice with Imi led to the activation of the MAPK-pathway accompanied with increased apoptosis. Moreover, in primary dermal cultures we found increased IL-6 levels after Imi treatment, whereas apoptosis was not affected. Interestingly, TLR7 and MyD88 expression is required for the tumoricidal effect of Imi since melanoma regression was not observed in TLR7<sup>-/-</sup> and MyD88<sup>-/-</sup> mice. Furthermore, results obtained in bone marrow chimeras demonstrate that the anti-tumor effect of Imi critically depends on the presence of TLR7 or MyD88 in bone-marrow derived cells. To investigate which leukocyte population is responsible for tumor regression after Imi treatment various immune cells, such as CD4<sup>+</sup>, CD8<sup>+</sup>, CD25<sup>+</sup> and NK1.1<sup>+</sup>-cells, were depleted in melanoma-bearing mice. We could show that the anti-tumor effect mediated by Imiquimod is impaired in the absence of CD4<sup>+</sup> and CD8<sup>+</sup>-cells, whereas depletion of NK-cells still resulted in a strong Imi effect. Moreover spontaneous tumor regression after CD4<sup>+</sup> or CD25<sup>+</sup> cell depletion was observed, even in the absence of Imi.

In summary we could show that the recruitment of immune cells to the site of Imi treatment may be mediated by IL-6 independently of TLR7 expression, whereas the tumoricidal effect of Imi is strictly depending on TLR7 and MyD88 expression on immune cells. Moreover, it seems that CD4<sup>+</sup> and CD8<sup>+</sup>-cells are required for the anti-tumor response mediated by Imi. Whether pDCs are essential for Imi action is currently under investigation.

**PD09/30 IN VITRO STIMULATION WITH AUTOLOGOUS EBV-TRANSFORMED B CELLS REACTIVATES CD4<sup>+</sup> T CELL RESPONSES THAT RECOGNISE HUMAN B LYMPHOMA-ASSOCIATED CELLULAR ANTIGENS**H.M. Long<sup>1</sup>, J. Zuo<sup>1</sup>, A. Leese<sup>1</sup>, N. Gudgeon<sup>1</sup>, H. Jia<sup>1</sup>, G. Taylor<sup>1</sup>, A. Rickinson<sup>1</sup><sup>1</sup>University of Birmingham, School of Cancer Sciences, Birmingham, United Kingdom

Epstein-Barr virus (EBV)-specific T cell preparations, generated by stimulating immune donor lymphocytes with the autologous EBV-transformed B lymphoblastoid cell line (LCL) *in vitro*, are being used to target EBV-positive malignancies. Whilst these preparations are enriched for EBV antigen-specific CD8<sup>+</sup> T cells, most contain a small CD4<sup>+</sup> T cell population whose specificity is unknown.

As one might anticipate of an EBV antigen-specific response, CD4<sup>+</sup> T cells generated in this way recognise EBV-transformed LCLs and not mitogen-activated B or T lymphoblasts or dendritic cells, and at the clonal level are classically restricted to one particular HLA-DR, -DQ or -DP allele. However, three pieces of evidence show that these LCL-stimulated CD4<sup>+</sup> effectors are not EBV-specific. Firstly they do not map to any known EBV antigen or epitope in recombinant vaccinia or peptide sensitisation assays. Secondly they can be raised from EBV-naïve as well as from EBV-immune individuals. Thirdly, they are capable of recognising a broad range of human B-lymphoma-derived cell lines irrespective of EBV genome status, providing those lines express the relevant HLA class II allele. Importantly, while all of the CD4<sup>+</sup> clones produce IFN $\gamma$ , many are also cytotoxic and can control the outgrowth of their target cells in co-cultivation assays.



We infer that such CD4<sup>+</sup> T cells are capable of recognising cellular antigens whose expression is specifically up-regulated in normal B cells by EBV-infection. These same cellular antigens also appear to be up-regulated in human B lymphoma cells, where their presentation via the HLA class II pathway renders these cells open to recognition by LCL-stimulated effectors. The identity of these antigens and their means of presentation are under investigation.

**PD09/31 TUMOR ASSOCIATED ANTIGEN-LOADED ERYTHROCYTES TARGETING *IN SITU* THE DC AND INDUCING A CYTOTOXIC T CELL RESPONSES: A NOVEL CONCEPT FOR CANCER IMMUNOTHERAPY**

A. Banz<sup>1</sup>, M. Creml<sup>1</sup>, A. Rembert<sup>1</sup>, Y. Godfrin<sup>1</sup>

<sup>1</sup>ERYtech Pharma, Lyon, France

**Objectives:** The aim of this study was to validate our novel cancer vaccine approach. Efficient targeted delivery of tumor-associated antigens (TAAs) to dendritic cells (DCs) *in vivo* represents a major step toward the development of more effective tumor vaccines. Red blood cells (RBCs) were used as carriers for antigen to target antigen presenting cells (APCs) and generate T cell immune responses.

**Methods:** Murine RBCs were loaded with ovalbumin (OVA) by a hypotonic lysis process. The coating with anti-TER119 antibody was tested to improve the trapping by the DC. OVA-loaded RBC (RBCOVA) were injected with Poly (I:C) by intravenous route into C57BL/6 mice. Phagocytosis of RBCOVA and CD4 and CD8 T cell responses were analyzed by flow cytometry.

**Results:** We observed that macrophages, plasmacytoid and myeloid DCs from the spleen were able to up-take RBCOVA and antibody treatment enhanced their up-take. Evaluation of T cell responses showed that RBCOVA injection induced a significant increase in the percentage of OVA-specific CD4 T cells compared to the injection of free OVA. Mice injected with RBCOVA had also a significantly higher percentage of OVA-specific CD8 T cells (12 versus 2%). These CD8 T cells were cytotoxic, since they produced gIFN, expressed the CD107 marker associated with degranulation, and were able to induce OVA-specific cellular lysis *in vivo* at a higher rate than those from mice injected with OVA (83 versus 53%). Despite a higher up-take by APC, coating of RBC with antibodies did not significantly increase T cell responses. Finally, we observed that the T cell response was dose dependent of the amount of antigen entrapped into erythrocytes and could be maintained for up to 30 days.

**Conclusion:** Our results lend support the concept of using RBCs as carriers for antigen. Efficient antigen entrapment into healthy erythrocytes and their phagocytosis by macrophages and DCs led to the generation of antigen-specific CD4 and CD8 effector T cells.

**PD09/32 THERAPEUTIC USE OF AVIDIN IS NOT HAMPERED BY HUMAN ANTI AVIDIN ANTIBODIES**

F. Petronzelli<sup>1</sup>, A. Pelliccia<sup>1</sup>, A. M. Anastasi<sup>1</sup>, R. F. Lindstedt<sup>1</sup>, S. Manganelli<sup>1</sup>, L. Ferrari<sup>1</sup>, C. Albertoni<sup>1</sup>, A. Rosi<sup>1</sup>, B. Leoni<sup>1</sup>, P. Carminati<sup>1</sup>, G. Paganelli<sup>2</sup>, R. De Santis<sup>1</sup>

<sup>1</sup>Sigma-tau, R&D Immunology, Pomezia, Italy, <sup>2</sup>European Institute of Oncology, Milan, Italy

**Objectives:** Since the therapeutic use of the xenogenic glycoprotein hen egg white avidin, as part of multifactor treatments, is spreading, we addressed immunogenicity issues to get insights useful to risk/benefit evaluations.

**Methods:** Human sera from 14 normal subjects, 104 oncologic patients and 21 glioma patients enrolled in avidin-based therapeutic treatments were analysed, by ELISA, for natural occurring Human Anti-Avidin Antibodies (HAVA) and post-avidin-treatment HAVA titers. The effect of anti-avidin antibodies on the safety and efficacy of avidin-based treatments was evaluated in animal models.

**Results:** The presence of HAVA in human sera was confirmed with titers lognormal distributed in the range of 3-13 (log<sub>2</sub> dilution giving 0.5 O.D.), with mean value of 7.1 ± 2.04. A mean titer increase of 3.72 log<sub>2</sub> (range 1-8) was consistently found in patients two months after avidin treatment (PAGRIT<sup>®</sup>), however it was neither related to avidin dose nor to the basal HAVA titer. HAVA-related clinical symptoms were not observed. In a IART<sup>®</sup> animal model, low or high HAVA titers, induced in mice by immunization, did not reduced biotin uptake and did not induce clinical symptoms. Additionally, in an animal model simulating the use of avidin as an antidote for the rapid clearance of biotinylated drug, high HAVA titers did not affect avidin chasing capacity.

**Conclusion:** The presence of anti-avidin antibodies is a common event in humans. However, neither efficacy nor tolerability of avidin appear to be affected by HAVA. Animal models confirm human observations and legitimize the use of avidin as therapeutic agent.

**PD09/33 PEPTIDE INHIBITORS OF TRANSFORMING GROWTH FACTOR-BETA ENHANCE THE EFFICACY OF ANTI-TUMOR IMMUNOTHERAPY**

D. L. Llopiz<sup>1</sup>, J. Dotor<sup>2</sup>, N. Casares<sup>1</sup>, J. Bezunartea<sup>1</sup>, N. Díaz-Valdés<sup>1</sup>, M. Ruiz<sup>1</sup>, P. Berraondo<sup>1</sup>, J. Prieto<sup>1</sup>, J. J. Lasarte<sup>1</sup>, F. Borrás-Cuesta<sup>1</sup>, P. Sarobe<sup>1</sup>

<sup>1</sup>Center for Applied Medical Research (CIMA), University of Navarra, Division of Hepatology and Gene Therapy, Pamplona, Spain, <sup>2</sup>DIGNA Biotech, Madrid, Spain

Transforming growth factor-beta (TGF-beta) is a cytokine with potent immunosuppressive effects which is over-expressed in many tumors. Therefore, development of molecules able to inhibit TGF-beta is of paramount importance to improve the efficacy of anti-tumor immunotherapy. TGF-beta inhibitor peptides P144 and P17 were combined with administration of adjuvant molecules poly(I:C) and agonistic anti-CD40 antibodies, and their effect on the growth of E.G7-OVA established tumors and on anti-tumor immune response was evaluated. Tumor rejection efficacy of a single administration of adjuvants was enhanced from 15% to 70% when combined with repeated injections of TGF-beta inhibitor peptides. Simultaneous administration of adjuvants and TGF-beta inhibitor peptides was required for maximal therapeutic efficacy. Although tumor cells produced TGF-beta, it was found that the beneficial effect of peptide administration was mainly due to inhibition of TGF-beta produced by regulatory CD4<sup>+</sup>CD25<sup>+</sup> T cells rather than by tumor cells. The enhanced anti-tumor effect was accompanied by a higher activity of dendritic cells, NK cells and tumor antigen-specific T cells, as well as by a decrease in the number of myeloid derived suppressor cells. In conclusion, administration of peptide inhibitors of TGF-beta in therapeutic vaccination enhances the efficacy of immunotherapy by increasing anti-tumor immune responses. These peptide inhibitors may have important applications for current immunotherapeutic strategies.

**PD09/34 IDENTIFICATION OF HUMAN MINOR HISTOCOMPATIBILITY ANTIGENS EXPRESSED BY EPITHELIAL CANCERS: IMPLICATION FOR GRAFT-VERSUS-SOLID TUMOR IMMUNITY**

K. Broen<sup>1</sup>, J. Vos<sup>1</sup>, H. Levensga<sup>2</sup>, A. van Horsen-Zoetbrood<sup>1</sup>, T. de Witte<sup>1,2</sup>, H. Dolstra<sup>1</sup>

<sup>1</sup>Radboud University Nijmegen Medical Centre, Central Hematology Laboratory, Nijmegen, Netherlands, <sup>2</sup>Radboud University Nijmegen Medical Centre, Department of Hematology, Nijmegen, Netherlands

**Objectives:** Allogeneic stem cell transplantation (SCT) combined with donor lymphocyte infusion (DLI) is explored as an experimental treatment for patients with metastatic solid tumors. The therapeutic effect is dependent on the graft-versus-tumor (GVT) response during which alloreactive donor T cells eliminate malignant cells via recognition of minor histocompatibility antigens (MiHA). Unfortunately, a beneficial GVT response is often associated by graft-versus-host disease (GVHD). To reduce accompanying GVHD, it is therefore crucial to identify MiHA selectively co-expressed on hematopoietic cells and solid tumor cells, in order to selectively induce GVT reactivity by vaccination or T cell therapy. The aim of this project is to exploit MiHA-specific cytotoxic T lymphocytes (CTL) to determine the expression of known and unknown MiHA on epithelial tumor cells and to identify tumor-expressed MiHA by molecular immunology strategies.

**Results:** To investigate which MiHA are able to induce CD8<sup>+</sup> T cell responses against metastatic renal cell carcinoma, we isolated a CTL, termed B1, from a patient with tumor regression following DLI. Interestingly, CTL B1 recognized a novel HLA-B7-restricted MiHA expressed on RCC cell lines. Furthermore, 40% of a panel of unrelated HLA-B7-restricted EBV-transformed B cell lines are recognized by CTL B1. The tissue specificity of CTL B1 against tumor targets and normal cell types has been tested extensively. Non-malignant primary cells (i.e. fibroblast, iDC, mDC and PHA T cell blasts) of HLA-B7+ individuals are not recognized by CTL B1. Several cell lines such as RCC, retinal epithelial and malignant cell lines from the hematopoietic system are also killed by CTL B1. In addition, 3D multicellular tumor spheroids are generated and killing potential of CTL B1 towards this cancer metastasis model is assessed.

To identify the RCC-expressed MiHA, we have phenotyped four informative CEPH (Centre d'Etude du Polymorphisme Humain) pedigrees by testing IFN $\gamma$  production of CTL B1 against their EBV-transformed B cell lines. This yielded segregation patterns are currently used for genome-wide linkage analysis in order to map the chromosomal region of this new MiHA.

**Conclusion:** Alloreactive CTL can be exploited to characterize MiHA expressed by solid tumors, which can be used as targets for tumor-specific immunotherapy after allogeneic SCT.

**PD09/35 KNOCKDOWN OF MGAT5 INHIBITS BREAST CANCER CELL GROWTH WITH ACTIVATION OF CD4<sup>+</sup>T CELLS AND MACROPHAGES**

X.-L. Zhang<sup>1</sup>

<sup>1</sup>Wuhan University School of Medicine, Immunology, Wuhan, China

N-acetylglucosaminyltransferase V (Mgat5, or GnT-V) is an enzyme that catalyzes b1-6 branching of N-acetylglucosamine on asparagine (N)-linked oligosaccharides (N-glycan) of cell proteins. The levels of Mgat5 glycan products commonly are increased in malignancies. Although Mgat5 is known to be important in tumor metastases, the effects of Mgat5 on host immune responses are not fully defined. In this study, a Mgat5 specific-short hairpin RNA (shRNA) vector was transfected into murine mammary adenocarcinoma MA782 cells to assess the effects of Mgat5 on tumor cell growth and T cells and macrophages following inoculation of mice with shRNA-transfected cancer cells. The results showed that blocking expression of Mgat5-modified glycans in MA782 cells significantly suppressed tumor progression both *in vivo* and *in vitro*, and also strongly stimulated Th1 cytokine production, and enhanced opsonophagocytic capability of macrophages *in vivo*. Importantly, reduction of complex N-glycans on MA782 tumor cells by Mgat5-shRNA resulted in significantly increased proliferation and CD45 surface expression of CD4<sup>+</sup> T cells. Our data suggest Mgat5-shRNA could serve as a useful tool to treat breast cancer as well as a powerful tool for the functional investigation of N-glycans and glycoprotein synthesis. Our data suggest that knockdown of Mgat5 inhibits breast cancer cells growth with activation of CD4<sup>+</sup> T cells and macrophages.

**PD09/36 MECHANISMS OF NKT CELL DEFECTS IN MURINE PROSTATE CANCER**M. Nowak<sup>1</sup>, P. Ilyinski<sup>1</sup>, S. M. Arredouani<sup>1</sup>, M. Sanda<sup>1</sup>, S. P. Balk<sup>1</sup>, M. A. Exley<sup>1</sup><sup>1</sup>Beth Israel Deaconess Medical Center/Harvard Medical School, Hematology/Oncology, Boston, United States

Breaking tolerance to self-antigens represents a promising treatment approach, especially for noncritical organ cancers. Invariant natural killer T (iNKT) cells recognize glycolipids presented on CD1d molecules by dendritic and related cells and regulate immune reactions. In numerous model studies  $\alpha$ -GalCer administration activates iNKT cells to inhibit existing and spontaneous as well as subsequent tumor growth and metastases. Importantly, in multiples types of model and human cancer numerical iNKT defects have been found. Moreover, the decreased iNKT numbers were accompanied by reversible functional defects, such as a decrease in IFN- $\gamma$  production by iNKT in advanced prostate cancer and multiple other malignancies, such as progressive multiple myeloma, resulting in a detrimental Th2 cytokine profile of iNKT in such patients. In the murine transgenic adenocarcinoma of the mouse prostate model (TRAMP) probasin-directed expression of SV40 T antigen drives the sequential development of dysplasia (prostatic intraepithelial dysplasia, PIN), locally invasive tumors, and metastatic disease resembling features of human disease.

We have identified numerical and functional NKT cell defects and their underlying mechanisms in the murine TRAMP prostate cancer model. Interestingly, iNKT infiltrate the tumors, but show aberrant activation and the tumors surprisingly express CD1d, providing a mechanism for iNKT defects. Importantly, however, iNKT defects could be reversed, demonstrating the value of this cancer model to optimize therapeutic approaches.

**PD09/37 TRUNCATED IL-6, A NATURAL INHIBITOR OF IL-6?**A. Mansuy<sup>1</sup>, L. Alberti<sup>1</sup>, O. Subiger<sup>2</sup>, A. Duc<sup>1</sup>, L. Bourdin<sup>2</sup>, A. Gougelet<sup>1</sup>, H. Rouquette<sup>2</sup>, C. Vermot-Desroches<sup>2</sup>, J.-Y. Blay<sup>1</sup><sup>1</sup>Centre Léon Bérard INSERM U590, Lyon, France, <sup>2</sup>IDD Biotech, Dardilly, France

The interleukin-6 (IL-6) is a multifunctional cytokine that plays a role in the proliferation of hematopoietic progenitors, differentiation and growth of B, T cells or macrophages. A high level of expression is observed for many tumors such as breast cell carcinoma, renal cell carcinoma (RCC) or multiple myeloma. Neutralize IL-6 circulating with anti IL-6 or anti IL-6R monoclonal antibodies now seems applicable to a wide scale in human therapy.

Along with this approach, a truncated IL-6 (tIL-6) was discovered from human mononuclear cells of peripheral blood, bronchial or lung epithelial cells or renal carcinoma cells. tIL-6 is translated from alternative splicing mRNA of the wild type IL-6 (wtIL-6) in which the exon 2 has disappeared. Thus, tIL-6 possesses the necessary amino acids to bind to a gp80 and one gp130, while wtIL-6 binds to a gp80 and two gp130. So, tIL-6 could inhibit IL-6 inducing signal activation by IL-6 receptors saturation.

The aim of the present study was to demonstrate *in vitro* the potential antagonistic tIL-6 activity. Firstly, to generate purified tIL-6, two expression systems were tested by using CHO cells or E coli bacteria. Expression of tIL-6 was demonstrated by Western blot and by ELISA. By flow cytometry, wtIL-6 or tIL-6 binding on IL-6R positive U266 and TF1 cells was observed. Identification of tIL-6 receptor(s) interaction is in progress. Comparative biological activity of tIL-6 versus wtIL-6 was evaluated on IL-6 induced cell proliferation and also on IL-6 induced Stat3 phosphorylation (tyrosine 705). Preliminary results revealed antagonistic effects on IL-6 induced P-Stat3 or on IL-6 triggered cell proliferation according tIL-6 source (CHO or E Coli). Further studies are on going to more argue on the proof of concept of tIL-6 as an enough efficient natural antagonistic.

The study was supported by Cancéropole Lyon Auvergne Rhone-Alpes (CLARA).

**PD09/38 A NOVEL ROLE FOR B CELLS IN THE INDUCTION OF ANTI-TUMOUR IMMUNITY**M. Ciechomska<sup>1</sup>, J. Kirby<sup>1</sup>, T. Lennard<sup>1</sup>, A. Knight<sup>1</sup><sup>1</sup>Newcastle University, Institute of Cellular Medicine, Newcastle, United Kingdom

**Introduction:** As many tumours are poorly immunogenic, one approach for cancer therapy aims to improve natural anti-tumour immunity. Recently it has been shown that anthracyclin-induced apoptosis in tumour cells results in the relocation of the ER resident chaperone calreticulin to the cell surface. Interestingly, this relocation directly stimulates T cell-mediated anti-tumour immunity following tumour cell phagocytosis by dendritic cells. In addition, evidence from medullary carcinoma of the breast patients shows a correlation between tumour-infiltrating B cells and a more favorable prognosis. Surprisingly many of these infiltrating B cells recognize proteins relocated from their normal intracellular distribution to the tumour cell surface, also as a result of apoptosis. Taken together, these findings support the concept that antigens displayed on the surface of cancer cells as a result of certain types of apoptosis are able to trigger local anti-tumour immunity.

**Aim:** This study aimed to test the hypothesis that apoptotic tumour cells can be recognised by antigen-specific B cells as a result of modification of normal protein localization and this recognition can lead to the activation of tumour-specific T cells.

**Results:** To test this hypothesis we have introduced a fluorescent model antigen into distinct sub-cellular compartments (mitochondria, cytosol, and ER) in HeLa cells and studied its localization following apoptosis induction. We show that the intracellular localization of antigen targeted to these compartments is altered following apoptosis. In particular we show that following anthracyclin and UV-C but not staurosporin treatment, ER targeted antigen is relocated to the cell surface. Using these transfectants we have also established an assay demonstrating contact-dependent extraction of plasma membrane antigen by antigen-specific B cells. These findings raise the distinct possibility that the induction of selective tumour apoptosis may be used to relocate intracellular 'neo' tumour antigens to the cell surface allowing antigen-specific B cells to act as APC in the generation of anti-tumour immunity.

**PD09/39 SUCCESSFUL PREVENTION OF SURGERY-INDUCED LIVER METASTASES DEVELOPMENT AFTER ANTI-TUMOR MONOCLONAL ANTIBODY THERAPY IS MEDIATED BY THE INNATE MONONUCLEAR PHAGOCYTE NETWORK**M. Bögel<sup>1</sup>, G. J. van der Bij<sup>1</sup>, M. A. Otten<sup>1</sup>, S. J. Oosterling<sup>2</sup>, S. Meijer<sup>2</sup>, R. H. J. Beelen<sup>1</sup>, M. van Egmond<sup>1</sup><sup>1</sup>VU Medical Center, Molecular Cell Biology and Immunology, Amsterdam, Netherlands, <sup>2</sup>VU Medical Center, Department of Surgery, Amsterdam, Netherlands

**Introduction:** Liver metastases are a frequent complication of colorectal cancer (CRC), often even after successful resection of the primary tumor. Post-operative adjuvant treatment, aimed to eliminate residual disseminated tumor cells, may help prevent secondary disease. As antibody therapy has been acknowledged as a successful strategy to treat malignancies, we studied the potential of therapeutic antibodies to prevent outgrowth of liver metastases.

**Methods:** Liver metastases were induced in mice or rats via injection of murine B16F10 melanoma cells into the spleen or by injection of rat CC531s colon carcinoma cells into the mesenteric vein, respectively. Wild type, Fc $\gamma$ RII<sup>-/-</sup>, Fc $\gamma$ RIII<sup>-/-</sup>, Fc $\gamma$ RI/III<sup>-/-</sup> or Fc $\gamma$ RI/II/III<sup>-/-</sup> mice were treated with anti-gp75 antibody. Rats were treated with a low or high dose of anti-CC531s antibodies of different isotypes. Kupffer cells (KC) were depleted in mice and rats through i.v. clodronate liposome injection. Animals were sacrificed 3wks or 24h after injection of (fluorescently-labeled) tumor cells.

**Results:** Anti-tumor antibodies efficiently prevented liver metastases outgrowth in mice and rats. Efficacy of antibody therapy was dependent on the presence of IgG receptors Fc $\gamma$ RI and Fc $\gamma$ IV. Because these receptors are only expressed by mononuclear cells, the role of monocytes and m $\phi$  in antibody therapy was investigated. Less tumor cells were present in livers of control rats treated with antibody 24 hours after tumor cells injection. After KC depletion less tumor cells were eliminated following antibody treatment, supporting a prominent role for KC. Furthermore, treatment with a low antibody dose was sufficient to prevent liver metastases outgrowth in control rats, but not in KC depleted animals. However, high doses antibody treatment still partly prevented metastases outgrowth in KC depleted rats, which was due to phagocytosis of tumor cells by recruited monocytes.

**Conclusion:** Antibody treatment after surgery can efficiently prevent the development of liver metastases. The protective effect of antibodies is mainly mediated by KC, although monocytes are able to partly replace KC when high doses of antibody are administered. The discovery that KC and monocytes can eliminate tumor cells after surgery through antibody-dependent cellular cytotoxicity has promising clinical implications for designing new adjuvant therapies for patients with CRC.

**PD09/40 REGULATION OF INDOLEAMINE 2,3-DIOXYGENASE (IDO) EXPRESSION IN TUMOUR CELLS IN AN IMMUNOTHERAPY MODEL FOR EWING FAMILY TUMOURS (EFT)**D. Max<sup>1</sup>, C. D. Kühnöl<sup>1</sup>, S. Burdach<sup>2</sup>, M. S. Staeger<sup>1</sup>, J. Föll<sup>2</sup><sup>1</sup>Martin-Luther-University Halle-Wittenberg, Department of Pediatrics, Halle, Germany, <sup>2</sup>TU Munich, Department of Pediatrics, Munich, Germany, <sup>3</sup>University of Regensburg, Department of Pediatric Hematology and Oncology, Regensburg, Germany

**Objectives:** Interleukin 2 (IL2) transgenic EFT cells induce an EFT directed T cell response and inhibit tumour growth *in vitro* and *in vivo* (Staeger *et al.*, Pediatr. Blood Cancer 2004; 43:23-34). However, tumour growth is not completely inhibited under these conditions. In order to improve this immunotherapy, we analyzed the co-stimulatory activity of the CD137/CD137L system. Furthermore, we analyzed expression of immunosuppressive IDO in EFT cells under these conditions.

**Methods:** NOD/scid mice were injected with tumour cells in the presence or absence of IL2 transgenic cells, CD137L transgenic cells, and HLA matched peripheral blood mononuclear cells (PBMCs). In addition, PBMCs were co-cultivated with IL2 transgenic cells *in vitro* and induction of IDO in wild type tumour cells was analyzed in transwell assays. During co-cultivation *in vitro* agonistic antibodies against CD137 were present or absent. RNA was isolated from established tumours and *in vitro* cultivated tumour cells and expression of IDO was analyzed by reverse transcriptase-polymerase chain reaction. Secretion of IL2 and interferon gamma (IFN $\gamma$ ) was determined by cytometric bead array.

**Results:** In the mouse model, we observed expression of IDO only in tumours in the presence of IL2 transgenic cells. Similarly, we observed expression of IDO in wild type tumour cells after co-culture with PBMCs and IL2 transgenic cells *in vitro*. Interestingly, the presence of CD137L transgenic cells inhibited expression of IDO *in vivo*. *In vitro* we observed lower expression of IDO in co-cultures in the presence of agonistic antibodies against CD137. However, expression of IDO in *in vitro* cultivated tumour cells showed high variability. Trough determination of IL2 and IFN $\gamma$  in culture supernatants we were able to show that the expression of IDO goes hand in hand with high concentrations of both cytokines. High concentration of IL2 in the absence of IFN $\gamma$  secretion did not lead to increased IDO expression. The effects of antibodies against CD137 on IDO expression were also dependent on inhibition of IFN $\gamma$  secretion.

**Conclusion:** It seems possible that agonistic antibodies against CD137 modulate expression of immunosuppressive IDO in EFT via regulation of IFN $\gamma$  secretion. However, the exact mechanisms of this phenomenon require further investigation.

**PD09/41 CD4<sup>+</sup> T CELLS ARE REQUIRED FOR GENERATION AND MAINTENANCE OF ATRA + DNA COMBINED THERAPY INDUCED ANTI-LEUKEMIC EFFECT**K. Pokorna<sup>1,2</sup>, M. Reboul<sup>1</sup>, C. le Pogam<sup>1</sup>, M. Aoki<sup>1</sup>, M. Chopin<sup>3</sup>, H. Moins-Teisserenc<sup>1</sup>, V. Holan<sup>2</sup>, D. Charron<sup>1</sup>, C. Chomienne<sup>1</sup>, R.A. Padua<sup>1</sup>, M. Pla<sup>1</sup><sup>1</sup>INSERM UMR-S 940, Institut Universitaire d'Hématologie, Hôpital Saint-Louis, Paris, France, <sup>2</sup>Institute of Molecular Genetics, Czech Academy of Sciences, Prague, Czech Republic, <sup>3</sup>Institut Universitaire d'Hématologie, Hôpital Saint-Louis, Département d'Expérimentation Animale, Paris, France

Acute promyelocytic leukemia (APL) is characterized by a specific translocation t(15;17) that encodes a fusion of the promyelocytic leukemia (PML) and retinoic acid receptor- $\alpha$  (RARA) proteins. APL is a unique model in that two therapeutic agents retinoic acid (ATRA) and arsenic induce in vivo leukemia differentiation and apoptosis respectively. However relapses still occur and immunotherapy provides an alternative approach. Using a DNA vaccine in combination with ATRA therapy we have previously shown (Nature Med 9:1413, 2003) that we can rescue 50% of APL mice from relapse and death giving rise to long term survivors. The survival advantage is concomitant with time-dependent antibody production and the high CD4<sup>+</sup> counts in peripheral blood of combined therapy treated mice are predictive of long survival. The main aim of the present study was to evaluate in vivo the implication of CD4<sup>+</sup> T cells in the anti-leukemic effect of the combined therapy. For this, leukemic mice were depleted of either CD4<sup>+</sup> or CD8<sup>+</sup> T cells by repeated injections of subset-specific monoclonal antibodies (mAb). Two experimental settings were performed. In the first setting, mAb injections started from day 10 after leukemia engraftment (one day after the first DNA vaccination). The CD4 or CD8 depletion did not affect the survival of either untreated or ATRA-treated leukemic mice. On the contrary, the depletion of CD4 or CD8 subsets completely abolished the anti-leukemic effect of the combined therapy. In the second setting, the depletion was conducted using long term survivors resulting from the ATRA+DNA treatment. The mAb injection was started on the day 106 or 263 after engraftment of leukemic cells. The depletion of CD4 T cells subset leads to relapse of disease and all mice died between 16 to 83 days after the beginning of depletion. All CD8-depleted long term survivors were alive on day 180 after depletion. Taken together, our results demonstrate the need of both CD4 and CD8 subsets for the generation of DNA vaccine driven anti-leukemic immune responses and underscore a crucial role of CD4<sup>+</sup> memory cells in the maintenance of durable remissions.

**PD09/42 CD8<sup>+</sup> EFFECTOR T CELLS LACKING IFN- $\gamma$ , TNF- $\alpha$  OR FAS-LIGAND INDUCE REGRESSION BUT NOT LONG-TERM CONTROL OF TUMORS WITHOUT SELECTION OF ANTIGEN LOSS VARIANTS**J.J. Listopad<sup>1</sup>, G. Willmsky<sup>2</sup>, T. Kammertöns<sup>3</sup>, T. Blankenstein<sup>1,2</sup><sup>1</sup>Max Delbrück Center for Molecular Medicine, Berlin, Germany, <sup>2</sup>Institute of Immunology, Charité Campus Benjamin Franklin, Berlin, Germany, <sup>3</sup>Institute of Immunology, Charité Campus Benjamin Franklin, Berlin, Germany

The knowledge of the effector mechanisms by which cytotoxic T lymphocytes (CTL) reject large established tumors is critical for successful adoptive T cell therapy. Previous models using tumor cells transfected to express large amounts of a surrogate tumor antigen showed that Interferon- $\gamma$  (IFN- $\gamma$ ), Tumor Necrosis Factor- $\alpha$  (TNF- $\alpha$ ) and perforin (pfp) were necessary for tumor rejection. The absence of either molecule by CTL resulted in the selection and growth of antigen loss variants (ALV). It remained unclear, however, which effector molecules are required for rejection of established tumors, if the target antigen is malignancy-associated and cannot be easily selected against. With tumor cells, derived from LoxP-Tag mice as a result of sporadic activation of SV40 large T (Tag), we show that immune C57BL/6 (B6) effector CD8<sup>+</sup> T cell (T<sub>E</sub>) reject large tumors. IFN- $\gamma$ , TNF- $\alpha$  or Fas-ligand knock-out (KO) T<sub>E</sub> caused initial regression followed by progressive tumor growth despite persistent Tag-specific in vivo CTL activity. These tumors still expressed TAG, the rejection antigen, and were rejected by Tag-specific T<sub>E</sub> after secondary transplantation. In contrast, pfp-KO T<sub>E</sub> rejected large tumors. Our data demonstrate that T<sub>E</sub> that lack either IFN- $\gamma$  or TNF- $\alpha$  or Fas-L cannot long-term prevent the growth of cancer cells, which retain the rejection and remain susceptible to rejection.

**PD09/43 THE IMMUNOSUPPRESSIVE EFFECTS OF HUMAN MESENCHYMAL STEM CELLS IN HUMAN GRAFT-VERSUS-HOST DISEASE-LIKE SYNDROME OF NOD/SCID MICE**Y.H. Kao<sup>1</sup>, B.-Y. Tsai<sup>1</sup>, B.-L. Chiang<sup>2</sup><sup>1</sup>Graduate Institute of Clinical Medicine, Taipei, Taiwan, Republic of China, <sup>2</sup>Department of Pediatrics, National Taiwan University Hospital, College of Medicine, National Taiwan University, Taipei, Taiwan, Republic of China

**Background:** Graft-versus-host disease (GVHD) is the major cause of morbidity and mortality following allogeneic hematopoietic stem cell transplantation, which has been the primary limitation to the wider application of allogeneic bone marrow transplantation (BMT). The immunomodulatory properties of mesenchymal stem cells (MSCs) have been considered as a potential application for treatment of GVHD. Some studies show the immunosuppressive effects for therapy of GVHD, however, the mechanism is still unclear.

**Methods:** To investigate the effect of MSC therapy for GVHD, we treated the human GVHD-like syndrome NOO-SCID, that is a model for xenogeneic GVHD (X-GVHD) by intraperitoneal injected 50x10<sup>6</sup> human peripheral blood cells into the mice NOD/SCID mice, with CFSE stained human bone marrow derived MSC and analyzed different cells population and condition.

**Results:** In comparison to the control mice, MSCs treated GVHD-like mice prolong the survival time and lower rate of body weight loss. In addition, flowcytometry analysis shows MSC blocking monocyte differentiated into dendritic cells (DCs) and inhibit upregulation of CD80, CD86 and MHCII of DCs. Furthermore, IL-10 increased but IL-2 level decreased in the serum of MSC treated mice. Moreover, T cells proliferation in MSC treated mice was inhibited as well.

**Conclusion:** Our data supported that the MSC attenuate GVHD-like syndrome through inhibiting DC maturation and activation of T cells. It also advanced the possible therapeutic effects of MSCs for GVHD.

**PD09/44 IMMUNOTHERAPEUTIC STRATEGY AGAINST EBV LATENCY II MALIGNANCY**O. Morales<sup>1</sup>, S. Depil<sup>1</sup>, C. Miroux<sup>1</sup>, F. Dufosse<sup>2</sup>, C. Aurialt<sup>3</sup>, V. Pancré<sup>1</sup>, N. Dlehem<sup>1</sup><sup>1</sup>CNRS UMR 8161, Institut de Biologie de Lille, Immunothérapie et Immunopathologie des cancers viro Induits, Lille, France, <sup>2</sup>CHRU de Lille, Service d'Immunologie, HLA, Transplantation, Lille, France, <sup>3</sup>Institut de Pharmacologie Cellulaire, Sophia-Antipolis, France

The Epstein-Barr virus (EBV) is associated with several malignant diseases which can be distinguished by their patterns of viral latent gene expression. The latency II (lat.II) program is limited to the expression of the non-immunodominant antigens EBNA-1, LMP-1 and LMP-2, and is particularly associated with Hodgkin's disease, nasopharyngeal carcinomas and peripheral T/NK-cell lymphomas. Knowing that CD4<sup>+</sup> T lymphocytes play a crucial role in controlling these EBV malignancies, we favoured an approach of immunotherapy, based on the stimulation of a specific CD4<sup>+</sup> T cell response.

We used the TEPITOPE software to predict promiscuous MHC class II epitopes derived from the latency II antigens EBNA-1, LMP-1 and LMP-2. The predicted peptides were then submitted to peptide-binding assay on HLA II purified molecules, which allowed the selection of 6 peptides (EBNA-1: 3, LMP-1: 1, LMP-2: 2) with a highly promiscuous capability of binding. The peptide cocktail is highly immunogenic in  $\beta$ 2<sup>-</sup>DR1 transgenic mice, leading to a specific cellular and humoral Th1 response. All the peptides used in the cocktail or individually were also recognized by human CD4<sup>+</sup> memory T cells from healthy donors expressing various HLA II genotypes and from patients with Hodgkin's lymphoma (HL). We have then generated peptide-specific CD4 cell lines, and assessed their cytotoxic potential to lyse lymphoblastoid cell lines (LCLs, Lat.III), or other EBV expressing cell lines such as T cell line (NC5, Lat.II) and monocyte cell lines (TE1, Lat.II). Finally, any changes in CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cell activity were observed in response to the peptide cocktail; avoiding the risk of aggravation of the pre-existing immunosuppressive environment, already known in EBV<sup>+</sup> associated malignancies.

Thus, this promiscuous peptide cocktail could be used in immunotherapeutic approaches against EBV latency II malignancies. It could be used as peptide-based vaccine or cellular therapy, with the hope of controlling the residual disease after classical treatment or to decrease the risks of relapse.

**PD09/45 IDENTIFICATION OF TUMOR-DERIVED MHC CLASS I PHOSPHOLIGANDS AND NATURALLY PRESENTED PHOSPHORYLATED MHC CLASS II PEPTIDES**V.S. Meyer<sup>1</sup>, O. Drews<sup>1</sup>, M. Günder<sup>1</sup>, H.-G. Rammensee<sup>1</sup>, S. Stevanović<sup>1</sup><sup>1</sup>Institute for Cell Biology, University of Tübingen, Department of Immunology, Tübingen, Germany

Major histocompatibility complex molecules present peptides for recognition by the T-cell receptor of T lymphocytes. Peptides produced endogenously are degraded as 8- to 12mers by the proteasome and are presented by MHC class I molecules. In contrast, MHC class II molecules bind peptides of a more variable length of 9 to 25 amino acids. In order to analyse naturally presented phosphorylated peptides, we precipitated the MHC molecule-peptide-complexes from solid renal cell carcinoma as well as several cell lines (RCC68, JY, MaMel-8a) by immunoaffinity chromatography and enriched posttranslationally phosphorylated peptides with an offline titaniumdioxide-based centrifugation technique. For the identification of potentially tumor-restricted phosphopeptides we compared the repertoire of phosphorylated peptides presented by a patient's tumor tissue to the one presented by the same patient's healthy renal tissue (RCC) by differential stable isotope labeling and mass spectrometric analysis.

We were able to identify a total of 75 posttranslationally phosphorylated peptides, 28 presented on MHC class I and 47 presented on MHC class II. Most of the class II-presented peptides were found in differing length variants. Nine out of 16 ligands were derived from significant transmembrane receptors such as Frizzled 6, CXCR4 and CD20. Additionally, we detected one MHC class I peptide that is known as tumor-specific (RPRLQHSFpSPF), derived from the protein Butyrate response factor 1. As it is known that at least CD8<sup>+</sup> T cells are able to discriminate between phosphorylated and non-modified peptides *in vivo*, MHC ligands containing phosphorylations specifically relevant for the proliferation of tumor cells may represent potential targets for T cell-based cancer immunotherapy.



**PD09/46 CYTOTOXICITY, ROI AND RNI PRODUCTION BY NOVEL CD14<sup>+</sup>CD16<sup>+</sup> AND CD14<sup>++</sup>CD16<sup>+</sup> MONOCYTE SUBPOPULATIONS GENERATED FROM CORD BLOOD HAEMATOPOIETIC PROGENITOR CD34<sup>+</sup> CELLS**

M. Stec<sup>1</sup>, J. Baran<sup>1</sup>, B. Mytar<sup>1</sup>, M. Zembala<sup>1</sup>

<sup>1</sup>Jagiellonian University Medical College, Immunology, Krakow, Poland

CD14<sup>++</sup>CD16<sup>+</sup> and CD14<sup>+</sup>CD16<sup>+</sup> are two main subpopulations of human monocytes in the peripheral blood. Recently, we have shown that expansion and differentiation of cord blood CD34<sup>+</sup> haematopoietic stem cells to monocytes/macrophages lead to generation of CD14<sup>+</sup>CD16<sup>+</sup> and CD14<sup>++</sup>CD16<sup>+</sup> monocyte subsets. Their immunophenotype and biological functions distinguish them from blood monocytes. In addition, CD14<sup>+</sup>CD16<sup>+</sup> and CD14<sup>++</sup>CD16<sup>+</sup> subpopulations differ in the release of cytokines/chemokines and chemotactic activity following stimulation with tumour cells. It suggested that CD14<sup>++</sup>CD16<sup>+</sup> monocytes exhibit an increased antitumour response in comparison to CD14<sup>+</sup>CD16<sup>+</sup> subpopulation. Aim of the present study was to compare cytotoxic activity against tumour cells, production of ROI (reactive oxygen intermediates) and RNI (reactive nitrogen intermediates) by CD14<sup>+</sup>CD16<sup>+</sup> and CD14<sup>++</sup>CD16<sup>+</sup> monocyte subsets generated from CD34<sup>+</sup> cord blood cells following stimulation with cancer cells. Immunomagnetically isolated CD34<sup>+</sup> cells were used for generation of monocytes by two-step approach: 7–10 days expansion in X-VIVO10 medium supplemented with FBS, SCF, FLT-3L, IL-3, TPO and then 7–10 days differentiation in IMDM medium in the presence of FBS, SCF, FLT-3L, IL-3 and M-CSF. Cytotoxic activity against cancer cells was assessed by MTT test, while the intracellular level of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) was measured by flow cytometry with the use of fluorescence indicator dihydrorhodamine 123 (DHR), whereas NO production was measured by 4,5 Diaminofluorescein (DAF). Monocyte subsets were isolated by FACS sorting. CD14<sup>++</sup>CD16<sup>+</sup> monocytes exhibited significantly higher cytotoxic activity against tumour cells *in vitro* as compared to CD14<sup>+</sup>CD16<sup>+</sup> cells (34.04% vs. 15.56%). However, following contact with tumour cells, CD14<sup>+</sup>CD16<sup>+</sup> monocytes produced more NO and ROI as compared to CD14<sup>++</sup>CD16<sup>+</sup> subpopulation (mean 37% vs. 19% and mean 5.4% vs. 1.6%, respectively). This may suggest that in contrast to blood monocyte subsets, ROI and RNI are not involved in the cytotoxic activity of monocytes generated from CD34<sup>+</sup> haematopoietic progenitors. Supported by the State Committee for Scientific Research (grant no. 3560/PO1/2007).

**PD09/47 NK CELLS RECOGNIZE AND KILL HUMAN GLIOBLASTOMA CELLS WITH STEM CELL-LIKE PROPERTIES**

A. Dondero<sup>1</sup>, R. Castriconi<sup>1</sup>, A. Daga<sup>2</sup>, G. Zona<sup>3</sup>, P.L. Poliani<sup>4</sup>, A. Melotti<sup>2,5</sup>, F. Griffero<sup>2,5</sup>, D. Marubbi<sup>2,5</sup>, R. Spaziant<sup>3</sup>, F. Bellora<sup>1</sup>, L. Moretta<sup>1,6,7</sup>, A. Moretta<sup>1,6</sup>, G. Corte<sup>2,5</sup>, C. Bottino<sup>1,7</sup>

<sup>1</sup>Università degli Studi di Genova, Dipartimento di Medicina Sperimentale, Genova, Italy, <sup>2</sup>Istituto Nazionale per la Ricerca sul Cancro, Genova, Italy, <sup>3</sup>Università degli Studi di Genova, Dipartimento di Neuroscienze, Oftalmologia e Genetica, Genova, Italy, <sup>4</sup>Università di Brescia, Brescia, Italy, <sup>5</sup>Università degli Studi di Genova, Dipartimento di Oncologia, Biologia e Genetica, Genova, Italy, <sup>6</sup>Centro di Eccellenza per le Ricerche Biomediche, Genova, Italy, <sup>7</sup>Istituto Giannina Gaslini, Genova, Italy

**Aims:** Glioblastomas (GBM) are poorly differentiated astrocytic tumors. Despite aggressive treatments combining surgery, irradiation and chemotherapy, the prognosis remains poor with a median survival time of 14 months. Thus different innovative therapies have been proposed including Natural Killer (NK)-based immunotherapy. Indeed among cytolytic lymphocyte populations, NK cells represent the most efficient effectors against different tumors. Aim of this study was to evaluate the cytolytic activity of human NK cells against ex-vivo derived GBM cells.

**Methods:** In this study, we isolated cancer cells from tumor specimens of nine different GBM patients. GBM cells were expanded *in vitro* in stem cell medium and analyzed for the expression of Neural Stem Cell (NSC) markers, multilineage differentiation and tumorigenicity in immunodeficient mice. Stem cell-cultured GBM cells were analyzed for susceptibility to lysis mediated by resting or lymphokine-activated NK cells both in allogeneic and autologous settings. Moreover, we measured the type and number of receptor/ligand interactions involved in the NK-mediated recognition of cancer cells.

**Results:** GBM cells, cultured under suitable culture conditions, displayed markers typical of neural stem cells, were capable of partial multilineage differentiation *in vitro*, and gave origin to infiltrating tumors when orthotopically injected in NOD/SCID mice. About the phenotypic analysis in terms of ligands specific for inhibitory or triggering NK receptors, cytofluorimetric analysis showed that GBM cells did not express protective amounts of HLA class I molecules while expressed various ligands specific for activating NK receptors. GBM cells, although resistant to freshly isolated NK cells, were highly susceptible to lysis mediated by both allogeneic and autologous IL-2 (or IL-15) activated NK cells. NKG2D and DNAM-1 triggering NK receptors played a predominant role in recognition and killing of GBM cells with stem cell-like properties.

**Conclusions:** In conclusion, our present study provides relevant information on the susceptibility of ex-vivo derived GBM with stem cell-like properties to NK mediated killing. These results may justify novel therapeutic approaches based on the use of activated NK cells as adjuvant, in an attempt to eradicate tumor cells residual after surgery.

**PD09/48 CANCER CELL DESTRUCTION *IN VITRO* AND IN PATIENTS BY LYMPHOCYTES PRIMED WITHOUT TUMOR CELLS OR IDENTIFIED TUMOR PEPTIDES**

B. Laumbacher<sup>1,2</sup>, S. Gu<sup>1</sup>, R. Wank<sup>1,2</sup>

<sup>1</sup>Forschungszentrum Immunzelltherapie, München, Germany, <sup>2</sup>Immunis e.V., München, Germany

Until now adoptive immunotherapy has required definition of tumor-specific immunogenic material. Using immune cells from the peripheral blood we achieved with a novel method of activation, that a cellular quartett of monocytes, dendritic cells, T helper and T cytotoxic cells lysed efficiently different autologous cancer cells *in vitro*. Such cells primed with this method increased significantly five year survival rates of patients in an adjuvant intention-to-treat setting. We first stimulated monocytes and dendritic cells by CD3-activation of T cells. In the next step we added unstimulated T cells since CD3-activated T cells internalize the antigen-specific T cell receptor. CD14<sup>+</sup> monocytes, indispensable for the cascade priming (CAPRI) process like T cells, showed after 24 hours increased maturation of CD14<sup>+</sup> monocytes to CD14<sup>+</sup>, CD83<sup>+</sup> dendritic cells in CAPRI cultures, numbers of CD4<sup>+</sup> CD25<sup>high</sup> FOXP3<sup>+</sup> regulating cells decreased. CAPRI cells enhanced HLA class I and class II expression in cocultured cancer cells of different types, crucial for MHC-restricted lysis by CAPRI helper and cytotoxic T lymphocytes. *In vitro* results indicate that the CAPRI method can be used for many cancers.

**PD09/49 TGF-BETA NEUTRALIZATION IN TUMOR-BEARING HOSTS RESTORES ANTIBODY PRODUCTION BUT REDUCES CYTOTOXIC T-CELL RESPONSES**

E.P. Kwiatkowski<sup>1,2</sup>, A. Mackiewicz<sup>1,2</sup>, D.W. Kowalczyk<sup>1,2</sup>

<sup>1</sup>Poznan University of Medical Sciences, Department of Cancer Immunology, Poznan, Poland, <sup>2</sup>Great Poland Cancer Center, Department of Cancer Diagnostic and Immunology, Poznan, Poland

**Objectives:** Patients with advanced neoplastic disease often display immunosuppression and fail to respond to active immunotherapy approaches. Therefore, tumor induced immunosuppression may contribute to the limited success of immunotherapy for the treatment of cancer. However, only a few studies so far have tried to reverse tumor induced immune dysfunctions. Since an important role in tumor induced immunosuppression has been assigned to transforming growth factor-beta we analyzed the effect of TGF- $\beta$  neutralization on induction of specific immune responses in tumor-bearing mice.

**Methods:** For *in vivo* experiment GL261 tumor model was used in C56BL/6 mice. For *in vivo* TGF- $\beta$  neutralization sT $\beta$ R1-Fc was used at 30 mg per mouse. For *in vitro* studies the sT $\beta$ R1-Fc was used at 1 mg/ml concentration. Specific CD8<sup>+</sup> T cells were detected by intracellular IFN- $\gamma$  staining. For T helper responses cytokine production and proliferation were analyzed. Specific anti- $\beta$ -galactosidase antibodies were measured by ELISA. T cell apoptosis was measured using the colorimetric CaspACE Assay System. CD95 expression was analyzed by flow cytometry.

**Results:** We showed that the long tumor presence inhibited specific T-cell and humoral adaptive immunity to exogenous antigen. TGF- $\beta$  neutralization with chimeric soluble TGF- $\beta$  Type II Receptor fused to the Fc fragment of IgG resulted in considerable restoration and augmentation of specific antibody responses. However, despite marked improvement in humoral response TGF- $\beta$  neutralization was not effective in restoration of cytotoxic T-cell mediated responses. In the T-cell compartment TGF- $\beta$  neutralization induced massive TNF- $\alpha$  and Fas-dependent T-cell apoptosis and reduced specific cytotoxic T-cell responses.

**Conclusion:** Our studies show that although long lived tumors induce profound immunosuppression, TGF- $\beta$  neutralization restores B-cell reactivity and antibody production in the presence of a tumor. However due to inhibitory effect on CTL responses this strategy may require additional measures to achieve full immune restoration in cancer patients.

**PD09/50 THE ROLE OF CHEMOTHERAPY IN ENHANCING ADOPTIVE T CELL THERAPY, VISUALIZED BY *IN VIVO* IMAGING**

C. Perez<sup>1</sup>, A. Jukica<sup>1</sup>, J. Charo<sup>1</sup>, T. Blankenstein<sup>1</sup>

<sup>1</sup>Max-Delbrueck Center for Molecular Medicine, Berlin, Germany

It is not clear why chemotherapy improves adoptive T cell therapy of cancer. Chemotherapy might act by killing tumor cells, stroma cells or both. To distinguish between these possibilities, we established a bioluminescence imaging (BLI) model with stroma and tumor cells expressing renilla (RLuc) or firefly (FLuc) luciferases, respectively, which can be discriminated by their substrates utilization and the emitted light spectrum. Tumor fragments composed of the ovalbumin-expressing FLuc-EG.7 tumor and RLuc-stroma were grafted to C57BL/6 mice. When the tumor size reached a mean diameter of  $\geq 10$  mm, mice were treated by chemotherapy followed by activated OT-I T cells, or either treatment alone and tumor versus resident stroma cell elimination was analyzed by BLI. Only the combined treatment led to complete tumor rejection. The rapid decrease of tumor cell-derived Fluc-signal directly reflected tumor regression. The resident stroma cell-derived RLuc-signal in all treatment groups diminished after tumor regression, suggesting that tumor cells were targeted first by the combined chemotherapy. Currently, we analyze the stroma cell types that reside in the tumor at the time of treatment and thereafter.

**PD09/51 THE ABSOLUTE NUMBER AS WELL AS THE ACTIVATION KINETICS OF REGULATORY T CELLS DETERMINES THE SUCCESSFUL GENERATION OF PRIMARY ANTI-TUMOR AND PATHOGEN-SPECIFIC IMMUNE RESPONSES FROM THE NAÏVE DONOR T CELL REPERTOIRE**

I. Jedema<sup>1</sup>, J. Pots<sup>1</sup>, M. van de Meent<sup>1</sup>, R. Willemze<sup>1</sup>, J.H.F. Falkenburg<sup>1</sup>  
<sup>1</sup>Leiden University Medical Center, Hematology, Leiden, Netherlands

Effective and specific control of disease relapses and/or infectious complications after allogeneic stem cell transplantation may be accomplished by the administration of in-vitro selected tumor- or pathogen-specific T cells. Although the in-vitro induction of primary immune responses against leukemic cells, minor histocompatibility antigens and viral antigens has been shown to be feasible by us and others, the robustness of the procedure remains limited, hampering large scale clinical application. We hypothesized that the major complication is the unfavorable balance between the number and activation of antigen-specific precursor T cells (Tprec), estimated to be < 0.001% of the naive donor T cells, and the number of regulatory T cells (Treg) capable of inhibiting the activation of antigen-specific Tprec locally at the site of the priming of the immune response, e.g. the antigen presenting cell (APC). Therefore, we determined the activation kinetics of naive donor T cells and CD4+CD25+CD127-FoxP3+ Treg upon stimulation with anti-CD3/28 antibodies. Whereas it took 2-4 days to get maximal activation of all naive CD4 and CD8+ T cells, Treg were uniformly activated already after 24 hours, resulting in expression of CD137 and CD69 on the whole population. Similar activation of Treg was seen upon exposure to autologous mature monocyte-derived APC, coincided by an increased survival of Treg. Next, we determined the inhibitory capacity of unstimulated Treg and Treg stimulated with autologous APC. Whereas on average a 1/1 ratio of unstimulated Treg was necessary to obtain 50% inhibition of CD3/28 induced Tresp activation and proliferation, activation of the Treg by APC significantly increased their inhibitory potential resulting in 50% inhibition of Tresp activation at a median frequency of 10% added Treg. In conclusion, these data demonstrate that Treg are activated with much faster kinetics compared to naive antigen-specific precursor T cells. Moreover, this activation further increased their inhibitory capacity. This may shift the balance locally at the APC site further towards Treg-induced inhibition. In accordance with this, depletion of CD45RO+ responder T cells, containing the majority of Treg, significantly increased the ability to induce primary immune responses in-vitro, facilitating the reproducible induction of virus- and tumor-specific T cells for adoptive transfer.

**PD09/52 ESSENTIAL ROLE OF THE SPLEEN FOR THE GENERATION OF CD8 T-CELL RESPONSES AGAINST TUMORS**

F. Jüngerkes<sup>1</sup>, T. Schwandt<sup>1</sup>, B. Schumak<sup>1</sup>, F. Schildberg<sup>1</sup>, S. Burgdorf<sup>1</sup>, T. Tüting<sup>2</sup>, J.J. Hernando<sup>3</sup>, G. Hämmerling<sup>4</sup>, A. Limmer<sup>1</sup>

<sup>1</sup>University Hospital Bonn, Institute for Molecular Medicine and Experimental Immunology (IMMEI), Bonn, Germany, <sup>2</sup>University Hospital Bonn, Department of Dermatology, Bonn, Germany, <sup>3</sup>University Hospital Bonn, Department of Obstetrics and Gynaecology, Bonn, Germany, <sup>4</sup>German Cancer Research Center (DKFZ), Department of Tumor Immunology, Heidelberg, Germany

T cell mediated anti-cancer therapy gained in importance during the last years. Vaccination strategies based on recombinant Adenovirus (AdV) vehicles, antigen-pulsed dendritic cells (DC) or recombinant/transgenic Listeria monocytogenes (LM) rate among the common methods for the induction of adaptive immune responses directed against tumors. Here, we emphasize the importance of the spleen in systemic vaccination protocols. We applied our findings in tumor vaccination strategies directed against organ-specific tumors. Although systemic vaccination based on AdV, DCs or LM primarily targeted the liver, the induction of efficient antigen-specific cytotoxic T cell (CTL) responses required the spleen. Nevertheless, splenectomized mice were capable to generate adaptive immune responses upon local immunization. However, locally induced CTL responses in splenectomized mice were not as strong as in systemically vaccinated non-splenectomized littermates.

Tumor growth of intravenously injected B16 melanoma cells expressing luciferase in mice was monitored by in vivo imaging. We determined a strong correlation between the anatomic location of the tumor, its rejection and the route of vaccination. Tumors resident in lung or liver were rejected within two weeks after systemic vaccination using AdV, DC or LM expressing tumor antigens. Importantly, tumor growth was neither inhibited in immunized asplenic mice nor in non-immunized mice. Therefore, we conclude a central role of the spleen for the generation of powerful immune responses against certain tumors.

**PD09/53 COMPARATIVE STUDY ON ANTI-TUMOR ACTIVITY OF EX VIVO DIFFERENTIATED DENDRITIC CELLS AND DENDRITIC CELL LINE JAWS II, TRANSDUCED WITH IL-12 GENES**

J. Rossowska<sup>1</sup>, E. Pajtasz-Piasecka<sup>1</sup>, J. Wojas<sup>1</sup>, D. Duś<sup>1</sup>

<sup>1</sup>Ludwik Hirsfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland

**Aim:** The purpose of the study was to analyze the migration ability and anti-tumor activity of IL-12 transduced mouse bone marrow derived-dendritic cells differentiated ex vivo (BM-DC/IL-12) and an established JAWS II dendritic cell line (JAWS II/IL-12). After transduction, BM-DC/IL-12 produced 3-5ng/ml and JAWS II/IL-12 – 5-10ng/ml of IL-12.

**Methods:** In the first part of the study MC38 colon carcinoma bearing mice were injected peritumorally with fluorochrome (CFSE)-labeled BM-DC/IL-12 or JAWS II/IL-12 cells. On the first, third, fifth and seventh day after cell vaccine injection, sLN, spleens and tumors were dissected and examined. The presence of CFSE-labeled dendritic cells (DC) in the injection site and in sentinel lymph nodes (sLN) as well as intensity of T lymphocytes infiltration to tumor tissue were examined using confocal microscope, whereas spleen cells cytotoxic activity was measured by flow cytometry. In the second part of the study, the influence of multiple peritumoral injections of IL-12 transduced DC alone or in combination with tumor antigen treated DC (BM-DC/Tag or JAWS II/Tag) on the MC38 tumor growth inhibition as well as spleen cells cytotoxic activity was estimated.

**Results:** It was observed that both BM-DC/IL-12 and JAWS II/IL-12 migrated from the injection site to the sLN more effectively than unmodified cells. Analysis of tumor tissues and spleens demonstrated that single injection of BM-DC/IL-12 primed stronger cellular anti-tumor response than JAWS II/IL-12. BM-DC/IL-12 caused higher influx of CD4<sup>+</sup> lymphocytes into tumor tissues. Moreover, these cells induced increased number of cytotoxic CD8<sup>+</sup>CD107a<sup>+</sup> and CD49b<sup>+</sup>CD107a<sup>+</sup> cells in spleen and enhanced the ability of spleen cells to IFN-gamma production. Unexpectedly, multiple injection of BM-DC/IL-12 alone or with BM-DC/Tag moderately affected tumor growth reduction, whereas the best results were obtained after application of JAWS II/IL-12 together with JAWS II/Tag.

**Conclusions:** Obtained results suggest that BM-DC/IL-12 cells, producing lower amount of IL-12, are more efficient activators of cellular anti-tumor response than JAWS II/IL-12. However, in advanced tumor disease, application of JAWS II/IL-12 with higher ability to IL-12 production, seems to be more profitable.

**This work was supported by the Ministry of Science and Higher Education (grants: N N401235334, N40114432/2798).**

**PD09/54 RNOS-INDUCED CHEMOKINE INACTIVATION IN CANCER**

B. Molon<sup>1</sup>, T. Kasic<sup>2</sup>, B. Savino<sup>3</sup>, C. Soldani<sup>4</sup>, A. De Palma<sup>4</sup>, R. Bonecchi<sup>3</sup>, P. Mauri<sup>4</sup>, V. Bronte<sup>1,2</sup>, A. Viola<sup>3</sup>

<sup>1</sup>Venetian Oncological Institute I.R.C.C.S., Padua, Italy, <sup>2</sup>Venetian Institute of Molecular Medicine, Padua, Italy, <sup>3</sup>Humanitas Clinical Institute, Milan, Italy, <sup>4</sup>I.T.B. Institute of Biomedical Technologies, Milan, Italy

The immune system possesses the requisites to act as a powerful weapon against cancer. However, results from clinical trials have shown that the efficacy of different immunotherapeutic approaches is inadequate for an immediate and widespread transfer to cancer patients. T lymphocytes activated by vaccination are virtually competent to attack neoplastic cells. Inefficacy of active immunotherapy likely depends either on the inability of sufficient lymphocyte numbers to reach the site of cancer growth or on the countermeasures orchestrated by tumor cells. In general, however, tumor microenvironment does not appear to be suitable for T lymphocyte functions and indeed a number of reports indicate that tumor-infiltrating lymphocytes (TIL) are impaired in both signal transduction and effector systems. At the tumor site, the reactions of NO with oxygen (O<sub>2</sub>) or oxygen-related reactive intermediates yield numerous reactive nitrogen as well as oxygen species (RNOS). One of the most intensively studied reaction is the one between NO and superoxide anions yielding peroxynitrite (ONOO<sup>-</sup>), which is a potent oxidant. In the past, we provided data showing that RNOS are involved in tumor-induced immunosuppression and we speculated that RNOS might block T lymphocyte infiltration in the tumor. We decided to study the role of RNS in tumor-induced immunosuppression. Typically, TILs are unable to reach the core of the tumor mass, and they concentrate at the border of the neoplastic lesion. We speculated that RNS modify chemokine biology and keep TILs distant from the tumor. Chemokines are small cytokines with selective chemoattractant properties, coordinating the homeostatic circulation of leukocytes as well as their movement to sites of inflammation or injury. Dysregulated expression of chemokines and/or their receptors is involved in the development of many human diseases, including autoimmune and chronic inflammatory diseases as well as immunodeficiency and cancer. We found that the chemoattractants CXCL12, CCL21 and CCL2 lose their ability to recruit T lymphocytes if exposed to peroxynitrite. However, the modified chemokine CCL2 retains its capacity of recruiting myeloid-derived suppressor cells. These data suggest that the modified chemokines may modify the tumor microenvironment and favor immunosuppression.

**PD09/55 THE ROLE OF CD11C<sup>+</sup> CD8α<sup>+</sup> DENDRITIC CELLS PULSED WITH HEAT SHOCKED TUMOR LYSATE IN THE REGRESSION OF FIBROSARCOMA**

A. Azadmehr<sup>1</sup>, A.A. Pourfathollah<sup>1</sup>, Z. Amirghofran<sup>2</sup>

<sup>1</sup>Tarbiat Modares University, Faculty of Medical Sciences, Immunology Department, Tehran, Iran, Islamic Republic of, <sup>2</sup>Shiraz University of Medical Sciences, Immunology Department, Medicinal & Natural Products Chemistry Research Center and Autoimmunity Diseases Research Center, Shiraz, Iran, Islamic Republic of

**Objectives:** The use of dendritic cells (DCs) as a cellular adjuvant provides a promising approach in immunotherapy of cancer. We investigated the effect of the subset lymphoid (CD8α<sup>+</sup>) DCs loaded with heat-treated tumor lysate (HTL) as a vaccine in tumor immunotherapy.

**Methods:** The Balb/c mice were injected subcutaneously in the right flank with Wehi-164 fibrosarcoma cells 10 days before immunization with the DCs. Then hsp70 expression in the HTL was detected by using western blot analysis. The mice spleen CD8α<sup>+</sup> DCs subset were isolated by magnetic cell sorting (MACS). Then the HTL pulsed CD8α<sup>+</sup> DCs, TL pulsed CD8α<sup>+</sup> DCs and unpulsed CD8α<sup>+</sup> DCs were injected subcutaneously around the tumor site. Tumor growth rate, survival, cytotoxic assay and lymphocyte subpopulation in the tumor tissue were measured in both case and control groups.

**Results:** The results showed that HTL-CD8 $\alpha^+$  DCs vaccine significantly induced the tumor growth suppression and longer survival than the other immunized mice. Immunotherapy with HTL-CD8 $\alpha^+$  DCs led to a significant increase in the activity and the number of cytotoxic T cells in the tumor tissue.

**Conclusion:** The current study suggests that specific anti-tumor immune responses against the fibrosarcoma can be induced by the subset of mouse spleen CD11c $^+$ CD8 $\alpha^+$  DCs pulsed with heat-treated tumor lysate and may provide a useful therapeutic approach for cancer treatment.

#### PD09/56 INTRATUMORAL TREATMENT WITH DENDRITIC CELLS ENGINEERED TO EXPRESS CD40L OR CD40L AND IL-2 INDUCES INHIBITION OF SUBCUTANEOUS HEPATOCELLULAR TUMOR GROWTH IN MICE.

A. Vogt<sup>1</sup>, W. Demmer<sup>1</sup>, T. Sauerbruch<sup>1</sup>, M. A. Gonzalez-Carmona<sup>1</sup>

<sup>1</sup>University of Bonn, Department of Internal Medicine I, Bonn, Germany

**Background:** Dendritic cells (DC) are professional antigen presenting cells able to prime T-cells against tumor-associated antigens. CD40/CD40L-interaction is essential for DC-activation and induction of antigen-specific T-cells. IL-2 is an essential cytokine for antigen-specific immune persistence. Previous studies have shown synergistic antitumoral effects by co-stimulation on CD40/CD40L-interaction and IL-2. In this study, we analyzed the impact of intratumoral (i. t.) treatment with DC transduced with adenoviruses encoding CD40L and IL-2 on tumor-growth in subcutaneous HCC.

**Methods:** DC were obtained from bone marrow of C3H-mice and adenoviral transduced on day 6 with Ad-IL-2, Ad-CD40L or Ad-LacZ (control). After stimulation with mab CD3, IL-2, IFN-gamma and IL-1-beta, mouse splenocytes were co-cultured with DC. Cytotoxicity of murine HCC-cells (Hepa129) was determined in a <sup>51</sup>Cr-release assay. Subcutaneous HCC were induced by inoculation of 10<sup>6</sup> Hepa129-cells into the right flank of mice. When tumor volume was 100-150mm<sup>3</sup>, 10<sup>6</sup> CD40L-, IL-2-, CD40L/IL-2- or LacZ-expressing DC were injected i. t. twice with a weekly interval. After the second DC-injection, tumors were analyzed by flow cytometry.

**Results:** Co-culture with Ad-CD40L-transduced DC enhanced significantly the cytotoxicity against Hepa129-cells of splenocytes in comparison to co-culture with Ad-IL-2 or Ad-LacZ-transduced DC (p< 0.05). Combination of CD40L/IL-2-expressing DC inhibited the tumor-growth significantly compared to mice treated with control DC or IL-2-DC (p< 0.05). However, the antitumoral activity of the combination was not stronger compared to DC transduced only with Ad-CD40L. Furthermore, CD40L-DC showed even a stronger inhibition of tumor growth than the combination of CD40L/IL-2-DC: the mean tumor volume was 361.4 $\pm$ 118.5mm<sup>3</sup> in CD40L-DC-treated animals vs. 868.2 $\pm$ 510.5 in CD40L/IL-2- or 1805 $\pm$ 309.7mm<sup>3</sup> in LacZ-DC-treated mice. Flow cytometry of tumors treated with CD40L-expressing DC revealed higher tumor-infiltration with CD4 $^{+}$ , CD8 $^{+}$ -T-cells and NK-cells than tumors treated with CD40L/IL-2 DC.

**Conclusions:** The combination of CD40L/IL-2-expressing DC can significantly inhibit the tumor-growth, activating both acquired and innate immunity. However, this effect seemed not to be synergistic, as treatments with CD40L-DC seemed to have even stronger antitumoral effects than the combination of CD40L- and IL-2-DC. These data underline the potential of the co-stimulation on CD40/CD40L-interaction in DC-based approaches against tumor disease.

#### PD09/57 DENDRITIC CELLS ENGINEERED TO EXPRESS CD40L AND AFP INDUCE TUMOR REGRESSION OF SUBCUTANEOUS HEPATOCELLULAR TUMORS BY ACTIVATING INNATE AND ADAPTIVE IMMUNITY AND BY INDUCING APOPTOSIS

M. A. Gonzalez-Carmona<sup>1</sup>, C. Schneider<sup>1</sup>, A. Vogt<sup>1</sup>, T. Sauerbruch<sup>1</sup>, W. H. Caselmann<sup>2</sup>

<sup>1</sup>University of Bonn, Department of Internal Medicine I, Bonn, Germany, <sup>2</sup>Bavarian State Ministry of the Environment, Public Health and Consumer Protection, Munich, Germany

**Background:** Dendritic cells (DC) are professional antigen presenting cells able to prime T-cells against tumor-associated antigens such as  $\alpha$ -fetoprotein (AFP), but their potential to induce HCC regression is limited. The interaction CD40ligand (CD40L) and CD40 is essential for initiation of antigen-specific T-cells responses. In this study, treatment with DC engineered to express CD40L and AFP induced a significant tumor regression in mice.

**Methods:** Three adenoviruses were used: Ad-mAFP (encoding murine AFP), Ad-CD40L (murine CD40L) and Ad-LacZ (control). Bone marrow-derived DC were cultured with GM-CSF and IL-4 and transduced with adenoviruses (MOI 250) on day 6. Expressions of CD80 and CD86 were assayed by flow cytometry, and IL-12 by ELISA. Apoptosis assessment in tumor cells was performed by detection of the sub-G1-fraction by flow cytometry. Subcutaneous hepatocellular tumors were induced by inoculation of 10<sup>6</sup> AFP-expressing Hepa129-cells into the right flank of mice. When tumor volume was >150 mm<sup>3</sup>, DC were injected into the tumor twice with a weekly interval. Tumors were collected and analyzed by flow cytometry. Apoptosis was analyzed by detection of caspase-3 activity.

**Results:** Expression of co-stimulatory molecules (CD80/CD86) and of IL-12 was significantly higher in CD40L-transduced DC than in Ad-AFP-transduced or control DC (>750 pg/ml/10<sup>6</sup> IL-12 in CD40L-DC vs. no detectable levels in control cells). Interestingly, tumor cells cultured with the supernatant of CD40L-expressing DC showed a significant increase of apoptosis: the sub-G1-fraction was increased up to 2-fold compared to tumor cells cultured with supernatant of control DC. Intratumoral treatment of hepatocellular tumors with Ad-AFP/Ad-CD40L-DC inhibited the tumor-growth significantly compared to mice treated with Ad-AFP/Ad-LacZ-DC (p=0.03). Flow cytometry of tumors revealed a significant tumor-infiltration with CD4 $^{+}$ , CD8 $^{+}$ -T-cells and NK-cells. Furthermore, higher caspase-3 activities were found in tumors treated with CD40L-expressing DC than in tumors treated with control DC or in untreated tumors (p< 0.05).

**Conclusions:** Transduction of DC with Ad-CD40L increases significantly the DC-stimulatory capacity. CD40L/AFP-expressing DC can significantly inhibit the tumor-growth of subcutaneous AFP-positive hepatocellular tumors, activating both acquired and innate immunity. Furthermore, CD40L-expressing DC seem to directly induce apoptosis in tumor cells as a further mechanism of the antitumoral potential of CD40L.

#### PD09/58 PROCESS OF TUMOR ERADICATION BY ADOPTIVE T CELL THERAPY VISUALIZED BY IN VIVO IMAGING

J. Charo<sup>1</sup>, C. Buschow<sup>1</sup>, C. Perez<sup>1</sup>, K. Anders<sup>1</sup>, A. Jukica<sup>1</sup>, T. Blankenstein<sup>1</sup>

<sup>1</sup>MDC, Berlin, Germany

In some models, adoptively transferred T cells effectively can reject tumors; in others they fail for poorly understood reasons. Therefore, methods like bioluminescence imaging, which allow to follow expansion, location and contraction phases of transferred T cells are desirable. We established Renilla luciferase (RLuc) transgenic mice, which, after crossing to T cell receptor transgenic mice, served as source for RLuc $^{+}$  tumor-specific T cells. T cells homed preferentially to antigen $^{+}$  tumors, which correlated with their rejection. The population dynamics of the adoptively transferred tumor-specific T cells went through three distinct stages, which identified parallel stages in tumor rejection. Using chemotherapy as a pretreatment to adoptive T cell therapy, we modulated the homing of these T cells, allowing an earlier access of T cells to the antigen $^{+}$  tumor. This model provides additional insight, in vivo, into the behavior of tumor-specific T cells during tumor rejection.

#### PD09/59 THE EFFECT OF LOCALLY PRODUCED IFN $\gamma$ ON ESTABLISHED TUMORS

D. Briesemeister<sup>1,2</sup>, D. Sommermeyer<sup>2</sup>, W. Uckert<sup>2</sup>, T. Kammertöns<sup>1</sup>, T. Blankenstein<sup>1,2</sup>

<sup>1</sup>Charité – Campus Benjamin Franklin, Institut für Immunologie, Berlin, Germany, <sup>2</sup>Max Delbrück Center for Molecular Medicine, Berlin, Germany

It has been shown that injecting a suspension of Interferon- $\gamma$  (IFN $\gamma$ )-secreting tumor cells results in their rejection. Probably, this effect is mediated by IFN $\gamma$  acting on developing stroma cells but not a direct effect on the cancer cells. However, it is not known, which influence locally produced IFN $\gamma$  has on established tumors. To address the question, whether a high local level of IFN $\gamma$  is sufficient to reject established tumors, we transfected the plasmacytoma cell line J558L with plasmids encoding a tetracycline-inducible IFN $\gamma$  gene. After the injection of tumor cells into mice, cytokine production was induced at different time points by administering doxycycline via the drinking water. Forty-eight hours after doxycycline treatment high local levels of IFN $\gamma$  were detected in the serum, which correlated with tumor burden (e. g. 700 mm<sup>3</sup> tumors produced 248 ng/ml IFN $\gamma$ ). Tumors did not grow within 100 days of observation when inducing IFN $\gamma$  expression immediately after tumor cell inoculation. Induction at a later time point, when the stroma was established (day 12-21, tumor volume 350-1600 mm<sup>3</sup>) resulted in hemorrhagic necrosis, growth delay but also lethal toxicity.

With the tetracycline system we found a tool which permits to switch IFN $\gamma$  on and off *in vivo* in established tumors. Currently we try to find a therapeutic window using different strategies such as dose titration of IFN $\gamma$  and intermittent treatment. Also, we are analyzing whether IFN $\gamma$  release induces the destruction of blood vessels in established tumors.

#### PD09/60 NEW INSIGHTS INTO THE MECHANISMS UNDERLYING CHEMO-IMMUNOTHERAPY ANTITUMOR EFFICACY BY MICROARRAY ANALYSIS

F. Moschella<sup>1</sup>, M. Valentini<sup>1</sup>, P. Sestili<sup>1</sup>, E. Arico<sup>1</sup>, F. Belardelli<sup>1</sup>, E. Proietti<sup>1</sup>

<sup>1</sup>Istituto Superiore di Sanità, Cell Biology and Neurosciences Department, Rome, Italy

Although cancer chemotherapy has been usually considered as immunosuppressive, some chemotherapeutic agents were recently proved to modulate the immune response and enhance the antitumor efficacy of either adoptive or active immunotherapy. Nowadays, cyclophosphamide (CTX) represents the gold standard chemotherapeutic agent able to potentiate the effectiveness of cancer immunotherapy and to induce tumor rejection in mice bearing highly metastatic tumor implants. Different mechanisms have been proposed to explain the immunomodulatory activity of CTX, including reduction of regulatory T cell number, increased tumor infiltration by lymphocytes, homeostatic proliferation and functional activation of B and T cells. Nevertheless, the molecular mechanisms underlying CTX ability to potentiate the immune response still need to be clarified. An high throughput technology, such as microarray, may represent an invaluable tool to gain insights into the mechanisms associated with the efficacy of cancer chemo-immunotherapies. In a previous study we proposed that the immunomodulatory properties of CTX largely depend on the induction of a "cytokine storm", occurring primarily in the bone marrow of treated mice. To deepen our knowledge of this phenomenon, we analyzed the effect of CTX treatment on the gene expression profiles of bone marrow as well as of spleen and peripheral blood cells of tumor-bearing mice by microarray analysis. This study showed that CTX profoundly affects the gene expression of these cells at early time points after treatment (already 1 day after chemotherapy), inducing, on one side, the up-regulation of several genes involved in cell differentiation, cell migration and regulation of the immune response, and, on the other hand, the decrease of biological functions, such as biosynthetic/metabolic processes, ribosome assembly and cell cycle. These findings provide new insights into the comprehension of the complex mechanisms underlying the ability of certain chemotherapeutic drugs to enhance the efficacy of immunotherapy and open new opportunities for the design of innovative chemo-immunotherapeutic strategies for cancer treatment.



**PD09/61 OPTIMIZATION OF FUNCTIONS AND SURVIVAL OF EFFECTOR CD8 T CELLS FOR ANTI-TUMOR ADOPTIVE THERAPIES**

M. Grange<sup>1</sup>, M. Buferne<sup>1</sup>, A.-M. Schmitt-Verhulst<sup>1</sup>, N. Auphan-Anezin<sup>1</sup>  
<sup>1</sup>Centre d'Immunologie de Marseille-Luminy, Marseille, France

Adoptive transfer of T lymphocytes (TL) specific for tumor antigens is undergoing evaluation for treatment of patients bearing solid tumors such as melanoma. This therapeutic approach involves an extensive in vitro expansion of anti-tumor TL before their adoptive transfer, therefore restricting this application to a small number of identified tumor antigens (and consequently of patients) to be used for efficient in vitro TL reactivation. Moreover, both in vitro culture and TL infusion require high doses of IL-2 to allow for prolonged maintenance of transferred TL. This cytokine has adverse effects on patients and may activate suppressive T regulatory lymphocytes.

In mouse models, we test a new strategy to

- (1) optimize selectively the activity of anti-tumor effector CD8 T cells;
- (2) favor their migration in tissues;
- (3) enhance their survival in vitro and in vivo;
- (4) reduce their sensitivity to immunosuppressive mechanisms;
- (5) avoid bystander activation of T regulatory cells.

This scheme relies on the constitutive activation of a cytokine-driven signaling pathway that allows for specific activation of genes, the products of which are involved in cytotoxic activity and homing of CD8 TL. This approach also confers a survival advantage on the modified TL, which allows for expansion of TL specific for low avidity tumor antigens. The molecular mechanisms that underlie this survival advantage are presently being studied to ensure the lack of deleterious effects of the introduced manipulation, a point that is also being evaluated in adoptive transfer studies. The capacity of manipulated anti-tumor CD8 TL to eliminate established tumors is under assessment in appropriate mouse models.

**PD09/62 DEFINING NOVEL MOLECULES TO RESCUE IMMUNITY AGAINST CANCER: MOLECULAR AND BIOLOGICAL BASIS FOR NEW THERAPIES**

T. Kasic<sup>1</sup>, F. Delposso<sup>1</sup>, C. Soldani<sup>2</sup>, R. Fruttera<sup>3</sup>, A. Gasco<sup>3</sup>, V. Bronte<sup>1,4</sup>, A. Viola<sup>1,2</sup>

<sup>1</sup>Venetian Institute of Molecular Medicine, Padova, Italy, <sup>2</sup>Clinical Institute Humanitas I.R.C.C.S., Milan, Italy, <sup>3</sup>University of Turin, Department of Drugs Technology, Turin, Italy, <sup>4</sup>Venetian Oncological Institute I.R.C.C.S., Padova, Italy

Prostate cancer (PCa) is the second leading cause of malignancy-related mortality in males in the Western world. While radical prostatectomy and local radiotherapy are largely successful for patients with localized cancer, available treatments for metastatic PCa have demonstrated weak curative efficacy. It is therefore necessary to find alternative therapeutic approaches to hormone-refractory metastatic PCa. Immunotherapy may provide a valid alternative therapy but the success of this approach depends on the ability of CTL to kill tumor cells. However, if the tumor environment exerts a suppressive action on antigen-specific TIL, immunotherapy will achieve little, if any, success. Thus, it is paramount to understand modulation of TIL responses by the tumor environment. To analyze the role of the prostate tumor environment we have started in our laboratory a study based on the use of collagen gel-matrix supported organ cultures which allowed us to demonstrate that human PCa are infiltrated by terminally differentiated CTL that are completely unresponsive. The steady-state regulation of the dormant state is dependent on the enhanced intratumoral metabolism of L-Arg, because the addition of ARG- and NOS- specific inhibitors was sufficient to activate them and recover their functions. These results identify a mechanism by which PCa induces in situ immunosuppression and suggest novel strategies for the tumor immunotherapy.

Based on our findings, drugs controlling ARG and NOS might be useful to aid immunotherapeutic approaches for the treatment of cancer by creating a favorable tumor environment for the T lymphocyte effector program. On these bases, we have developed and tested a new class of NO-donor molecules characterized by having a furoxan moiety linked through an ester bridge to the carboxy function of aspirin. The data obtained indicate that these compounds can normalize the immune status of tumor-bearing hosts and restore mice lymphocyte responsiveness, in terms of proliferation and effector functions in both in vitro and in vivo assays. Moreover, we observed that the newly synthesized NO-releasing COX-inhibitor restore TIL responsiveness in human PCa in vitro. Thus, this compounds could be consider potential candidates as adjuvants in the antitumor immunity elicited by cancer vaccination.

**PD09/63 EVALUATION OF CYTOTOXIC EFFECT OF TUMOR CELL LYSATE COMPLEX WITH HEAT SHOCK PROTEINS (NANO PARTICLE) PULSED WITH CD11c<sup>+</sup> CD8α<sup>+</sup> DENDRITIC CELLS IN THE REGRESSION OF FIBROSARCOMA**

A. Azadmehr<sup>1</sup>, A.A. Pourfathollah<sup>1</sup>, Z. Amirghofran<sup>2</sup>

<sup>1</sup>Tarbiat Modares University, Faculty of Medical Sciences, Immunology Department, Tehran, Iran, Islamic Republic of, <sup>2</sup>Shiraz University of Medical Sciences, Immunology Department, Medicinal & Natural Products Chemistry Research Center and Autoimmunity Diseases Research Center, Shiraz, Iran, Islamic Republic of

**Objectives:** The use of dendritic cells (DCs) as a cellular adjuvant provides a promising approach in immunotherapy of cancer. We investigated the effect of the subset lymphoid (CD8α<sup>+</sup>) DCs loaded with heat-treated tumor lysate (HTL) as a vaccine in tumor immunotherapy.

**Methods:** The Balb/c mice were injected subcutaneously in the right flank with Wehi-164 fibrosarcoma cells 10 days before immunization with the DCs. Then hsp70 (nano particle) expression in the HTL was detected by using western blot analysis. The mice spleen CD8α<sup>+</sup> DCs subset were isolated by magnetic cell sorting (MACS). Then the HTL pulsed CD8α<sup>+</sup> DCs, tumor lysate pulsed CD8α<sup>+</sup> DCs and unpulsed CD8α<sup>+</sup> DCs were injected subcutaneously around the tumor site. Tumor growth rate, survival, cytotoxic assay and lymphocyte subpopulation in the tumor tissue were measured in both case and control groups.

**Results:** The results showed that HTL-CD8α<sup>+</sup> DCs vaccine significantly induced the tumor growth suppression and longer survival than the other immunized mice. Immunotherapy with HTL-CD8α<sup>+</sup> DCs led to a significant increase in the activity and the number of cytotoxic T cells in the tumor tissue.

**Conclusion:** The current study suggests that specific anti-tumor immune responses against the fibrosarcoma can be induced by the subset of mouse spleen CD11c<sup>+</sup>CD8α<sup>+</sup> DCs pulsed with tumor lysate complex with heat shock proteins (as nano particle) and may provide a useful therapeutic approach for cancer treatment.

**Keywords:** Heat shock proteins, CD11c<sup>+</sup>CD8α<sup>+</sup> dendritic cells, Tumor cell lysate, Fibrosarcoma, Immunotherapy.

**PD09/64 ASSOCIATION OF BTLA GENE POLYMORPHISMS WITH THE RISK OF MALIGNANT BREAST CANCER IN HEILONGJIANG PROVINCE OF CHINESE WOMEN**

Z. Fu<sup>1</sup>, D. Li<sup>1</sup>

<sup>1</sup>Harbin Medical University, Immunology Department, Harbin, China

**Background:** The immunity and hereditary factors play the important roles in development of breast cancer. B and T lymphocyte attenuator (BTLA) is a new CD28-family member expressed on T cell, B cell and DCs. BTLA is also an immuno-inhibitory receptor with the ability to deliver inhibitory signals for suppressing lymphocyte activation. To identify the influences of BTLA gene polymorphisms on the risk of breast cancer, a case control group was conducted in Northeastern women of China, Heilongjiang Province.

**Methods:** We genotyped five SNPs in BTLA gene among exons and introns. Our research groups consist of 550 patients with breast cancer and 506 age/sex-matched healthy controls. Genotypes were determined by the polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) and polymerase chain reaction with confronting two-pair primer (PCR-CTPP) methods. Data was analyzed using the Chisquare test by EXCEL and Haploview software.

**Results:** The frequencies of BTLA rs1844089CT and rs2705535AG were higher in patients than in controls (P=0.0164, OR=1.344, 95% CI 1.055-1.711; P=0.0031, OR=1.492, 95% CI 1.144-1.946, respectively), and rs rs1844089 CC genotype, rs2705535 GG genotype and rs9288952 CC genotype had lower incidences in patients than in controls (P=0.0483, OR=0.784, 95% CI 0.616-0.998; P=0.0098, OR=0.709, 95% CI 0.547-0.921; P=0.0400, OR=0.594, 95% CI 0.360-0.981, respectively). The frequency of haplotype, CAAAT was significantly higher in patients (P=0.0112, OR=3.133, 95% CI 1.259-7.792). Strong association showed between five SNPs of BTLA gene and tumor size, estrogen receptor (ER), progesterone receptor (PR), C-erbB-2 and P53 statuses. A strong association was observed between tumor size, ER, PR, P53 and the CAGAT(P=0.012), TAAAT(P=0.0378), CAGAT(P=0.0013), CAAGT(P=0.0373) and CAAAT(P=0.0306) haplotypes.

**Conclusion:** These results firstly suggested that BTLA gene polymorphisms may affect the sporadic breast cancer risk and prognosis in northeast of Chinese women, Heilongjiang Province.

**PD09/65 ANTITUMOR ACTIVITY OF RECOMBINANT HYBRID PROTEIN INTERFERON-GAMMA – TUMOR NECROSIS FACTOR-ALPHA ISOLATED AND PURIFIED FROM ESCHERICHIA COLI STRAIN-PRODUCER**

A.A. Denisov<sup>1</sup>, P.L. Gnedenkov<sup>1</sup>, A.V. Tret'yakova<sup>1</sup>, L.V. Mikhina<sup>1</sup>, O.A. Karpova<sup>1</sup>, S.V. Melnikova<sup>1</sup>, S.A. Popova<sup>1</sup>, Y.S. Korobovtseva<sup>1</sup>

<sup>1</sup>Research Center For Toxicology and Hygienic Regulation of Biopreparations, Molecular Genetics & Immunology, Serpukhov, Russian Federation

The goal of the study was constructing of a strain-producer for recombinant hybrid protein interferon-gamma- tumor necrosis factor-alfa (IFNγ-TNFα), developing of the method for protein isolation and purification, and investigating of its anti-tumor activity.

As a source of fused infy-tnfα gene the earlier obtained pLFT 313 plasmid, which determined low level of hybrid protein IFNγ-TNFα synthesis in E.coli cells, was used. To enhance the level of protein production, infy-tnfα gene was re-cloned into pQE30 plasmid, and a new recombinant strain – E.coli SG20050/pQE30::infy-tnfα was obtained. This allowed using the obtained strain-producer for isolation and purification of the hybrid protein.

To isolate the hybrid protein, cells of the strain-producer were cultivated in Luria broth containing ampicillin (100 µg/ml) and tetracycline (10 µg/ml) at 37°C and moderate aeration for 24 hours. Biomass was disintegrated by ultrasound. Purification was carried out by low-pressure column chromatography using ion-exchange sorbents (Macro-Prep DEAE Support, BioRad Co. and CM Sephadex C-50, Amersham Co.). Specific activity of purified hybrid protein evaluated by cytotoxicity test on L929 mouse fibroblasts line was 2 × 10<sup>6</sup> IU/mg.

Purified hybrid IFN $\gamma$ -TNF $\alpha$  protein was used for evaluation of antitumor activity in in vitro and in vivo experiments on re-inoculated murine tumor cells – lymphocytic leukemia P 388, lymphadenosis L 5178Y, and carcinoma of human large intestine HT-29. In in vivo experiments antitumor effects were evaluated by tumor growth inhibiting (decrease of tumor sizes or ascitic fluid volumes) or animals' life expectancy. In experiments in vitro optic density of cellular monolayer after adding of hybrid protein was compared to control. In vitro experiments antitumor activity of hybrid IFN $\gamma$ -TNF $\alpha$  protein was shown on all tested re-inoculated murine tumor cells: lymphocytic leukemia P 388, lymphadenosis L 5178Y, and carcinoma of human large intestine HT-29. Results of in vivo experiments demonstrated antitumor activity of hybrid protein towards lymphadenosis L 5178Y and carcinoma of human large intestine HT-29.

#### PD09/66 A NEW STRATEGY TO INDUCE EFFECTIVE ANTITUMOR RESPONSE IN VITRO AND IN VIVO

M. Wang<sup>1</sup>, Z. Xie<sup>2</sup>, M. Shi<sup>3</sup>, H. Lu<sup>1</sup>, M. Yu<sup>3</sup>, M. Hu<sup>3</sup>, F. Lu<sup>3</sup>, Y. Ma<sup>1</sup>, B. Shen<sup>3</sup>, N. Guo<sup>3</sup>

<sup>1</sup>Henan University, Kaifeng, China, <sup>2</sup>National University Hospital and National University of Singapore, Singapore, Singapore, <sup>3</sup>Institute of Basic Medical Sciences, Beijing, China

To induce Her2-specific cell immune response, we used xenogeneic antigen rat neu L2-S2 domains as the vaccine antigen. The antigenic protein was engineered as a chimeric protein with human IgG1 Fc region (neu-Fc). Neu-Fc could stimulate the cell proliferation in MLR effectively. Simultaneous neu-Fc and IFN- $\gamma$  stimulation dramatically elevated IL-12 secretion and reduced IL-10 production in PBMCs. To further augment the activating effects on Th1-type response, BCG was utilized as a non-specific stimulus. Neu-Fc, IFN- $\gamma$  and BCG costimulation exhibited the most conspicuous effects on the reversal of the Th1-type inhibitory effects by MCF-7 cell supernatant compared with neu-Fc alone or IFN- $\gamma$  and BCG costimulation. The lytic activity of effector cells to Her2 overexpressing cells was greatly promoted by neu-Fc, IFN- $\gamma$  and BCG stimulation simultaneously. Neu-Fc led to considerable retardation in EMT6/Her2 tumor growth in Balb/C mice. IFN- $\gamma$  and BCG efficiently enhanced the antitumor activity. A large amount of inflammatory cells were found to be accumulated in the tumor tissues or surrounded tumors in mice treated with neu-Fc, IFN- $\gamma$  and BCG but no inflammatory cell infiltration was observed in control tumors, indicating that the strategy is potent enough to support the initiation and propagation of tumor-specific immune response in an established tumor and generate a proinflammatory environment.

#### PD09/67 PERIPHERAL BLOOD CD8+ LYMPHOCYTE LEVELS PREDICT RECURRENCE-FREE PERIOD OF BLADDER CARCINOMA AFTER TUR PLUS IL-2 INSTILLATIONS

D. Characiejus<sup>1</sup>, V. Pašukonienė<sup>2</sup>, J. J. L. Jacobs<sup>3</sup>, M. Mauricas<sup>1</sup>, W. Den Otter<sup>3</sup>

<sup>1</sup>Institute of Immunology, Vilnius University, Vilnius, Lithuania, <sup>2</sup>Institute of Oncology, Vilnius University, Vilnius, Lithuania, <sup>3</sup>VU Medical Centre, Amsterdam, Netherlands

**Aim:** To determine the role of peripheral blood CD8+ lymphocytes including CD8highCD57+, CD8highCD57- and CD8low subsets in recurrences of superficial bladder carcinoma after transurethral resection (TUR) and intravesical interleukin-2 (IL-2) instillations.

**Methods:** Immunological parameters and recurrence-free period were analysed in 22 patients with superficial bladder carcinoma, treated with intravesical instillations of IL-2. Recombinant IL-2 (Proleukin<sup>®</sup>, Chiron, nowadays Novartis) was instilled in doses of 9x10<sup>6</sup> IU on 5 consecutive days, beginning on the second day after TUR. Samples of peripheral blood were taken prior TUR and analysed on a FACSsort<sup>®</sup> (Becton Dickinson) flow cytometer. Absolute counts and percentages of lymphocyte subsets were examined to find a cut-off that would categorize IL-2 treated patients into subgroups with the highest hazard ratio for recurrence.

**Results:** Low absolute counts of peripheral blood CD8+ lymphocytes were found to have the predictive significance for long recurrence-free period. For patients with < 877 versus > 877 CD8+ lymphocytes/ $\mu$ L, the hazard ratio for recurrence was 5.17 (P< 0.001). Median recurrence-free period of patients with < 877 and with > 877 CD8+ lymphocytes/ $\mu$ L was 339 and 62 days, respectively. The negative influence of CD8+ lymphocytes on the recurrence-free period was largely due to CD8highCD57+ and CD8low subsets with cut-off values of 244 and 170 cells/ $\mu$ L and hazard ratios for recurrence 3.09 and 3.07, respectively (P=0.001). The highest predictive significance was found when combined counts of these regulatory/suppressor lymphocyte subsets were analysed (cut-off value 591 cells/ $\mu$ L, hazard ratio for recurrence 5.67, P=0.001).

**Conclusion:** Peripheral blood CD8+ lymphocytes have negative predictive significance on the recurrence-free period of superficial bladder carcinoma patients after TUR and intravesical IL-2 instillations. This negative predictive significance may be due to adverse influence of CD8highCD57+ and CD8low regulatory/suppressor subsets.

#### PD09/68 INDUCTION OF TUMOR CELL DEATH THROUGH TARGETED INTRODUCTION OF THE PRO-APOPTOTIC GENE, TSMAC

J. Pen<sup>1</sup>, P. Emeagi<sup>1</sup>, C. Heirman<sup>1</sup>, J. Aerts<sup>1</sup>, K. Thielemans<sup>1</sup>, K. Breckpot<sup>1</sup>, Laboratorium voor Moleculaire en Cellulaire Therapie

<sup>1</sup>Vrije Universiteit Brussel, Jette, Belgium

**Introduction:** Recent data demonstrate that PD-L1 expressing tumor cells suppress effector T cells and are resistant to tumor infiltrating lymphocytes (TIL) mediated apoptosis, posing a challenge for immunotherapy.

**Objectives:** To evaluate whether PD-L1 expressed on human melanoma cells, is hampering the anti-tumor T cell response and whether induction of tumor cell death through lentivirus-delivered tSMAC (truncated second mitochondria-derived activator of caspases), can support this T cell response.

**Results:** We evaluated the expression of PD-L1 on 624-mel and 1087-mel (HLA-A2), which were untreated, treated with IFN- $\gamma$  or lentivirally transduced (PD-L1), demonstrating upregulation of PD-L1 on the latter two. Co-cultures with TIL recognizing Melan-A in HLA-A2, demonstrated that this PD-L1 expression hampered the production of IFN- $\gamma$  by TIL. Moreover, we observed that lysis of PD-L1 expressing 624-mel was decreased when compared to their counterparts. This was not observed for 1087-mel, challenging our current knowledge on the anti-apoptotic effect of PD-L1.

Next, we evaluated the capacity of tSMAC to induce apoptosis. Therefore, tumor cells were transduced with tNGFR or tSMAC-Ires-tNGFR encoding lentiviruses. Induction of tumor cell death was determined by flow cytometry and was observed 72 hours after transduction in the tumor cells transduced with tSMAC encoding lentiviruses, but not control lentiviruses. Addition of the caspase inhibitor, Z-VAD-fmk partially reversed the effect. Since the expression of tSMAC has to be limited to the melanoma cells, we evaluated whether melanoma-specific expression could be obtained using the human tyrosinase promoter (huTYR). We compared the expression of eGFP driven by the Cytomegalovirus promoter (CMV) or the huTYR in non-melanoma cells and different melanoma cell lines, demonstrating CMV driven eGFP expression in all cell lines, whereas we only observed huTYR driven eGFP expression in melanoma cell lines. We further cloned the tSMAC gene into the huTYR containing lentiviral vector.

**Conclusion:** Lentiviruses encoding tSMAC controlled by the huTYR will be evaluated to induce apoptosis. We will further evaluate whether the resulting apoptotic bodies (containing tumor antigens) are taken up and processed by immature dendritic cells and whether this results in activation (lentiviral component) and presentation of the tumor antigens to effector T cells.

#### PD09/69 CHARACTERISATION AND TARGETING IMMUNE REGULATORY PATHWAYS IN OVARIAN CANCER

H. Abdul Aziz<sup>1</sup>, C. Maine<sup>2</sup>, C. Hayford<sup>1</sup>, Q. Ding<sup>1</sup>, R. Dina<sup>3</sup>, H. Gabra<sup>1</sup>, A. George<sup>2</sup>, S. Ghaem-Maghami<sup>1</sup>

<sup>1</sup>Imperial College, Surgery, Oncology, Anaesthetics, Reproductive Biology, Ovarian Cancer Action Centre, London, United Kingdom, <sup>2</sup>Imperial College London, Immunology, London, United Kingdom, <sup>3</sup>Imperial College London, Investigative Sciences, London, United Kingdom

**Background:** Tumour cells and antigen presenting cells (APCs) take advantage of inhibitory molecules and enzymes to promote tumour tolerance. This study aims to investigate the role of programmed death-1 (PD-1)/ programmed death ligand-1 (PD-L1), indoleamine 2,3-dioxygenase (IDO) and/or arginase enzymes in ovarian cancer.

**Methods:** We identified patients with ovarian cancer prior to their primary surgery and collected peripheral blood (n = 50), ascites (n = 60) and tumour tissues (n = 120) from them. Plasma and ascitic supernatants were used for cytokines measurement using Luminex assay. For comparison, peripheral blood mononuclear cells (PBMCs) from healthy women and ascites cells from benign and borderline ovarian cancer cases were used as controls. Immune cells isolated from blood and ascites were used for phenotypic analysis, functional and enzymatic assays to further understand the role of these molecules in tumour tolerance. Ovarian cancer tissue samples were used for immunohistochemistry (IHC) and for RNA extraction to screen immune related genes by quantitative real time PCR (qRT-PCR).

**Results:** In the ascites of patients with ovarian cancer, we have shown considerable upregulation of PD-1 expression on T cells and a high percentage of activated monocytes and dendritic cells which express PD-L1. In contrast, we found minimal expression of PD-L1 by similar cells in patients with benign and borderline disease. To investigate the relevance of PD-1 and PD-L1 expression, we have investigated PD-1 and PDL1 blockade and have observed an increase in T cell proliferation in cells from these patients. Significantly higher levels of IDO and arginase enzyme activity as well as expression at mRNA level of these enzymes were found in samples from patients with ovarian cancer compared to control cases. Ascitic supernatants from ovarian cancer patients are highly inhibitory to activated T cells. Our high throughput cytokine analysis on patients' plasma and ascitic supernatants may explain the immunosuppressive property demonstrated when using this supernatant to culture activated T cells. We are validating our findings using IHC on formalin-fixed paraffin embedded ovarian cancer using panel of immune markers.

#### PD09/70 CANCER VACCINE ON THE BASIS OF GLYCOPEPTIDES OF TUMOR CELLS

O. Mazur<sup>1</sup>, V. Shlyakhovenko<sup>1</sup>, V. Mosienko<sup>1</sup>, J. Janish<sup>1</sup>, V. Kozak<sup>1</sup>, V. Milnevska<sup>1</sup>, A. Verbinenko<sup>1</sup>, O. Karnaushenko<sup>1</sup>

<sup>1</sup>R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology NAS of Ukraine, Kyiv, Ukraine

**Introduction:** Tumor cells produce a plenty of tumorspecific (TSA) and tumorassociated (TAA) antigens which support tumor cells in a condition of a malignant transformation and provide them series of metabolic advantages in comparison to the surrounding normal cells. At the same time these antigens are the ground for diagnostics or targets for creation of various antitumor treatment. One of approaches is the biotherapy, particularly cancer vaccinotherapy. Among set of

approaches in creation of cancer vaccines the important place belongs to vaccines on the basis of proteins and peptides isolated from various structures of tumor cells.

**Materials & methods:** Mice C57Bl/6 transplanted cells of melanoma B16 or Lewis lung carcinoma subcutaneously. By a controlled proteolysis and the subsequent fractionating and purification from a tumor tissue the fraction of glycopeptides of molecular mass 50 kDa was obtained.

**Results:** We have developed technology for glycopeptides preparation from membranes of cancer cells. By a controlled proteolysis followed subsequent fractionation and purification the fraction of glycopeptides of molecular mass of 50 kDa was received. By immunization of animals the delay of tumor growth of transplanted tumours and decrease of metastatic potential are marked. Application of a contrasuppression by means of administration the small doses of cyclophosphamide considerably increases the antitumor efficiency of a vaccine.

**Conclusion:** the technology of creation of original cancer vaccine on the basis of fraction of glycopeptides isolated from tumor cells is developed. The opportunity of the further rising of efficiency glycopeptide vaccines is discussed.

**Keywords:** tumorspecific (TSA), tumorassociated (TAA) antigens, glycopeptides, cancer vaccines.

#### PD09/71 CHARACTERIZATION OF TUMOR ANTIGEN PEPTIDE-SPECIFIC T CELLS ISOLATED FROM THE NEOPLASTIC TISSUE OF PATIENTS WITH GASTRIC ADENOCARCINOMA

A. Amedei<sup>1</sup>, E. Niccolai<sup>2</sup>, C. Della Bella<sup>2</sup>, M. Benagiano<sup>2</sup>, L. Bencini<sup>3</sup>, F. Cianchi<sup>3</sup>, R. Moretti<sup>3</sup>, G. Del Prete<sup>2</sup>, M.M. D'Elia<sup>2</sup>

<sup>1</sup>University of Florence, Internal Medicine, Firenze, Italy, <sup>2</sup>University of Florence, Firenze, Italy, <sup>3</sup>Azienda Ospedaliero-Universitaria Careggi, Firenze, Italy

**Objectives:** Gastric cancer is a significant cause of morbidity and mortality worldwide. Surgical resection remains the primary curative treatment for gastric adenocarcinoma, but the poor (15-35%) survival rate at 5 years has prompted many studies for new therapeutic strategies, such as specific immunotherapy. The aim of this study was to analyze the functional properties of the T-cell response to different antigen peptides related to gastric cancer (GCAA) in patients with gastric adenocarcinoma (GA).

**Methods:** To this purpose, we have cloned and characterized tumor-infiltrating T cells (TILs) isolated from the neoplastic gastric tissue samples of 20 GA patients (8 cases with diffuse type cancer and 12 with the intestinal type) selected for their HLA-A02 and/or -A24 alleles. Also we have evaluated by cytofluorimetric analysis the cytokine profile of GCAA-specific T cell present in the peripheral blood of the same patients.

**Results:** A T-cell response specific to different peptides of gastric cancer antigens tested was documented in 17 out of 20 GA patients; among the 316 CD8<sup>+</sup> T-cell clones generated from the TILs of gastric cancer, 59 (18%) proliferated to GCAA peptides: namely, 29 CD8<sup>+</sup> clones responded to peptides restricted by HLA-A24 and 30 to peptides restricted by HLA-A02. Most of the cancer peptide-specific TILs (75%) expressed a Tc1 profile and cytotoxic activity (100%) against target cells. The majority of peripheral blood peptide-specific T cells (15%) also expressed the Tc1 functional profile.

**Conclusion:** In this study we have demonstrated that in most of patients with gastric adenocarcinoma, a specific type-1 T-cell response to gastric cancer antigens was detectable and would have the potential of hamper tumor cell growth. However, in order to get tumor cell killing in vivo, the activity and the number of cancer peptide-specific cells probably need to be enhanced by vaccination with the appropriate cancer antigenic peptides or by injection of the autologous tumor peptide-specific T cells expanded in vitro.

#### PD09/72 TOLL-LIKE RECEPTORS ON B-CLL CELLS: EXPRESSION AND FUNCTIONAL CONSEQUENCES OF THEIR STIMULATION

D. Rožková<sup>1</sup>, L. Novotná<sup>1</sup>, R. Pytlík<sup>2</sup>, I. Hochová<sup>3</sup>, T. Kozák<sup>4</sup>, J. Bartůňková<sup>1</sup>, R. Špišek<sup>1</sup>

<sup>1</sup>Charles University, 2nd Faculty of Medicine, University Hospital Motol, Institute of Immunology, Prague, Czech Republic, <sup>2</sup>Charles University, 1st Faculty of Medicine, General University Hospital, First Department of Medicine, Prague, Czech Republic, <sup>3</sup>Charles University, 2nd Faculty of Medicine, University Hospital Motol, Department of Hematology, Prague, Czech Republic, <sup>4</sup>Charles University, 3rd Faculty of Medicine, University Hospital Královské Vinohrady, Department of Clinical Hematology, Prague, Czech Republic

Toll-like receptors (TLRs) stimulation plays a crucial role in the homeostasis of human B cells. We investigated the expression of TLRs 1-10 on the cells of B-cell chronic lymphocytic leukemia (B-CLL) and analyzed the functional consequences of TLR stimulation on leukemic cells. We show that B-CLL cells express identical set of TLRs as memory B cells of healthy donors, i.e. TLR-1, TLR-2, TLR-6, TLR-7 and TLR-9. Expression of TLRs correlates with their capacity to respond to specific TLR agonists. At the level of phenotype, ODN2006 (TLR-9 agonist) is the most potent stimulus. B-CLL cells also respond to the stimulation with loxoribine, Pam3CSK4 and MALP-2 (TLR-7, TLR1/TLR2 and TLR2/TLR6 agonists, respectively). TLR-7 and TLR-9 stimulation induces production of IL-6 and TNF. In 47% of tested patients, treatment with ODN2006, MALP-2 and Pam3CSK4 reduced leukemic cells survival. Stimulation of B-CLL cells with TLR-9 agonists, loxoribine, MALP-2 and Pam3CSK4 induces significant proliferation. We report that TLR stimulation induces expression of CD38, a negative prognostic marker, on B-CLL cells. Expression of CD38 is induced by direct stimulation of B-CLL cells through TLR-7 and TLR-9 or CD38 can be induced on B-CLL cells indirectly by a soluble factor induced in non-B-CLL cells after stimulation with TLR-2, TLR-3 or TLR-5 agonists. Nature of this factor remains unknown. Our results argue for cautious evaluation of immunointervention strategies based on the administration of TLRs agonists in the treatment of B-CLL as their effects on B-CLL cells may be tumor promoting as well as tumor suppressing.

#### PD09/73 ISOLATION AND CHARACTERIZATION OF TUMOR CELLS FOR PATIENT-TAILORED DENDRITIC CELL-BASED IMMUNOTHERAPY OF OVARIAN CARCINOMA

H. Tišerová<sup>1</sup>, T. Brtnický<sup>2</sup>, L. Rob<sup>2</sup>, J. Bartůňková<sup>1</sup>, R. Špišek<sup>1</sup>, D. Rožková<sup>1</sup>

<sup>1</sup>Charles University, 2nd Faculty of Medicine, University Hospital Motol, Department of Immunology, Prague, Czech Republic, <sup>2</sup>Charles University, 2nd Faculty of Medicine, University Hospital Motol, Department of Obstetrics and Gynaecology, Prague, Czech Republic

Surgery and chemotherapy represent the standard treatment modalities for ovarian cancer. Despite good patients' responsiveness to surgery and chemotherapy, the long term prognosis is poor. The majority of patients are diagnosed at the advanced stages of the disease and their five year survival rate is below 25%. Moreover, 30-40% of early stage patients relapse after initially successful chemotherapy. Immunotherapy at the stage of minimal residual disease after the radical surgery could have the potential to restore the balance between the tumor growth and anti-tumor immune response and improve the prognosis of ovarian cancer. We explore the use of dendritic cells pulsed with killed tumor cells for the immunotherapy of ovarian cancer. We sought to define the optimal source and procedure for the isolation of high numbers autologous tumour cells under GMP condition. Yield and purity of tumor cells was significantly higher when the resected tumor tissue was processed in comparison to ascitic fluid. We present the procedure for isolation of tumor cells from tumor mass using automated mechanical dissociation together with the enzymatic treatment. However, in some patients this method does not yield the sufficient number of tumor cells for the generation of dendritic cell based vaccine. In those patients, we use allogeneic tumor cells lines (OV-90, OVCAR-3, TOV-21G, SK-OV-3) as an alternative source of tumor antigens.

Precise characterization of tumor-specific or associated antigens is essential for the analysis of the immune response to DC-based vaccines when whole tumor cells are used. Using real-time quantitative PCR we analyzed the expression of six major tumor associated antigens- HER-2/neu, MAGE-A12, MAGE-A3, NY-ESO-1, FBP and CA-125 in established tumor cell lines and tumor cells from patients.

This approach allows successful generation of adjuvant dendritic cell based vaccine for the majority of ovarian cancer patients after the reduction of tumor burden by surgery.

This project was supported by project MSM 0021620812 from the Czech Ministry of Education and grant GACR 310/08/0838.

#### PD09/74 DENDRITIC CELL MEDIATED REACTIVATION OF FUNCTIONALLY IMPAIRED MEMORY T CELLS TO IMPROVE GRAFT-VERSUS-TUMOR RESPONSES AFTER STEM CELL TRANSPLANTATION

W. Hobo<sup>1</sup>, N. Adisty<sup>1</sup>, F. Maas<sup>1</sup>, T. de Witte<sup>1,2</sup>, R. van der Voort<sup>1</sup>, H. Dolstra<sup>1</sup>

<sup>1</sup>Radboud University Nijmegen Medical Centre, Central Hematology Laboratory, Nijmegen, Netherlands, <sup>2</sup>Radboud University Nijmegen Medical Centre, Department of Hematology, Nijmegen, Netherlands

**Objectives:** Tumor relapses after allogeneic stem cell transplantation are still a major problem, despite the long-term presence of minor histocompatibility antigen (MiHA)-specific memory T cells. These relapses correlate with the absence of expanding MiHA-specific CD8<sup>+</sup> memory T cells, suggesting that they become inactive over time. As described in chronic viral infections, the co-inhibitory PD-1/PD-L pathway might be responsible for impaired memory T cell function. Mature dendritic cells (DCs) have the potential to revive these PD-1-exhausted T cells, but have high PD-L1 and PD-L2 expression, which might impair the response when they encounter PD-1 expressing alloreactive CD8<sup>+</sup> T cells. To study the role of PD-L1 and PD-L2 on DCs we knock down PD-L protein expression and investigate if this results in improved memory T cell activation.

**Methods:** Healthy donor monocyte-derived DCs (immature, 1 day mature, 2 day mature) were electroporated with only one of 3 different PD-L1 or PD-L2 siRNAs and subsequently cultured in conventional maturation medium containing IL-4, GM-CSF, IL-1 $\beta$ , IL-6, TNF- $\alpha$  and PGE2. The extent and duration of the siRNA-mediated knockdown in these DCs was examined using flow cytometry and Q-PCR.

**Results:** Two days after electroporation of immature DCs (iDCs), specific knockdown ranging from 24% to 76% for PD-L1 and 87% to 92% for PD-L2 was observed for the three different siRNAs. No effects of the siRNAs on other co-stimulatory molecules like CD80, CD86 and CD83 were observed. In all experiments, PD-L1 and PD-L2 siRNA number 2 gave the most pronounced down regulation compared to the other siRNAs tested. The siRNA-mediated knockdown of PD-L1 and PD-L2 molecules in iDCs lasted for at least 5 days following maturation. When we compared siRNA electroporation of iDCs versus 1 or 2 day matured DCs, we found that 2 days after electroporation PD-L down regulation was less efficient in previously matured DCs than in iDCs, though knockdown was still considerable.

**Conclusion:** Electroporation with either PD-L1 or PD-L2 siRNA results in profound specific down regulation of the respective molecule on the DC cell surface. Currently, we are investigating whether PD-L knockdown results in improved T cell stimulation in an allogeneic mixed lymphocyte reaction.



**PD09/75 AUTOLOGOUS ANTI-TUMOR ACTIVITY OF NK CELLS EXPANDED FROM ACUTE LYMPHOBLASTIC LEUKEMIA PATIENTS**B.-Y. Tsai<sup>1</sup>, S.-T. Huang<sup>2</sup>, S.-M. Shou<sup>2</sup>, Y.-H. Kao<sup>2</sup>, J.-L. Tang<sup>3</sup>, C.-C. Li<sup>3</sup>, C.-T. Lin<sup>3</sup>, B.-L. Chiang<sup>4</sup><sup>1</sup>YongLin Healthcare Foundation, Taipei, Taiwan, Republic of China, <sup>2</sup>Graduate Institute of Clinical Medicine, National Taiwan University, Taipei, Taiwan, Republic of China, <sup>3</sup>Tai Cheng Stem Cell Therapy Center, National Taiwan University, Taipei, Taiwan, Republic of China, <sup>4</sup>National Taiwan University Hospital, College of Medicine, National Taiwan University, Department of Pediatrics, Taipei, Taiwan, Republic of China**Background:** Acute myeloid leukemia (AML) is characterized based on the myeloid progenitor cells fail to mature and accumulation. The current therapeutic methods are combinations of transplantation, targeted therapy, and immunotherapy. Cell-based immunotherapy is one of the safest way of cancer treatment, but with limited efficacy. Natural killer (NK) cells are interesting candidates for tumor immunotherapy; however, their potential clinical use in AML is still not clear.**Methods:** In this study, we expand NK cells from peripheral blood of 5 newly diagnosed and untreated patients with AML. The cells were cultured with the added cytokines and analyzed for their purity with flow cytometric analysis.**Results:** After 3 weeks of culture, the number of NK cells from the AML patients had expanded from 300 to 800 fold. Furthermore, expanded NK cells showed significant cytotoxicity against primary autologous AML cells not only *in vitro* but also in NOD-SCID human tumor model. In the future, we also like to study the effect of allogeneic NK cells for the killing leukemic cells.**Conclusion:** In terms of these findings, it is suggested that autologous NK cells expanded *ex vivo* could be considered as a possible treatment modality for AML. We plan to set up the standard operation protocol for the NK cells culture and apply for the treatment for leukemic patients in the near future.**PD09/76 EFFECT OF IN VITRO CULTURE CONDITIONS ON MURINE CAR-EXPRESSING T CELLS FOR CANCER IMMUNOTHERAPY**V. Watson<sup>1</sup>, V. Sheard<sup>1</sup>, A. O'Neill<sup>1</sup>, R.E. Hawkins<sup>1</sup>, D.E. Gilham<sup>1</sup><sup>1</sup>University of Manchester, Cell Therapy Group, Medical Oncology, Manchester, United Kingdom**Objectives:** In this study, we have investigated the effects of *ex vivo* culture conditions, including cell density, addition of cytokines and length of time in culture on the phenotype and function of genetically modified murine T cells expressing chimeric antigen receptors (CARs) consisting of a single chain antigen-binding domain (scFv) targeting carcinoembryonic antigen (CEA), fused with the CD3 $\zeta$  signalling domain.**Methods:** Pep 3 (CD45.1+) splenocytes were activated, transduced with a retroviral vector containing a CEA-specific CAR by spinfection, and expanded *in vitro* for up to 21 days, at cell densities of 0.3x10<sup>6</sup>/ml and 1x10<sup>6</sup>/ml in media supplemented with IL-2, IL-7 or IL-2+IL-7. Cell phenotype and function *in vitro* were assessed by flow cytometry, killing assay and cytokine release at various timepoints. *In vivo*, transduced cells were infused into conditioned C57BL/6 recipients (CD45.1-) with 1 day established CEA-expressing tumours. Engraftment of transferred cells was assessed in peripheral blood or spleen by flow cytometry.**Results:** Greatest cell expansion *in vitro* was achieved from low initial cell culture density combined with IL-2+IL-7. In short-term (3-8 day) assays, IL-7 appeared to support CAR CD8+ cell growth as compared to IL-2 alone. This translated into improved CD8+ gene-modified T cell engraftment *in vivo*. In longer-term cultures (up to 21 days), IL-7 culture resulted in no significant cell expansion; however, cell survival was enhanced and these cells were characterised by a more naïve cell surface phenotype (CD44-CD62L+), as compared to IL-2/IL-2+IL-7 cultures, which displayed a more central/effector memory phenotype (CD44+CD62L+/-). IL-7 also appeared to be important for driving full antigen-specific responses *in vitro*, as cells cultured in IL-2 alone displayed impaired killing and cytokine release when co-cultured with CEA-expressing target cells.**Conclusion:** *Ex vivo* culture conditions affect the *in vitro* phenotype and function of CAR-expressing cells. *In vivo*, engraftment level is significant for anti-tumour efficacy of transduced T cells, and can be enhanced with preconditioning. We are currently investigating whether any of the different *in vitro* culture conditions can further enhance engraftment and anti-tumour efficacy of CAR-expressing cells *in vivo*.**PD09/77 CHANGING OF THE HLA LIGANDOME OF RCC68 AFTER A 24 H TREATMENT WITH THE MULTI TYROSINE KINASE INHIBITOR SUNITINIB**M. Günder<sup>1</sup>, O. Drews<sup>1</sup>, H.-G. Rammensee<sup>1</sup>, S. Stevanović<sup>1</sup><sup>1</sup>Institut für Cell Biology, University of Tübingen, Department of Immunology, Tübingen, Germany

Angiogenesis, the formation of new blood vessels, is essential for the growth and spread of solid tumors. Thus blocking of this process can be an adequate strategy for chemotherapeutic therapy.

In this study we tested the influence of Sunitinib (Sutent), an orally administered multi tyrosine kinase inhibitor selective for vascular endothelial growth factor (VEGF), platelet growth factor (PDGF), KIT and FLT3, on the HLA ligandome of the tumor cell line RCC68. We isolated the HLA-presented peptides from cultured RCC68 cells treated with Sunitinib and from untreated controls. After performing differential isotope labelling with nicotinic acid (NIC) and deuterated nicotinic acid (dNIC), a method established in our group, we were able to detect these modified peptides after HPLC separation in a Q-TOF mass spectrometer. We were interested in finding peptides from tumor associated antigens (TAAs) and gathering more exact quantitative data about their presentation levels depending on Sunitinib treatment.

We could show that Sunitinib has an influence on the MHC presentation of various peptides. This indicates that Sunitinib, additionally to being a potent therapeutic agent in cancer treatment, might be also used to enhance the efficacy of adoptive T-cell transfer by modifying the MHC ligandome.

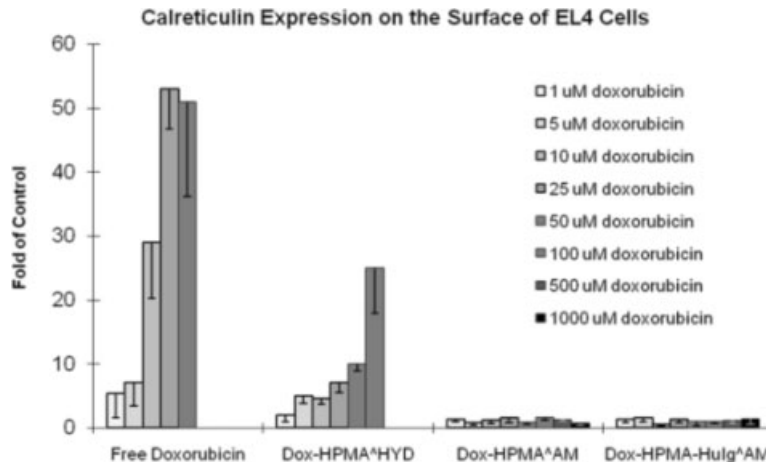
**PD09/78 VACCINATION STRATEGIES FOR THE IMMUNOTHERAPY OF RENAL CELL CARCINOMA**N. Herbert<sup>1</sup>, M. Zöller<sup>1</sup><sup>1</sup>Uniklinik Heidelberg, Experimentelle Chirurgie – Tumorzellbiologie -, Heidelberg, GermanyRenal cell carcinomas (RCC) are mostly radiation and chemotherapy resistant. Instead, they are supposed to be immunogenic. Thus, immunotherapeutic protocols are suggested to provide an alternative therapeutic option. Several immunogenic RCC-associated antigens are known. We selected MAGE-A9, RAGE-1 and G250 as tumor associated antigens (TAAs) to control in a murine model the efficacy of concomitant vaccination with tumor lysate or peptide pulsed DC plus orally applied attenuated *Salmonella typhimurium* (SL). For that purpose, we use TAA-transfected Renca cells as a murine model for RCC and *S. typhimurium* transformed with a eukaryotic expression vector containing the cDNA of RCC-autoantigens SNX6 and GOLGA4.The concomitant activation of autoantigen and TAA specific T cells has been suggested to strengthen the TAA specific response. In addition, while DCs more efficiently promote a T helper response, SL transferred cDNA vigorously initiate CTL activation, which could be confirmed using the TRAP assay (T cell recognition of antigen presenting cells) *ex vivo*. Thus, we could identify *ex vivo* antigen specific T cells in lymph node cells from vaccinated mice. Taken together our results reveal a combinatorial DNA and APC vaccination as a very efficient immunotherapeutic approach in RCC.The generation of DC for tumor vaccination in humans is hampered by the repeated requirement of blood samples from patients and is very cost-intensive. Thus, we aimed in parallel to use CD40-activated B cells as a source of APC. CD40-activated B cells can be easily obtained from peripheral blood and have the advantage of being expandable *in vitro* and can be maintained *in vitro* for at least 12 months. To optimize the generation of human CTL lines for adoptive transfer we investigated the improvement of antigen presentation of CD40-activated B cells by TAA-mRNA transfection, which revealed promising results *in vitro*. *In vivo* confirmation by adoptive transfer of *in vitro* generated antigen-specific CTLs by TAA-mRNA transfected autologous B cells in the SCID mouse model is in progress.**PD09/79 THE HUMAN CELL LINE NEMODDC IS AN EFFECTIVE VECTOR FOR DENDRITIC CELL BASED IMMUNOTHERAPY**C. Goletz<sup>1</sup>, A. Löffler<sup>1</sup>, H. Baumeister<sup>1</sup>, G. Steffen<sup>1</sup><sup>1</sup>Glycotope GmbH, Berlin, Germany

Dendritic cell (DC) based immunotherapy is a promising therapeutic approach against cancer and infectious diseases. To date, the majority uses autologous DCs as vector for presentation of disease relevant antigens. However, the use of autologous DCs is limited by restriction to only one patient, variation in quality and low numbers of DCs. As an alternative the well characterized human cell line NemodDC offers at the same time a high standardization and all characteristics of fully functional DCs.

The CD34<sup>+</sup>/CD14<sup>+</sup> NemodDC line represents precursor DCs (prec-NMDC), which can differentiate and mature into immature DCs (i-NMDC) and mature DCs (m-NMDC) with all phenotypic characteristics of human DCs shown by flow cytometry. i-NMDC and m-NMDC can be loaded with defined antigens and cell lysates, by transfection (viral vectors, DNA- or RNA-electroporation) or fused with tumor cells. Antigens are processed and presented after maturation in the context of MHCI, MHCII or CD1 thereby able to induce potent specific T cell responses *in vitro* measured by IFN $\gamma$ -ELISPOT, tetramer and cytotoxicity analyses. Furthermore, it was shown that NemodDC is expressing a series of TLR and chemokine receptors and is able to migrate in skin transplant assays.Based on these data, the HLA-A2<sup>+</sup>, -A3<sup>+</sup> and -B44<sup>+</sup> NemodDC line has a great potential as a novel immunotherapeutic for treatment of cancer and infectious diseases.**PD09/80 HPMA CONJUGATES AND IMMUNOGENIC CANCER CELL DEATH**L. Kovar<sup>1</sup>, T. Etrych<sup>2</sup>, M. Kabesova<sup>1</sup>, J. Strohalm<sup>2</sup>, K. Ulbrich<sup>2</sup>, B. Rihova<sup>1</sup><sup>1</sup>Institute of Microbiology ASCR, v.v.i., Prague, Czech Republic, <sup>2</sup>Institute of Macromolecular Chemistry ASCR v.v.i., Prague, Czech Republic**Objectives:** Doxorubicin is a routinely used anthracycline antibiotic for cancer treatment and it has a broad spectrum of applicability. Polymeric carriers of drugs, copolymers of *N*-(2-hydroxypropyl)methacrylamide (HPMA), have been developed to avoid the side-effects of free drug. These conjugates consist of HPMA copolymer backbone to which the drug is bound via amide, enzymatically degradable (Dox-HPMA<sup>AB</sup>) or pH sensitive, hydrolytically cleavable bond (Dox-HPMA<sup>HPD</sup>). Surface expression of calreticulin (CRT) is currently considered to be a key mechanism of action of free doxorubicin and is believed to determine the immunogenicity of doxorubicin-induced cell death *in vivo*.

**Methods:** To determine surface expression of calreticulin, standard flow cytometry was performed.

**Results:** Incubation of EL-4 cells (murine T-cell lymphoma) with a desired amount of free doxorubicin enhanced surface expression of calreticulin (CRT) as described previously. We were able to induce CRT expression with different amounts of free doxorubicin also in other human or murine cancer cell lines or with normal splenocytes. As doxorubicin is released from Dox-HPMA<sup>HYD</sup> conjugate intracellularly, the expression of CRT was enhanced after treatment of cells with this conjugate. We documented that doxorubicin is not released from the Dox-HPMA<sup>AM</sup> conjugate and this is supported by the fact that CRT expression is not enhanced after exposure of cells to Dox-HPMA<sup>AM</sup> conjugate. Data obtained with twenty other HPMA conjugates containing doxorubicin bound either by amide or hydrazone bonds demonstrate that free doxorubicin (it does not matter if released from HPMA conjugates of free itself) is crucial for CRT expression.



[Expression of CRT on the surface of EL-4 cells exp]

**Conclusion:** Though HPMA conjugates containing doxorubicin bound via enzymatically cleavable bond can cure mice and evoke anti-tumor resistance, they do not induce CRT expression that is currently considered to be a key factor for induction of anti-tumor immunity. This means that the expression of CRT is not the only mechanism which are responsible for anti-tumor immune response induced by selected chemotherapy.

#### PD09/81 CORRELATION OF EFFECTOR FUNCTION WITH PHENOTYPE AND POPULATION DOUBLINGS UNDERGONE AFTER *IN VITRO* DIFFERENTIATION OF NAÏVE MART-1-SPECIFIC CD8<sup>+</sup> T CELLS

J.G. Casado<sup>1</sup>, O. delaRosa<sup>2</sup>, G. Pawelec<sup>3</sup>, S. Morgado<sup>1</sup>, B. Sánchez-Correa<sup>1</sup>, E. Peralbo<sup>2</sup>, E. Durán<sup>4</sup>, F. Barahona<sup>5</sup>, R. Solana<sup>2,6</sup>, R. Tarazona<sup>1</sup>

<sup>1</sup>University of Extremadura, Immunology Unit, Cáceres, Spain, <sup>2</sup>University of Córdoba, Department of Cellular Biology, Physiology and Immunology, Córdoba, Spain, <sup>3</sup>University of Tübingen, Tübingen, Germany, <sup>4</sup>University of Extremadura, Anatomy and Comparative Pathological Anatomy Unit, Cáceres, Spain, <sup>5</sup>Centro de Biología Molecular, CSIC-UAM, Universidad Autónoma de Madrid, Madrid, Spain, <sup>6</sup>Instituto Maimónides de Investigación Biomédica de Córdoba, Córdoba, Spain

**Objectives:** Adoptive transfer of antigen-specific CD8<sup>+</sup> T cells may represent an effective strategy for immunotherapy of tumors such as melanoma, but is limited by the number and functionality of *in vitro* expanded T cells. The aim of the study was to characterize circulating and *in vitro* expanded ELAGIGILTV-specific T cells from healthy donors in relation to their differentiation status and cytotoxic capacity.

**Methods:** *In vitro* antigen-driven expansion of ELAGIGILTV-specific T cells was performed in healthy individuals with detectable percentages of ELAGIGILTV-specific CD8<sup>+</sup> T cells. The protocol used for *in vitro* expansion was efficient and high numbers of specific CTLs were obtained after 14 days of culture. Functional characteristics of *in vitro* expanded cells were tested by IFN- $\gamma$  secretion and cytotoxicity assays.

**Results:** After antigen-induced *in vitro* expansion, two distinct phenotypes correlating with cell proliferation rate emerged in the different donors. Those cultures achieving fewer cumulative population doublings (CPD) displayed an effector (CD45RA<sup>+</sup>CCR7<sup>-</sup>) phenotype and were cytotoxic. In contrast, cultures reaching higher CPD were non-cytotoxic CD45RA<sup>+</sup>CCR7<sup>+</sup> phenotype T cells.

**Conclusion:** The generation of larger numbers of ELAGIGILTV-specific CD8<sup>+</sup> T cells after *in vitro* stimulation with ELAGIGILTV peptide correlates negatively with the acquisition of a CD45RA<sup>+</sup>CCR7<sup>+</sup> phenotype and cytotoxic capacity. Although these results provide information on the differentiation and development of effector function, additional analysis will be necessary to figure out the mechanisms that contribute to the differentiation pathway of specific CD8<sup>+</sup> T cells and to learn how to manipulate this process.

#### PD09/82 THE ROLE OF REGULATORY T CELLS IN ALPHAVIRUS-BASED IMMUNIZATIONS

M. Walczak<sup>1</sup>, J. Regts<sup>1</sup>, L. Boon<sup>2</sup>, A.J. van Oosterhout<sup>3</sup>, H.W. Nijman<sup>4</sup>, J.C. Wilschut<sup>1</sup>, T. Daemen<sup>1</sup>

<sup>1</sup>University Medical Center Groningen, Department of Medical Microbiology, Molecular Virology Section, Groningen, Netherlands, <sup>2</sup>Bioceros, Utrecht, Netherlands, <sup>3</sup>University Medical Center Groningen, Department of Pathology and Medical Biology, Groningen, Netherlands, <sup>4</sup>University Medical Center Groningen, Department of Gynecology/Oncology, Groningen, Netherlands

In a previous study we demonstrated that in patients with (pre)malignant cervical lesions regulatory T cells (Tregs) are increased (Visser *J et al.*, Clin Exp Immunol, 2007). This immunosuppressive state may hamper the efficacy of immunotherapeutic vaccines.

In the present study we examined the role of Tregs on an immunotherapeutic strategy against HPV-induced cervical cancer. This immunization is based on a vector system derived from an alphavirus, the Semliki Forest virus (SFV). This vector encodes for the HPV oncoproteins E6 and E7 (SFVE6,7). We determined the effect of the *in vitro* and *in vivo* depletion/inactivation of Tregs on the efficacy of SFV-based immunizations and the effect of Tregs on cytotoxic T cell (CTL) responses in immunized mice.

The *in vivo* Treg-depletion/inactivation (using anti-TGF-beta+anti-IL-10 or anti-CD25 or anti-FR4 treatment) combined with SFVE6,7 immunizations did not enhance the frequency of HPV-specific CTLs nor their cytolytic activity. This observation was confirmed by *in vitro* cultures of spleen cells depleted from Treg cells, where no further increase in target cell lysis was noticed. Moreover using a newly established and optimized micro-CTL assay we showed that Treg cells are able to suppress HPV-specific CTLs expansion *in vitro* only at very high spleen to regulatory T cells ratios. The cytolytic activity of HPV-specific CTLs *in vitro* was not affected by the presence of Tregs.

In conclusion, decreasing the level of Tregs *in vitro* and *in vivo* does not further enhance the efficacy of SFVE6,7 immunizations. As literature suggests that Tregs may play a more prominent role in tumor-bearing animals we will, in the near future, also study the role of Tregs under these conditions.

This research was supported by an Ubbo Emmius grant from GUIDE, Groningen.

#### PD09/83 TUMOR TARGETED IL-2 AND TNF ALPHA TRIGGER A SYNERGISTIC T CELL-MEDIATED THERAPEUTIC EFFECT AGAINST MOUSE NEUROBLASTOMA

E. Balza<sup>1</sup>, B. Carnemolla<sup>1</sup>, L. Mortara<sup>2</sup>, P. Castellani<sup>1</sup>, D. Soncini<sup>1</sup>, R.S. Accolla<sup>2</sup>, L. Borsi<sup>1</sup>

<sup>1</sup>Istituto Nazionale per la Ricerca sul Cancro, Department of Translational Oncology, Genova, Italy, <sup>2</sup>University of Insubria, Department of Clinical and Biological Sciences, Varese, Italy

**Aims:** To evaluate the potential synergistic effects of tumor targeted IL-2 and TNF $\alpha$  in inducing a therapeutic immune response in two murine models of neuroblastoma (NB). Neuroblastoma (NB) is the most frequent extracranial solid tumor in young children. In the metastatic disease, conventional therapy results in poor survival rates. The induction of tumor protective immunity against NB remains a major challenge for active immunotherapy.

**Methods:** L19-IL2 and L19mTNF $\alpha$  are fusion proteins composed by the scFv L19, specific for the highly conserved ED-B domain of fibronectin, a tumor-associated antigen, and the inflammatory cytokines IL-2 and m(ouse)TNF $\alpha$ . We studied two experimental murine models of neuroblastoma: Neuro2A (N2A) and NIE-115 (NIE). These NB tumors were s.c. induced in syngeneic A/J mice. When tumor reached a volume of 0.1 cm<sup>3</sup>, the mice were daily injected with L19-IL2 (40 mg/mouse or 20 mg/mouse on day 3) for 5 days. On day 3, L19mTNF $\alpha$  (0.7 pmol/g) was co-injected.

**Results:** 80% of the N2A and 30% of NIE NB-bearing mice were cured by L19-IL2 and L19mTNF $\alpha$  combined treatment with respect to 30% and 20% for N2A when single L19-IL2 or L19mTNF $\alpha$  treatment was used alone, respectively, or 20% when the two recombinant cytokines were used. Protected mice further rejected a homologous tumor challenge. Immune splenocytes from cured mice fully protected naïve mice against NB challenge at an E/T ratio of 5/1 in Winn assay. Immune

CD4<sup>+</sup> and CD8<sup>+</sup> T cells accounted for 40% and 60% protection, respectively. A typical memory CTL response was observed after tumor challenge, three months after cure, together with inducible mixed antitumor Th1/Th2 type of response. *In vivo* depletion experiments indicated that CD4<sup>+</sup> and CD8<sup>+</sup> T cells play a major role in the tumor cure after therapy as compared to NK cells.

**Conclusions:** L19-IL2 and L19mTNF $\alpha$  combined treatment efficiently cooperate in curing tumor-bearing mice by generating a therapy-induced antitumor vaccination. In our experimental model tumor cure is strongly associated to the generation of adaptive immunity involving CD4<sup>+</sup> and CD8<sup>+</sup> T cells, as these cells from cured mice can transfer antitumor protection to naïve animals.

#### PD09/84 CANCER TARGETING WITH THE MODIFIED *SALMONELLA TYPHIMURIUM* VECTOR

P. Chorobik<sup>1,2</sup>, M. Gajda<sup>3</sup>, M. Bereta<sup>1</sup>

<sup>1</sup>Jagiellonian University Medical College, Department of Immunology, Krakow, Poland, <sup>2</sup>Jagiellonian University, Fac. of Biochem. Biophys. Biotech., Department of Cell Biochemistry, Krakow, Poland, <sup>3</sup>Jagiellonian University Medical College, Department of Histology, Krakow, Poland

Genetically modified *Salmonella typhimurium* VNP20009 is a useful vector for vaccine development. We modified VNP20009 to express carcinoembryonic (CEA)-specific single chain antibodies (scFv) fused to Lpp-OmpA on the bacterial surface to increase tumor targeting. Confocal and atomic force microscopy (AFM) revealed polar localization of the fusion protein on the surface of bacteria. Whole body imaging (courtesy of ADAMED, Poland) revealed intra tumor localization of red-fluorescent protein expressing bacteria (VNP-RFP) immediately after i.v. inoculation. Treatment of tumor-bearing mice (subcutaneous or lung model of transplanted tumors) with the modified VNP20009 showed substantial inhibition of tumor growth in the majority of mice. VNP20009-stimulated infiltration of cancerous tissue by leukocytes might be partially responsible for the observed anti-cancer effects of the vector.

#### PD09/85 RELEVANT ROLE OF REPEATED INTRAPERITONEALLY TREATMENT WITH CPG-ODN IN MICE BEARING ADVANCED-STAGE OF HUMAN OVARIAN CARCINOMAS

L. Sfondrini<sup>1</sup>, M. De Cesare<sup>2</sup>, M. Campiglio<sup>3</sup>, F. Bianchi<sup>3</sup>, P. Perego<sup>2</sup>, C. Rumio<sup>1</sup>, E. Tagliabue<sup>3</sup>, A. Balsari<sup>1,3</sup>

<sup>1</sup>University of Milan, Department of Human Morphology and Biomedical Sciences 'Città Studi', Milan, Italy, <sup>2</sup>Fondazione IRCCS Istituto Nazionale dei Tumori, Pre-clinical Chemotherapy and Pharmacology Unit, Milan, Italy, <sup>3</sup>Fondazione IRCCS Istituto Nazionale dei Tumori, Molecular Targeting Unit, Milan, Italy

Tumor cell growth, even in advanced stages of ovarian cancer, is nearly always restricted to the peritoneal cavity, therefore repeated intraperitoneal injections of CpG-ODN recruiting and activating innate effector cells throughout the abdominal cavity to the tumor site might control tumor cell growth and ascites formation. After a single CpG-ODN treatment, in IGROV-1 ovarian tumor ascites-bearing athymic mice, the number of tumor cells declined rapidly and markedly, and ascites volumes declined shortly after treatment (5 h), increasing thereafter at a slower rate than in controls. When administered every 7 days for 4 weeks, CpG-ODN had only a marginal effect on survival time, whereas administration 5 days/week for 3 or 4 weeks led to a significantly increased survival-time as compared to controls (P < 0.005) and completely controlled ascites growth without apparent toxicity, although a disorganization of lymphoid organs was observed. Bio-Plex-assay of cytokine levels in peritoneal fluid of ascites-bearing mice after CpG-ODN treatment revealed an increase in IL-6, -10, -12 and IFN- $\gamma$  at 24 h, which returned to control mice levels at 48-96 h, while the high levels of angiogenic factors remained unchanged. Depletion of NK or monocytes/macrophages only slightly influenced the CpG-ODN-induced reduction of ascites tumor cells, indicating that the antitumor activity might not be related to a specific cell/cytokine but rather to the repertoire of cells and cytokines accumulated in the peritoneal cavity.

Thus, our data suggest a relevant role for repeated activation of cells and cytokines of innate immunity in the therapy of ovarian cancer patients with malignant ascites.

#### PD09/86 NATURAL KILLER CELLS KILL HUMAN MELANOMA CELLS WITH PHENOTYPIC AND FUNCTIONAL CHARACTERISTICS OF CANCER STEM CELLS

C. Manzini<sup>1</sup>, G. Pietra<sup>1</sup>, S. Rivara<sup>1</sup>, M. Vitale<sup>2</sup>, M. Balsamo<sup>1</sup>, L. Moretta<sup>1,3</sup>, M.C. Mingari<sup>1,2</sup>

<sup>1</sup>University of Genoa, Dept. of Experimental Medicine-DIMES, Genoa, Italy, <sup>2</sup>National Institute for Cancer Research, Genoa, Italy, <sup>3</sup>Giannina Gaslini Institute, Genoa, Italy

**Objective:** Experimental and clinical data suggest that tumours harbour a cell population retaining stem cell characteristics that can drive tumorigenesis. CD133 has been shown to mark cancer stem cells (CSC) from various tissues and therefore it may represent a putative stem cell-associated marker shared by tumours cells belonging to different histotypes. In this context, it has been shown that CD133 is always co-expressed with ABCB5 drug resistance-related molecule in malignant melanoma stem cells (MMSC). No information is available on the ability of NK cells to recognize and lyse MMSC. In this study we assessed whether melanoma cell lines, characterized by stem cell-like features (including the expression of "stemness" markers, the ability to undergo self-renewal, the radio-resistance, the multi-lineage differentiation potential and the tumorigenicity), were susceptible to lysis by IL-2 activated NK cells.

**Methods:** Primary melanoma cultures were obtained from 7 subcutaneous and 1 lymph node metastasis. For growing both primary culture of melanoma cells and established melanoma cell lines the culture medium used was RPMI 1640 supplemented with 10% FBS. In order to obtain floating spheroids melanoma cell clusters (shown to be enriched in CSC), tumours cells were cultured in serum free medium containing DMEM/F12, B27 supplement, FGF-b and EGF. Enriched NK cells were isolated from healthy donors using the Human NK Cell Enrichment Cocktail-RosetteSep and then cultured on irradiated feeder cells in the presence of rIL-2 and PHA to obtain activated NK cells.

**Results:** We found that melanoma cell lines are highly heterogeneous for the expression of several stem cell-related markers including CD133, c-kit/CD117 and p75NTR/CD271. In addition, we showed that activated NK cells efficiently kill different melanoma cell subsets that were enriched in putative CSC by the use of both phenotypic and functional selection criteria (i.e. CD133 expression, radioresistance, or the ability to form melanospheres in stem cell supportive medium). NK cell-mediated recognition and lysis of melanoma cells involved different combinations of activating NK receptors.

**Conclusions:** Since CSC have been reported to be both drug- and radio-resistant, our present data suggest that NK-based adoptive immunotherapy could represent an effective therapeutic approach for metastatic melanoma.

#### PD09/87 IDENTIFICATION OF PUTATIVE CANCER/TESTIS ANTIGENS BY COMPARATIVE GENE EXPRESSION PROFILING

D. Max<sup>1</sup>, G. Weißflog<sup>1</sup>, C. Hutter<sup>1</sup>, J. Kaftan<sup>1</sup>, C. Mauz-Körholz<sup>1</sup>, J. Föll<sup>2</sup>, D. Körholz<sup>1</sup>, M.S. Staeger<sup>1</sup>

<sup>1</sup>Martin-Luther-University Halle-Wittenberg, Department of Pediatrics, Halle, Germany, <sup>2</sup>University of Regensburg, Department of Pediatric Hematology and Oncology, Regensburg, Germany

**Objectives:** DNA microarray-detected signal intensities of tumour associated genes are characterized by a high variance in the total group of samples (including normal and tumour samples) compared with the variance in normal tissues alone. Therefore, the ratio of the variance in normal tissues and the total variance (Wilks' lambda score, WLS) can be used for the identification of tumour specific genes. Vice versa, this approach can be used for the identification of tissue specific genes that are only expressed in a subset of normal samples. We used this method for the characterization of gene expression profiles of Ewing family tumours (EFT) and Hodgkin's lymphoma (HL).

**Methods:** We combined published microarray data from tumour samples and normal tissues from our own lab and from the GEO data base. WLS was used for the identification of tumour associated and tissue specific genes. Putative cancer/testis antigens (CTA) were further analyzed by reverse transcription-polymerase chain reaction.

**Results:** In our analysis of EFT we found the complete set of EFT associated genes that we had identified in our previous studies, indicating that the method gives reliable results. Similar results were obtained during analysis of HL samples. In these samples we observed high expression of interleukin 21 (IL21) and IL26, despite the fact that IL26 was expressed only in a subset of HL cell lines. In addition, we identified a set of tissue specific genes that were expressed only in small subsets of normal samples, e.g., insulin (expressed only in pancreas) or the Charcot-Leyden crystal protein (expressed in blood and bone marrow). Among these genes we found several testis specific genes (e.g., protamines or the glyceraldehyde-3-phosphate dehydrogenase S), as well as CTA, e.g., members of the GAGE (G antigen) and SPANX (sperm protein associated with the nucleus, X chromosome) families. In addition, the expression pattern of ZBTB32 (zinc finger and BTB domain containing 32) suggest that this is a new HL associated CTA.

**Conclusion:** Our results demonstrate that WLS can be used for the identification of tumour associated genes and for the identification of tissue specific genes including new cancer/testis antigens.

#### PD09/88 LONG-TERM LOW-DOSE INTERLEUKIN-2 (IL-2), ALFA-INTERFERON ( $\alpha$ -IFN) AND 13-CIS-RETINOIC ACID (RA) AS MAINTENANCE THERAPY IN CANCER PATIENTS. A PRELIMINARY STUDY

B. Vazquez<sup>1</sup>, J. Rebollo<sup>2</sup>, E. Martinez<sup>3</sup>, M. Sureda<sup>4</sup>, R. Gonzalez Manzano<sup>4</sup>

<sup>1</sup>Plataforma Oncología. USP Hospital San Jaime, Laboratorio Inmunología, Torrevieja, Spain, <sup>2</sup>Plataforma Oncología. USP Hospital San Jaime, Oncología Médica, Torrevieja, Spain, <sup>3</sup>Plataforma Oncología. USP Hospital San Jaime, Laboratorio Biología Molecular, Torrevieja, Spain, <sup>4</sup>Plataforma Oncología. USP Hospital San Jaime, Torrevieja, Spain

**Objectives:** To study the feasibility and efficacy of long-term therapy with low-dose IL-2,  $\alpha$ -IFN and RA in high risk cancer patients as a maintenance therapy after conventional treatment.

**Methods:** Patients with advanced and high-risk localized cancer after conventional therapy have been treated with s.c. IL-2 +/-  $\alpha$ -IFN (or Pegylated Interferon) +/- oral RA as has been described elsewhere by Recchia F et al (J Immunother 2007;30:448-454). Primary end points are tolerance and Progression Free Survival (PFS). Serum cytokines (IL-6, VEGF and sR $\alpha$ IL-2) and lymphocytes subpopulations have been determined before and during therapy. ANOVA test has been used to see mean differences.



**Results:** From June'06 to January'09, 30 patients (pts) (median age 54 years, range 31–77) have been treated. Seven pts had colo-rectal cancer, 7 non-small cell lung cancer, 5 melanoma, 4 breast cancer, 4 ovarian cancer, 2 gastric cancer and 1 sarcoma. Four pts had high-risk localized disease (LD) and 26 pts metastatic disease (MD). Twenty pts initiated maintenance therapy free of disease (NED) and 10 pts with active disease (AD). Two pts have reported grade 3 and 2 pts grade 2 asthenia, and arthralgia, headache, conjunctivitis and mucositis, 1 pt each. Four pts stopped therapy because of drug fever, asthenia and cholestasis. Median PFS has been 7.5+ months (range 2–17+). Pts with LD and NED has shown the longest PFS. No significant differences in serum cytokines and T4/T8 ratio has been shown during therapy. However, a significant increase in NK cells counts ( $P=5.166$ ;  $p=0.003$ ) have been observed.

**Discussion:** Maintenance immunotherapy with IL-2,  $\alpha$ -IFN and RA in poor prognosis cancer pts after conventional treatment is feasible and well tolerated. Some pts show prolonged PFS, especially those with NED at initiation of the maintenance therapy. NK cells count is increased but serum IL-6, VEGF and sIL-2 are not modified during treatment.

#### PD09/89 **IN VITRO ANALYSIS OF A TUMOR-SPECIFIC IMMUNE-RESPONSE INITIATED BY SENESCENT NEUROBLASTOMA CELLS**

S. Taschner-Mandl<sup>1</sup>, E. Bozsaky<sup>1</sup>, A. Kowalska<sup>1</sup>, D. Rieder<sup>2</sup>, Z. Trajanosky<sup>2</sup>, J. Khan<sup>3</sup>, F. Speleman<sup>4</sup>, I.M. Ambros<sup>1</sup>, P.F. Ambros<sup>1</sup>

<sup>1</sup>Childrens Cancer Research Institute (CCRI), Tumor Biology, Vienna, Austria, <sup>2</sup>Graz University of Technology, Graz, Austria, <sup>3</sup>NCI, NIH, Washington DC, United States, <sup>4</sup>Ghent University Hospital, Ghent, Belgium

In neuroblastoma (NB), amplification of the *MYCN* (MNA) proto-oncogene is a highly significant marker for a poor prognosis independent of other biological or clinical parameters. Lack of MHC I expression, defects of the antigen-presentation machinery, GD2-shedding and down-modulation of NKG2D-ligands were described, among others, as immunosuppressive mechanisms in patients and cell lines with MNA. Cellular senescence, a permanent state of proliferative arrest, has recently been described to occur *in vivo* and provides a *bona fide* barrier to tumorigenesis. We have previously described that NB cell lines, negative for MHC I, up-regulate MHC I surface expression spontaneously and in response to hydroxyurea (HU) – a substance that leads to the elimination of extra-chromosomally amplified *MYCN* and senescence induction in NB cell lines. Furthermore, the ganglioside GD2 – an NB-cell associated molecule that inhibits T-cell activation *in vitro* and *in vivo* – is down-regulated in these senescent NB cell lines. In order to characterize senescent versus non-senescent NB-cells in terms of gene expression profile, both cell types were analyzed by Affymetrix as well as competitive hybridization array followed by validation of a panel of genes by quantitative RT-PCR. We found that HU-treated senescent NB-cells up-regulate among others MHC I and other immune-response-related molecules, including IL-6, IL-8, components of the immunoproteasome complex, PSME1, PSMB10, calreticulin and tapasin and the activating natural killer (NK)-cell (NKG2D) ligand MICA. In line with these expression data, functional data suggest that – in contrast to non-senescent cells – senescent NB-cells (1) allow activation of cells of the innate and adaptive immune system (T-cell, NK and NKT-cell) and (2) reduce growth and tumor marker (GD2) expression of non-senescent NB-cells in co-cultures. Specifically, HU-induced senescent NB cell lines allow anti-CD3 mediated activation of allogeneic CD8<sup>+</sup> and CD4<sup>+</sup> T-cells as well as proliferation of CD56<sup>+</sup> and CD56<sup>+</sup>CD8<sup>+</sup> cells. Based on these findings we hypothesize that hydroxyurea induces a senescent, non-malignant, immunogenic state in neuroblasts. These data provide the basis for future studies using hydroxyurea or related drugs as senescence inducer in neuroblastoma patients to prevent tumor relapse originating from dormant tumor cells with persisting malignant potential.

#### PD09/90 **DOWN REGULATION OF PROSTATE SPECIFIC ANTIGEN BY IL-1 BETA IN PROSTATIC CANCER CELL LINE, LNCAP**

Y. Bouraoui<sup>1</sup>, Z. Culig<sup>2</sup>, R. Oueslati<sup>1</sup>

<sup>1</sup>Faculty of Sciences of Bizerte, Unit Immuno Microb Environnemental and Carcinogenesis (IMEC), Zarzouna, Tunisia, <sup>2</sup>Innsbruck Medical University, Department of Urology, Innsbruck, Austria

**Introduction:** Prostate Specific Antigen, PSA, is a clinically important marker used to monitor diagnosis, treatment response, prognosis and progression in patients with prostate cancer. The production of PSA in prostate epithelial cells is androgen dependant. But, some cytokines can have an effect on PSA expression in prostatic cell lines.

**Aim:** The aim of this work was to study the regulation of PSA's production by IL-1beta, the activation of NFkappaB and AKT pathways on LNCaP cell lines.

**Methods:** The LNCaP ATCC cells were treated with IL-1 beta for 48 hours. The number of total cells and viable cells was counted with CASY® cell counter and analyser system model TTC. PSA levels produced by LNCaP cells were measured by Advia Centaura, Bayer® auto analyser. In Western Blot analysis, NFkappaB and AKT were revealed by the Odyssey antibody and detected by the scanner LI-COR Odyssey®.

**Results:** The treatment of LNCaP cells with IL-1beta inhibited cell proliferation and decreased PSA production. Western Blot analysis showed that AKT pathway was not affected by IL-1beta; whereas the phosphorylation of NFkappaB was stimulated by this cytokine. PSA and LNCaP cells proliferation were down regulated by IL-1beta through its up-regulation of the transcription factor NFkappaB.

**Conclusion:** These finding, suggesting a cross talk between NFkappaB, PSA and others signaling pathways.

#### PD09/91 **SERUM INTERLEUKIN-8 AND INSULIN LIKE GROWTH FACTOR-1 IN EGYPTIAN BLADDER CANCER PATIENTS**

M. Mahmoud<sup>1</sup>, M. Ali<sup>2</sup>, H. Hassoba<sup>3</sup>, G. Elhadidy<sup>4</sup>

<sup>1</sup>Faculty of Medicine, Suez Canal University, Biochemistry Dept., Ismailia, Egypt, <sup>2</sup>Faculty of Medicine, Suez Canal University, Urology Dept., Ismailia, Egypt, <sup>3</sup>Faculty of Medicine, Suez Canal University, Clinical Pathology Dept., Ismailia, Egypt, <sup>4</sup>Faculty of Medicine, Suez Canal University, Microbiology Dept., Ismailia, Egypt

**Background:** Bladder cancer is a major health problem in Egypt as it represents the most common malignancy, constituting 30% of all cancers. The increasing morbidity and mortality rates of bladder cancer have forced the scientists to search for new unifying diagnostic and therapeutic methods that will improve treatment effects. Insulin-like growth factor-1 (IGF-1) and Interleukin-8 (IL-8) have been implicated in the development of various human cancers.

**Aim:** evaluation of the potential usefulness of serum IGF-1 and IL-8 in Egyptian bladder cancer patients.

**Patients and Methods:** serum levels of IGF-1 and IL-8 were determined in 51 patients with bladder cancer and 18 healthy controls using a chemiluminescence enzyme immunometric assay.

**Results:** Serum levels of IL-8 were significantly higher in the cancer patients than those of controls ( $P < 0.001$ ). The levels were significantly higher in patients with invasive cancer than those of superficial cancer ( $P < 0.01$ ). Also, IL-8 showed a significant elevation in relation to schistosomiasis infection ( $P=0.02$ ), however, it did not differ in relation to either pathological type of tumor or its grade ( $P>0.05$ ). Serum IGF-1 levels showed no significant difference between bladder cancer patients and controls ( $P > 0.05$ ). Also, there was no significant relationship between IGF-1 levels and clinicopathological parameters.

**Conclusion:** In Egyptian patients with cancer bladder, serum IL-8 is significantly elevated and its elevation is related to both tumor stage and associated schistosomiasis infection. Thus, serum IL-8 but not serum IGF-1 levels can help as a serum tumor marker for bladder cancer.

#### PD09/92 **ANTIBODIES AGAINST ENDOTHELIN-1 RECEPTORS (ETRS) GENERATED THROUGH ELECTROPORATION-AIDED DNA IMMUNIZATION: POSSIBLE TOOLS FOR CANCER IMMUNO-IMAGING AND IMMUNOTHERAPY**

B. Allard<sup>1,2</sup>, F. Priam<sup>1,3</sup>, D. Boquet<sup>1</sup>, A. Wijkhuisen<sup>1,3</sup>, J.-Y. Couraud<sup>1,3</sup>

<sup>1</sup>CEA Saclay, Service de Pharmacologie et d'Immunologie (SPI), Laboratoire d'Ingénierie des Anticorps pour la Santé (LIAS), Gif sur Yvette Cedex, France, <sup>2</sup>Université Paris 11, Châtenay-Malabry, France, <sup>3</sup>Université Paris 7 Denis Diderot, Paris, France

Endothelins are a family of three 21 amino acid peptides (ET-1, ET-2 and ET-3). ETs exert their action via two identified G-protein-coupled receptors (GPCR) subtypes: the ET<sub>A</sub> receptor (ET<sub>A</sub>R) and the ET<sub>B</sub> receptor (ET<sub>B</sub>R). In addition to their well-known vasoactive properties, it is now clearly demonstrated that ETs and their receptors are involved in the development of tumours (Nelson *et al.* 2003, Ghoul *et al.* 2007, Bagnato *et al.* 2008). Indeed, it has been shown that ET-1 and both receptor subtypes are overexpressed in many different cancers such as ovarian, prostate and nasopharyngeal carcinoma, melanoma, glioblastoma and others. More interestingly, this "endothelin axis" has recently been reported to promote key steps of tumour progression including cell proliferation, apoptosis escape, angiogenesis and metastatic dissemination, making ETs and their receptors very relevant targets for cancer therapy.

In this context, we generated murine polyclonal antibodies directed against human ETRs. We used the technique of DNA immunization combined with electroporation to immunize mice with expression vectors containing ETR cDNAs. Briefly, plasmids were injected into the tibialis anterior muscle of BALB/c mice; subsequently, the uptake of plasmid DNA by muscle cells was enhanced through *in vivo* electroporation. This technique has been used with success to produce antibodies able of recognizing the native conformation of membrane receptors (Costagliola *et al.* 1998, Tymciu *et al.* 2002, Kaptein *et al.* 2008).

Potent polyclonal antisera were obtained, as determined by FACS, immunofluorescence and ELISA assays, using CHO cells overexpressing ETRs (CHO-ET<sub>A</sub>R or CHO-ET<sub>B</sub>R). Pharmacological tests, based on the monitoring of intracellular calcium release upon ET-1 stimulation of CHO-ETR cells, revealed that polyclonal antisera were also able of inhibiting ETRs calcium signalling pathway triggered by ET-1 stimulation.

These preliminary results are encouraging for an upcoming production of monoclonal antibodies blocking ETRs and for further *in vivo* experiments on tumours models. DNA immunization appears to be a method of choice to produce pharmacologically-active antibodies against membrane receptors.

#### PD09/93 **DISTINCTION OF CELLULAR RESPONSES AND TARGET EPITOPES FOR THE ANTI-CD40 ANTIBODIES AF1.15 AND MAB89 ON NORMAL AND MALIGNANT B CELLS**

A.M. Mattila<sup>1</sup>, A. Pääri-Sampo<sup>1</sup>, S. Meri<sup>1</sup>

<sup>1</sup>University of Helsinki, Department of Bacteriology and Immunology, Helsinki, Finland

Anti-CD40 antibodies have varying anti-tumour effects and anti-CD40 antibody treatment is a new approach for the treatment of cancer. In the present study we determined the effects of a novel AF1.15 anti-CD40 monoclonal antibody compared to a known anti-CD40 monoclonal antibody Mab89. The effects of the anti-human-CD40 monoclonal antibodies on the proliferation of normal and malignant human B cells in the presence and absence of IL-4 were analyzed by a thymidine incorporation assay. Flow cytometric analysis was used to determine AF1.15 and Mab89 induced apoptosis in different lymphoma cell lines and the amount of AF1.15 and Mab89 binding to B cell surface CD40 molecules. The results show that AF1.15 increased the proliferation of B cells and peripheral blood lymphocytes. IL-4 potentiated the proliferation inducing effect of AF1.15. Mab89 did not induce proliferation in normal B cells. The proliferation of both follicular lymphoma cell

lines HF-1 and HF-4b was inhibited by AF1.15 in a dose dependent manner. However, Mab89 inhibited only the proliferation of HF-1 cells but not the proliferation of HF-4b cells. AF1.15 in combination with IL-4 and Mab89 alone both induced apoptosis in the HF-1 cells. Flow cytometric analysis showed that the amount and intensity of AF1.15 and Mab89 binding to HF-1 and HF-4b lymphoma cells was cell line dependent. Interestingly, the amount of monoclonal antibody binding to CD40 did not correlate with the proliferative effects. Unlabeled AF1.15 reduced the binding of directly labeled Mab89 less than the unlabeled Mab89 in flow cytometric analysis. In conclusion, the novel monoclonal anti-CD40 antibody AF1.15 stimulates the proliferation of normal B cells, inhibits the proliferation of malignant follicular lymphoma B cells and induces apoptosis in HF-1 follicular lymphoma cells. Flow cytometric analysis and binding competition assay results imply that AF1.15 and Mab89 have different epitopes on the CD40 molecule. The differences in cellular responses and binding profiles of these anti-CD40 antibodies suggest epitope-related differences in the function of the CD40 molecule. Analysis and characterization of novel anti-CD40 antibodies offers possibilities for not only finding new therapeutic monoclonal antibodies for immunotherapy of cancer but also for boosting B cell responses.

#### PD09/94 ANTITUMOR EFFECT OF PERTUSSIS TOXIN IN C6 GLIOMA

B. Pineda<sup>1</sup>, M. Orozco-Morales<sup>1</sup>, F.-J. Sánchez-García<sup>2</sup>, P. Guevara<sup>1</sup>, L. Sánchez-Chapul<sup>3</sup>, E. Rangel<sup>4</sup>, R. Pérez-Madrigrá<sup>1</sup>, J. Sotelo<sup>1</sup>

<sup>1</sup>Instituto Nacional de Neurología y Neurocirugía, Neuroinmunología, México, México, <sup>2</sup>Instituto Politécnico Nacional, Immunoregulacion, México, México, <sup>3</sup>Instituto Nacional de Rehabilitación, México, México, <sup>4</sup>Instituto Nacional de Neurología y Neurocirugía, Neuroinmunoenendocrino, México, México

**Objective:** To induce apoptosis in Treg cells using pertussis toxin in a glioblastoma multiform model as a way of immune intervention in anti-tumoral treatment. **Material and methods:** C6 glioma cells were implanted subcutaneously in 30 Wistar rats. PTx or saline solution was injected intraperitoneally when the tumor reached about 2 cm<sup>3</sup> of diameter. The tumoral volume was determined weekly. The number of infiltrated lymphocytes from tumor, spleen and blood as well as the induction of apoptosis with Annexin V/7AAD and sub Go Peak within the tumor were determined by flow cytometry. mRNA of granzyme and perforin were determined by real time PCR. The toxicity of PTx was evaluated *in vitro* using C6 glioma cell line.

**Results:** The group treated with PTx showed 75% reduction in tumor volume and a significant decrease in the percentage of CD56+ cells ( $2.2 \pm 0.76\%$  Vs  $0.92 \pm 0.04\%$ )  $p < 0.03$  and Treg cells ( $5.3 \pm 1.7\%$  Vs  $0.22 \pm 0.4\%$ ) in tumor ( $p < 0.04$ ). In spleen a significant decrease in the percentage of Treg cells was seen in the PTx group ( $3.1 \pm 0.1\%$  Vs  $0.5 \pm 0.2\%$ ) ( $p < 0.03$ ). In contrast, there was an increase in the percentage of tumor cells in apoptosis ( $68.5 \pm 7\%$  Vs  $43.1 \pm 5.3\%$ ) ( $p < 0.01$ ). A twofold increase in the relative expression of mRNA of granzyme and perforin was observed in the PTx group. We did not find a significant toxicity of PTx on C6 glioma cell line *in vitro*.

**Discussion and conclusion:** We suggest that PTx allows the induction of a cytotoxic immune response (as assessed by perforin and granzyme gene expression) against tumoral cells (apoptosis) through the elimination of Treg cells. Pertussis toxin (PTx) is commonly used to increase experimental autoimmune diseases because it causes brain blood barrier permeabilization and maturation of antigen presenting cells, activation of auto-reactive lymphocytes and apoptosis of Treg cells; affecting the balance between tolerance, inflammation and autoimmunity. Recently, it has been reported that Tregs cells negatively modulate tumoral microenvironment in several types of neoplasms and the depletion of these cells, using monoclonal antibodies, increases the therapeutic effect of anti-cancer vaccines.

#### PD09/95 LOCAL LOW DOSE IRRADIATION TRIGGERS TUMOR INFILTRATION BY ADOPTIVELY TRANSFERRED AND HOST T LYMPHOCYTES AND ENHANCES IMMUNOTHERAPY IN MICE

T. Seibel<sup>1</sup>, H. Prakash<sup>1</sup>, P. Beckhove<sup>1</sup>

<sup>1</sup>German Cancer Research Center, Translational Immunology, Heidelberg, Germany

The use of immunotherapeutic approaches for the treatment of cancer is limited because of the tumors intrinsic resistance to T lymphocyte infiltration and effector function. Insufficient infiltration can be overcome by inducing an activated tumor microenvironment utilizing whole body irradiation in mice. However, irradiation treatment of human cancer with high doses is not applicable in some patients due to high associated risk of organ toxicity. We hypothesized that locally applied low dose irradiation is sufficient to create a niche favoring immune effector cell entry to the tumor.

We employed the RIPTag transgenic mouse model expressing SV40 "Tag" as a model tumor antigen. Following *in vitro* activation, "Tag" specific TCRtg T lymphocytes derived from TagTCR1 and TCRCD8 mice were injected into RIPTag mice previously irradiated with doses ranging from 0.5Gy to 6Gy. Histological examination demonstrated that transfer of activated tumor-specific CD4+ or CD8+ T lymphocytes alone resulted in accumulation of low T lymphocyte numbers in the tumor tissue, whereas a combinational treatment including locally applied low dose irradiation and transfer boosted tumor infiltration. Transfer of activated tumor-specific CD4+ T lymphocytes allowed infiltration by host CD8+ T lymphocytes and was most effective with a dose of 2Gy. In contrast, host CD4+ T lymphocyte numbers were strongly elevated after a transfer of activated tumor-specific CD8+ T lymphocytes in mice irradiated with only 0.5Gy. Moreover, we have shown recently that regulatory T lymphocytes were found to be selectively accumulated in patient derived tumor tissue and display an increased capacity to transmigrate through tumor endothelial cells *in vitro*. In our model of combinational treatment, irradiation with more than 0.5Gy reduced the number of regulatory T lymphocytes in the tumor significantly.

This is the first demonstration, to our knowledge, of enhanced influx of immune effector cells and reduced influx of immune regulatory cells triggered by a combinational treatment with local low dose irradiation and adoptive T lymphocyte transfer. We believe this approach can be the basis for the development of a novel and promising anticancer therapy that utilizes an activated tumor microenvironment to selectively enrich immune effector cells facilitating immune-mediated tumor destruction.

#### PD09/96 HISTONE DEACETYLASE INHIBITORS BUT NOT PROTEASOME INHIBITORS SENSITIZE TUMOR CELLS FOR THE CYTOLYTIC IMMUNE EFFECTOR FUNCTIONS

N. Yaktapour<sup>1</sup>, M. Schmudde<sup>1</sup>, A. Braun<sup>1</sup>, E. Friebe<sup>1</sup>, J. Sonnemann<sup>2</sup>, J.F. Beck<sup>2</sup>, B.M. Bröker<sup>1</sup>

<sup>1</sup>University of Greifswald, Institute of Immunology and Transfusion Medicine, Greifswald, Germany, <sup>2</sup>University Children's Hospital Jena, Department of Pediatric Hematology and Oncology, Jena, Germany

**Objectives:** Treatment with histone deacetylase inhibitors (HDIs) leads to the upregulation of the stress proteins MIC-A and MIC-B on tumor cells and increases their sensitivity for the cytotoxic effects of IL-2 stimulated NK cells. The proteasome inhibitor Bortezomib also induces cell stress. Bortezomib acts in synergy with HDIs, because the latter interfere with a cellular rescue mechanism, aggresome formation. We hypothesized that Bortezomib alone or in combination with HDIs could also increase the susceptibility of tumor cells to the cytotoxic effector mechanisms of immune cells.

**Methods:** We used three tumor cell lines: medulloblastoma (DAOY), melanoma (SKMel) and prostatic cancer cells (PC3). Tumor cells were pre-treated with Bortezomib alone or in combination with HDIs and then confronted with IL2-stimulated PBMCs. Tumor cell death and cell surface markers were measured by flow cytometry.

**Results:** HDIs and Bortezomib were both tumortoxic on their own, and their combination was synergistic. The tumor cells responded to different HDIs with an increase of surface expression of MIC-A and MIC-B, and they became more sensitive to the cytotoxic effect of PBMCs that had been pre-activated with IL2 for 3 days. In this system cytotoxicity was mediated by NK cells, which, in contrast to T cells, released their granules and mobilized CD107a. Similarly, Bortezomib treatment caused upregulation of MIC-A/B and additionally of the TRAIL receptor DR5 on the tumor cells. However, this did not change tumor cell susceptibility to a subsequent challenge with activated PBMCs, neither when Bortezomib was applied alone, nor when it was used in combination with HDIs.

**Conclusion:** While HDIs as well as Bortezomib induced the expression of stress proteins by tumor cells to a similar extent, only HDIs increased their sensitivity to cytotoxic immune cells. Thus strong MIC-A and MIC-B expression is not sufficient for tumor cell killing by activated NK cells.

#### PD09/97 EXPRESSION OF CANCER-TESTIS ANTIGENS IS INCREASED IN CHRONIC MYELOID LEUKEMIA (CML) CELL LINES FOLLOWING EPIGENETIC TREATMENT BUT DOES NOT LEAD TO SPECIFIC HUMORAL IMMUNE RESPONSES IN PATIENTS WITH CML

T. Luetkens<sup>1</sup>, F. Uhlich<sup>1</sup>, T. Stasche<sup>1</sup>, R. Akbulak<sup>1</sup>, Y. Hildebrandt<sup>1</sup>, S. Kobold<sup>1</sup>, K. Bartels<sup>1</sup>, T. Brümmendorf<sup>1</sup>, P. Schafhausen<sup>1</sup>, N. Kröger<sup>1</sup>, C. Bokemeyer<sup>1</sup>, D. Atanackovic<sup>1</sup>

<sup>1</sup>University Medical Center Hamburg-Eppendorf, Department of Oncology/Hematology, Hamburg, Germany

**Background:** Limitations of current therapeutic options in CML, such as imatinib-resistance and persistence of minimal residual disease, require the evaluation of novel therapeutic approaches. In order to identify target structures for potential antigen-specific immunotherapies, we performed a comprehensive analysis of CT antigen expression, its dependence on epigenetic mechanisms, and associated humoral immune responses in CML.

**Methods:** 10 CML cell lines and bone marrow from 10 healthy donors were screened for the expression of 30 CT antigens by RT-PCR and Western blot. Cell lines were further evaluated by RT-PCR following stimulation with 5'-Aza-2'-Deoxycytidine and Trichostatin. Expression of 16 selected antigens was analyzed in BM and PBL samples from 60 patients with CML and sera from these patients were screened for antibodies against 15 CT antigens.

**Results:** RT-PCR showed expression of 9 of 30 CT antigens in BM samples from healthy donors. 16 of the remaining 21 antigens were expressed in at least one untreated cell line. Most consistently expressed on the RNA level in untreated cell lines were PRAME, MAGE-C2, and CRT2. Treatment with 5'-Aza-2'-Deoxycytidine led to a two-fold increase in the average number of antigens expressed per cell line while treatment with Trichostatin had a less pronounced effect. Investigating the expression of 15 antigens in BM and blood samples from patients with CML by RT-PCR, only PRAME was repeatedly detected in patients (32.1%) and expression correlated with disease stage ( $p = 0.005$ ), blast cell count ( $r = 0.38$ ;  $p = 0.01$ ), and reduced overall survival ( $p = 0.02$ ). Interestingly, none of the patients expressing PRAME showed a humoral immune response against this antigen, significant levels of antibodies were only detected against NY-ESO-1 in one patient.

**Conclusions:** CT antigens are commonly expressed in CML cell lines and expression was increased following application of a demethylating agent. We show for the first time that expression of PRAME, the only repeatedly detected CT antigen in patients, does not lead to a significant antibody response, but correlates with multiple clinicopathological parameters. Only one patient who had not expressed any of the investigated antigens showed a specific humoral response against NY-ESO-1, possibly indicating the eradication of clones expressing this antigen *in vivo*.

**PD09/98 THE EFFECTS OF ARSENIC TRIOXIDE AND ZOLEDRONIC ACID ON MALIGNANT PLASMA CELLS DERIVED FROM BONE MARROW CELLS OF MULTIPLE MYELOMA PATIENTS**

M. Yousefi<sup>1</sup>, A. H. Emami<sup>2</sup>, M. R. Khorramzadeh<sup>1</sup>, M. Momeny<sup>3</sup>

<sup>1</sup>School of Public Health, Tehran University of Medical Sciences, Immunology Department, Tehran, Iran, Islamic Republic of, <sup>2</sup>School of Medicine, Medical Sciences/University of Tehran, Tehran, Iran, Islamic Republic of, <sup>3</sup>Faculty of Medicine, Medical Sciences/ University of Tehran, Department of Medical Genetics, Tehran, Iran, Islamic Republic of

**Objectives:** Multiple myeloma (MM) is a disease of plasma cells that has fatal consequences. New agents associated with molecular targets have prompted clinical investigators to design new treatment strategies initially for advanced MM and later for newly diagnosed MM, with encouraging preliminary results. Here, we devised a project to assess the mechanisms of action of two drugs, Arsenic trioxide (ATO) and Zoledronic acid (Zometa) on Bone marrow mononuclear cells (BMMCs) derived from patients.

**Methods:** Bone marrow samples were collected from 10 patients after receipt of formal consent. BMMCs were collected from samples. In two parallel sets of experiments, BMMCs were treated with 0.5, 2, 6  $\mu$ M ATO and 0.1, 10, 100  $\mu$ M Zometa, for 72 hours. The following analyses were then performed on treated cells as compared to untreated cells (assumed as control): cytotoxicity using Micro culture tetrazolium test (MTT assay); matrix metalloproteinase-2 zymography; comparative gene expression analysis of IL-6, vascular endothelial growth factor (VEGF) and intercellular adhesion molecule-1 (ICAM-1).

**Results:** MTT assay showed significant proliferation inhibition in ATO high dose treatment (6  $\mu$ M). However, no significant inhibitory effect of Zometa was seen. Zymography analyses showed significant decrease in gelatinolytic activity in treated cells. Analyses of gene expression using Real-Time RT-PCR methodology showed significant decrease in IL-6, ICAM-1, and VEGF genes as normalized against Hypoxanthine phosphoribosyltransferase (HPRT) normalizer and as compared with untreated cells.

**Conclusion:** Both ATO and Zometa could significantly decrease MM cells critical phenotype and genotype. This finding could support the hypothesis that ATO or Zometa could inhibit growth and metastasis of malignant cells.

**PD09/99 MATURATION OF MONOCYTE DERIVED DENDRITIC CELLS WITH OK432 LEADS TO ALTERED EXPRESSION OF SURFACE MARKERS**

A.-O. Hovden<sup>1</sup>, D. A. Sandnes<sup>1</sup>, H. J. Aarstad<sup>2,3</sup>, S. Appel<sup>1</sup>

<sup>1</sup>University of Bergen, The Gade Institute, Bergen, Norway, <sup>2</sup>University of Bergen, Department of Surgical Sciences, Bergen, Norway, <sup>3</sup>Haukeland University Hospital, Department of Head and Neck Surgery, Bergen, Norway

**Background:** Design of tumour specific immunotherapies using the patients' own dendritic cells (DC) is a fast advancing scientific field. Critical to the success of immunotherapy is the functional qualities of the DC generated *in vitro*. OK432 is an immunotherapeutic agent derived from penicillin-killed *Streptococcus pyogenes* that has been used effectively to treat a variety of tumours.

**Methods:** DC were generated from monocytes isolated from buffy coat preparations from healthy blood donors. The monocytes were cultured with IL-4 and GM-CSF for 6 days to generate immature DC verified by microscopy and surface marker expression. The immature DC were incubated with different stimuli (OK432, cytokine cocktail and combinations of OK432 and the cytokine cocktail).

**Results:** DC matured with 0.1 KE OK432 showed a consistent lower surface expression of HLA-DR, CD86, CD83 and CD80 compared to cells stimulated with the cytokine cocktail. Combining the cytokine cocktail and 0.1 KE OK432 produced an intermediate phenotype of surface markers. Lowering the dosage of OK432 to 0.01KE reduced the expression of the surface markers further in a dose-dependent manner, whereas 0.01KE OK432 together with the cytokine cocktail resulted in surface expression similar to cytokine cocktail alone. OK432 stimulation induced a similar percentage of CD40 positive DC as the cytokine cocktail, but the median fluorescence intensity was 2-fold higher for OK432 DC. Surface expression of CCR7, a receptor important for the migration of the cells, was most pronounced in DC matured with 0.1KE OK432.

**Conclusion:** The clinical effects seen by the use of OK432 do not seem to result from a higher degree of activation of DC. Further work is needed to elucidate the mechanism behind OK432 as an immunotherapeutic agent.

**PD09/100 IMMUNOTHERAPY AND CHEMOIMMUNOTHERAPY IMPROVES SUCCESS OF CHEMOTHERAPY ALONE AND SELECT TUMOR WITH LOW MHC CLASS I EXPRESSION**

C. Garrido López<sup>1,2</sup>, I. Romero<sup>2</sup>, E. Berruguilla<sup>2</sup>, I. Linares<sup>2</sup>, I. Algarra<sup>3</sup>, A. Collado<sup>2</sup>, F. Garrido<sup>1,2</sup>, Á. M. García-Lora<sup>2</sup>

<sup>1</sup>Universidad de Granada, Bioquímica y Biología Molecular e Inmunología, Granada, Spain, <sup>2</sup>Hospital Universitario Virgen de las Nieves, Servicio de Análisis Clínicos, Granada, Spain, <sup>3</sup>Universidad de Jaén, Ciencias de la Salud, Jaén, Spain

**Objectives:** It is known that immune response and immunologic phenotype of tumour cells have great implications in the success of anticancer therapy. We pretend analyze the success of different types of immunotherapy and chemoimmunotherapy protocols on spontaneous metastases derived from a murine fibrosarcoma tumour clone and the MHC phenotype of these metastases.

**Methods:** A7 is a clone obtained from MCA-induced fibrosarcoma in Balb/c mice. It has positive surface expression of MHC class I molecules: K, D and L. In spontaneous metastasis assays, A7 generated 6 to 30 pulmonary metastases and 1 to 3 lymph node metastases per mouse. Metastases had a MHC class I expression different from A7 cells: 59% had downregulation of MHC class I molecules, 50% of which were negative for L molecule. Four different anticancer treatments were established:

- 1) CpG and A7-irradiated cells;
- 2) Protein-bound polysaccharide K (PSK);
- 3) Docetaxel;
- 4) PSK and Docetaxel.

All treatments were administered after extirpation of primary tumour, weekly during 6 weeks. To monitor the treatment, some mice were sacrificed at different time points.

**Results:** The number of metastases in all treated groups was lower than in the control group and MHC class I phenotype was different. All treatments were not toxic and, except for Docetaxel, they led to absence of any metastases by the end of the therapy. During the treatment, in CpG group 45% of mice developed metastases (one per mouse) showing losses of MHC class I expression. In PSK group, only one mouse presented metastases, all with downregulation of MHC class I molecules. No metastases were found when PSK and Docetaxel were used together. In Docetaxel group 75% of mice developed metastases. Metastases found during the treatment showed MHC phenotype similar to control and at the end of the treatment presented an increase in MHC class I expression.

**Conclusions:** In summary, we can conclude that immunotherapy reduces metastases development. Combination of chemotherapy and immunotherapy improves results, eliminating all metastases. Immunotherapy produced an immuno-stimulation and led to selection of metastases with low MHC class I expression. However, chemotherapy did not stop the progression of MHC class I positive metastases.

**PD09/101 THE EFFECT OF *T. SPIRALIS* ES ANTIGEN ON PROLIFERATION OF MELANOMA CELLS**

S. Vasilev<sup>1</sup>, Z. Zizak<sup>2</sup>, Z. Juranic<sup>2</sup>, N. Ilic<sup>1</sup>, A. Gruden-Movsesijan<sup>1</sup>, L. Sofronic-Milosavljevic<sup>1</sup>

<sup>1</sup>Institute for the Application of Nuclear Energy-INEP, University of Belgrade, Belgrade, Serbia, <sup>2</sup>Institute of Oncology and Radiology of Serbia, Clinical Center of Serbia, Belgrade, Serbia

It is well known that co infection with different pathogens, including helminths and in particular *Trichinella spiralis* (*T. spiralis*), can alter the progression of different diseases including malignant one. Melanoma is a very aggressive form of skin cancer that is resistant to conventional chemotherapy and represents an open field for new therapeutic approaches. We studied the effect of *T. spiralis* excretory-secretory muscle larvae (ES L1) antigen on the proliferation of the melanoma cells *in vitro*. Human melanoma (Fem-x), and mouse melanoma (B16) tumor cells were cultured as a monolayer and treated with five different concentrations (12.5-200  $\mu$ g/ml) of investigated compound (ES L1) in complete nutrient medium, except for the control cells. Cell survival was determined by MTT test 72 h after the addition of antigen. Obtained results indicated that *T. spiralis* ES L1 antigen in a statistically significant manner exerted a mild, dose dependent, anti-proliferation action toward target tumor cell lines. Stronger effect was determined toward human melanoma tumor cells compared with the effect on mouse melanoma cells. Immunomodulatory effects on malignant tumors progression, previously observed in chronic *T. spiralis* infection, could be ascribed to the presence of ES L1 in circulation of the infected host and that is why here presented results are promising.

(Ministry of Science and Technological Development, Serbia, Grants No: 143047 and 145006).

**PD09/102 ANALYSIS THE TREATMENT EFFECTS WITH RESVERATROL ON CELL CYCLE AND APOPTOSIS IN TUMOR LARYNGEAL CELLS**

M. Bostan<sup>1</sup>, G. G. Matei<sup>1</sup>, D. A. Manu<sup>2</sup>, L. G. Ghetea<sup>3</sup>, L. I. Brasoveanu<sup>1</sup>

<sup>1</sup>Institute of Virology Stefan S. Nicolau, Center of Immunology, Bucharest, Romania, <sup>2</sup>Ilfov Country Hospital, Bucharest, Romania, <sup>3</sup>Bucharest University, Institute of Genetic, Bucharest, Romania

Despite the recent advances in chemo radiotherapy and antibody therapy, the prognosis for patients with laryngeal cancer is still poor. Our purpose was to analyse the therapeutic value of the phytochemical agent – resveratrol in tumor cells culture obtained from primary tumor of patients with laryngeal cancer. We analysed using flow cytometry methods the effects of resveratrol alone or in combination with cisplatin on cell cycle progression and apoptotic activity of laryngeal tumor



cells. In our experiments, in the first cells were incubated separately with cisplatin or resveratrol for 6 and 24 h, in the second cisplatin addition was followed by subsequent resveratrol treatment. Our results shown, that not only cisplatin treatment (4ug/ml and 8ug/ml) but and resveratrol (20ug/ml and 50ug/ml) induced growth arrest of cells in G0/G1 phase. Data obtained after cisplatin treatment shown its pro-apoptotic activity on laryngeal carcinoma cells, so we tried to investigate how might influence the resveratrol treatment the apoptotic processes in tumor cells. Resveratrol treatment induced an increase apoptosis in a manner dependent of concentration. Preincubation of tumor cells with cisplatin followed by resveratrol treatment appeared to be most effective and was correlated with strong apoptosis activation. In conclusion, our results open the way for additional experiments to consider the molecular basis of this response and studies to determine the adequate concentrations of resveratrol can be applied to treat of laryngeal tumours.

**Key words:** laryngeal cancer, resveratrol, cisplatin, cell cycle, apoptosis

Acknowledgements: This study was supported by national program PNII 41-084/2007 in Romania

#### PD09/103 EFFECT OF STIMULI TREATMENT ON CELL SIGNALLING MECHANISMS ASSOCIATED TO COLORECTAL CELL LINES

M. Hirt<sup>1</sup>, C. Hotnog<sup>1</sup>, D. Hotnog<sup>1</sup>, L. I. Brasoveanu<sup>1</sup>

<sup>1</sup>Stefan S. Nicolau Institute of Virology, Center of Immunology, Bucharest, Romania

Colorectal cancers represent some malignancies with high incidence and mortality throughout world, their etiology involving many genetic, immunological and biochemical factors. The main obstacle against the success of therapy in many cancers seems to be the impossibility of eradication all tumor cells. A new therapeutic approach could be more useful for destruction of tumor cells: renewal of the cellular pathways that lead directly to apoptosis. Traditional anti-cancer therapies have limited effects, therefore the cytotoxic action exerted by drugs on tumors might be added by natural compounds and the signalling mechanisms influenced. Curcumin and quercetin seem to affect directly several major targets and the molecular mechanisms involved, such as activation of protein kinases. Phosphorylation/ dephosphorylation reactions represent the main mechanisms by which the proteins involved in signal transduction modify their activities. The present work focused on the study of signal transduction processes by evaluating phosphorylation status of AMPK- $\alpha$ , - $\beta$ , LKB1, TSC2, mTOR and S6K1 associated to colon (Colo201, Lovo, Caco2, SW1116) and rectal (SW837) adenocarcinoma cell lines induced by cytotoxic drugs (5-fluorouracil) in the presence or absence of natural compounds (quercetin and curcumin). Levels of antigen expression of phosphorylated vs non-phosphorylated proteins were evaluated by immunoblotting and/or flow-cytometry in the tumor cell lines under study. Membrane expression of antigens was detected by labeling specific monoclonal antibodies, while cellular protein expression was detected by SDS-PAGE and Western blotting using the chemiluminescence method. Our results showed a differential antigen expression of the molecules under study involved in signaling mechanisms involved in kinase activities depending on the association of stimuli and drugs. Protein expression alterations, resulting frequently from gene modifications, are responsible not only for the appearance of cancer, but also for the clinical responses of patients to chemotherapy. The knowledge of the relation between the implied molecules, of the mechanisms of regulation of the gene and protein expression, as well as their functions, of the effect of therapeutic agents (oncolytic agents, natural compounds) on proliferation, induction of apoptosis and tumor cell lysis has a potential diagnostic and prognostic value of the disease evolution, but also regarding the tumor response to immunotherapy.

#### PD09/104 VEGF, MMP2 AND IL-8 EXPRESSION PROFILE IN ADIPOSE-DERIVED MESENCHYMAL STEM CELLS (MSCs) OF PATIENTS WITH BREAST CANCER

M. Jaberipour<sup>1</sup>, M. Razmkhah<sup>1</sup>, B. Khalatbari<sup>2</sup>, A. Ghaderi<sup>1</sup>

<sup>1</sup>Shiraz Institute for Cancer Research, Shiraz University of Medical Sciences, Shiraz, Iran, Islamic Republic of, <sup>2</sup>Shiraz University of Medical Sciences, Plastic Surgery Department, Shiraz, Iran, Islamic Republic of

**Objectives:** Angiogenesis is an important process for progression and metastasis of most types of tumors such as breast cancer. Vascular endothelial growth factor (VEGF), Matrix Metalloproteinase (MMP) and Interleukine-8 (IL-8) plays crucial roles in cancer development through their angiogenic ability. It has been shown that MSCs might be recruited to the tumor microenvironment and may contribute to the production of proangiogenic factors. Herein, we investigated the expressions of VEGF, MMP-2 and IL-8 mRNA levels in adipose-derived MSCs of patients with breast cancer.

**Methods:** Twenty patients with breast cancer were examined for expression of VEGF, MMP-2 and IL-8 mRNAs in MSCs. For this, MSCs were isolated from fragments of breast adipose tissue after mincing and incubating with collagenase. The expression of extracted mRNAs was determined using real-time quantitative RT-PCR. Results were compared to fifty sex and age matched healthy controls.

**Results:** Relative Quantification (RQ) of VEGF was about 2.6 folds higher in patients than controls. RQ of MMP-2 was about 1.4 folds higher in patients than healthy women. No statistically significant difference was found in the expression of IL-8 between patients and controls. Furthermore, the expression of VEGF and IL-8 were higher in stage 3 compared to stage 1 and 2. These differences were not statistically significant.

**Conclusion:** These data suggest that the higher expression of VEGF and MMP2 by MSCs of breast cancer patients can probably change the prognosis and susceptibility of women to breast cancer. Thus, these molecules might be introduced as potential therapeutic targets for human breast cancer.

#### PD09/105 PROTEINS FROM A CALENDULA OFFICINALIS EXTRACT SHOW ANTITUMOR ACTIVITY AND IMMUNOSTIMULATION IN VITRO

E. Berruguilla<sup>1</sup>, I. Romero<sup>1</sup>, C. Garrido-López<sup>1</sup>, V. García-Cabrera<sup>1</sup>, A. García-Lora<sup>1</sup>, F. Garrido<sup>1</sup>

<sup>1</sup>Hospital Universitario Virgen de las Nieves, Análisis Clínicos e Inmunología, Granada, Spain

Previous studies of our group have shown a complementary dual activity in a Calendula officinalis crude water extract: cytotoxicity against cell lines derived from different tumor and immunomodulatory action in human peripheral blood lymphocytes (PBLs). When subpopulation analysis was performed, the lymphocytes proliferating were mainly NKT cells, T cells and B cells. Besides proliferation, activation of lymphocytes was detected. In this study, we obtained a calendula protein extract and tested its ability to induce in vitro cytotoxicity in MDA MB 231 (human breast carcinoma) and A7 (murine fibrosarcoma) cell lines. This activity was also tested in PBLs. When a dose-response cytotoxicity curve was performed, the crude extract (250  $\mu$ g/mL) produced 50% reduction of viability in MDA MB 231 cells. These cells, when treated with the protein extract (12  $\mu$ g/mL) showed a significantly higher (80%) viability reduction. In A7 cell line, the protein extract (12  $\mu$ g/mL) showed 75% viability reduction while no cytotoxicity was noted when the same cells were treated with the crude extract (250  $\mu$ g/mL). Proliferation activity was then assayed in PBLs in a dose-response manner. Stimulation index of PBLs treated with the protein extract at a dose of 1.5  $\mu$ g/mL was 2.65, practically identical to that obtained in PBLs treated with the crude extract at a higher dose of 500  $\mu$ g/mL. These results indicate that our protein extract clearly enhances the cytotoxicity activity of the crude extract. Moreover, the immunomodulatory activity in PBLs was also potentiated. Thus, proteins or glyco-proteins may be the active principles with anti tumour and immunomodulatory activities contained in the crude extract of Calendula officinalis.

#### PD09/106 THE EFFECTS OF CHEMOTHERAPEUTIC DRUGS (OXA AND 5-FU) ON THE EXPRESSION OF IMMUNOLOGICAL RELATED MOLECULES IN THE HUMAN COLORECTAL CANCER CELLS

T. Wan<sup>1</sup>, Y. Wu<sup>1</sup>, G. Chen<sup>1</sup>, X. Cao<sup>1</sup>

<sup>1</sup>Second Military Medical University, Institute of Immunology, Shanghai, China

**Objectives:** To determine the effects of chemotherapeutic drugs OXA and 5-Fu on the expression of immunological related molecules in human colorectal cancer cells.

**Methods:** Colorectal cancer cells SW480 ( $1 \times 10^6$ /ml) were treated with OXA ( $1 \times 10^{-5}$ M,  $5 \times 10^{-6}$ M,  $1 \times 10^{-6}$ M,  $1 \times 10^{-7}$ M) or 5-Fu ( $3 \times 10^{-3}$ M,  $1.5 \times 10^{-3}$ M,  $0.75 \times 10^{-3}$ M,  $1.5 \times 10^{-4}$ M). Then, SW480 cells and their supernatants were collected at 4, 12, 24 hours. Expressions of CD95 (Fas), HLA-A2, CD95L (Fas Ligand) on cell surface and proportion of 7-AAD labeled cells were detected by FACS. Secretions of IL-10 and TGF- $\beta$  in supernatants were measured using ELISA kit.

**Results:** The results showed that various concentrations of OXA or 5-Fu used to treat SW480 cells could upregulate CD95 (Fas) and HLA-A2 expressions in the dose and time-dependent manner. Secretions of IL-10 and TGF- $\beta$  by SW480 cells were significantly reduced after treatment with OXA or 5-Fu. There was no obvious difference in 7-AAD labeled cell counts of SW480 cells between the OXA or 5-Fu treated groups and the untreated control, indicating that reduction in IL-10 and TGF- $\beta$  secretion by cancer cells by the drug was not due to the death of cancer cells.

**Conclusion:** Chemotherapeutic drugs OXA and 5-Fu could upregulate surface expressions of CD95 (Fas) and HLA-A2, and downregulate secretion of immunosuppressive cytokines IL-10 and TGF- $\beta$  in human colorectal tumor cells. These results indicated that chemotherapy we used in combination with immunotherapy may contribute to the induction of antitumor immune response.

### PD09 – IMMUNOTHERAPY OF TUMORS

#### PD09/107 REDIRECTING T-CELLS AGAINST CANCER CELLS BY TRANSFER OF BROADLY TUMOR REACTIVE GDT-CELL RECEPTORS

V. Marcu-Malina<sup>1</sup>, M. Theobald<sup>1</sup>, J. Kuball<sup>1</sup>

<sup>1</sup>UMC Utrecht, Utrecht, Netherlands

Adoptive transfer of  $\alpha\beta$ -T-lymphocytes is a promising treatment for a variety of malignancies, but often not feasible due to difficulties in generating T-cells reactive with the targeted antigen from patients. To facilitate rapid generation of cells for therapy, T-cells can be programmed with genes encoding for an antigen-specific  $\alpha\beta$ -T-cell receptor (TCR). However, this concept is limited by the low affinity of most tumor-reactive  $\alpha\beta$ -TCR chains. Furthermore most MHC class I-restricted tumor-antigens are not exclusively expressed on tumor cells. One attractive alternative to mediate a selective anti-tumor-reactivity with a high-affinity TCR arises

from the ability of  $\gamma\delta$ T-cells to mediate anti-tumor-reactivity while ignoring healthy-environment. The  $\gamma\delta$ T-cell-receptor (TCR) itself is supposed to mediate this intriguing property.  $\gamma\delta$ 2TCRs recognize non-classical antigens including metabolites of the mevalonate pathway. To test the ability of  $\gamma\delta$ 2TCRs to redirect abT cells selectively against tumor cells, the  $\gamma\delta$ 2TCR was retrovirally transduced into human abT cells. Thereby, strong surface-expression of introduced  $\gamma\delta$ 2TCRs was observed while endogenous  $\alpha\beta$ TCR chains were down-modulated. Functional analysis revealed that a  $\gamma\delta$ 2TCR efficiently reprograms both, CD4+ and CD8+ T-cells against a broad panel of cancer cells while ignoring normal cells. Moreover, tumor-specific T-cell proliferation, cytokine secretion and killing were significantly enhanced by additional application of bisphosphonates. In summary,  $\gamma\delta$ 2TCRs are an attractive alternative to redirect  $\alpha\beta$ T cells against cancer cells with both, an improved efficacy and safety profile as compared to currently used  $\alpha\beta$ TCRs.

**PD09/108 TUMOR-SPECIFIC CROSSLINKING OF GLUCOCORTICOID-INDUCED TUMOR NECROSIS FACTOR-RELATED PROTEIN LIGAND COSTIMULATES CD8<sup>+</sup> EFFECTOR T CELLS AND INHIBITS CD4<sup>+</sup>CD25<sup>+</sup> REGULATORY T CELL ACTIVITY**

T.S. Burckhardt<sup>1</sup>, M. Thiel<sup>1</sup>, H. Nishikawa<sup>2</sup>, H. Shiku<sup>2</sup>, C. Renner<sup>1</sup>

<sup>1</sup>Department of Oncology, University Hospital Zurich, Zurich, Switzerland, <sup>2</sup>Department of Cancer Vaccine and Gene Therapy, Mie University Graduate School of Medicine, Mie, Japan

**Objectives:** Naturally occurring CD4<sup>+</sup>CD25<sup>+</sup>GITR<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells (Treg) actively suppress the activation and expansion of self-reactive T cells. The glucocorticoid-induced tumor necrosis factor-related protein (GITR) is a membrane associated receptor and application of its ligand (GITRL) is an approach that has been investigated in tumor therapy models. We propose to target tumor infiltrating Treg by using bispecific constructs where GITRL is linked to a single chain antiFAP (fibroblast activation protein) antibody. High expression of FAP is restricted to tumor-associated fibroblasts found in the stroma of over 90% of epithelial cancers; therefore, FAP is an attractive localization target.

**Methods:** We cloned antiFAP-mGITRL bispecific constructs and controls, produced them in mammalian cell lines and purified them via IMAC. Multimerization state was assessed by gel filtration. Affinity for the respective receptors was determined by surface plasmon resonance. Effect of the constructs on proliferation of murine CD8<sup>+</sup> cells and IFN $\gamma$  and IL-2 production was quantified by *in vitro* costimulation and suppression assays.

**Results:** AntiFAP-mGITRL mainly assembled as dimeric construct. Fitting of the 1:1 Langmuir binding model to the steady-state plateaus yielded a KD value of 1.2  $\mu$ M for GITR and 335 nM for FAP. *In vitro* murine proliferation assays revealed that application of antiFAP-mGITRL led to proliferation of CD4<sup>+</sup> and CD8<sup>+</sup> cells and restored proliferation of effector cells otherwise suppressed in the presence of Treg. Most importantly, presentation of the construct by FAP-transfected cells, thus allowing crosslinking of multiple GITR molecules on T cells, led to enhanced costimulation of effector T cells and enhanced inhibition of suppressive activity. Moreover, antiFAP-mGITRL restored IFN $\gamma$  and IL-2 production by CD8<sup>+</sup> cells in this setting.

**Conclusion:** We have shown that antiFAP-mGITRL acts favourably on effector T cells, especially in the context where they are being suppressed by Treg. The antibody directed delivery of GITRL opens a new path to positively act on the local T cell environment in the tumor. Accumulation of the construct in FAP-positive tumor stroma can be used to locally render CD8<sup>+</sup> effector cells resistant to suppression by Treg. Targeted delivery and thereby enhanced immunomodulatory effects of antiFAP-mGITRL represent a promising approach in antibody-based tumor immunotherapy.

**PD09/109 VG9VD2 T LYMPHOCYTES EFFICIENTLY KILL ZOLEDRONATE-SENSITIZED CHRONIC MYELOGENOUS LEUKEMIA CELLS**

M. D'Asaro<sup>1</sup>, C. La Mendola<sup>1</sup>, P. Vigneri<sup>2</sup>, A. Messina<sup>2</sup>, N. Caccamo<sup>1</sup>, A. Salerno<sup>1</sup>, F. Dieli<sup>1</sup>

<sup>1</sup>Università degli Studi di Palermo, Dipartimento di Biopatologia e Metodologie Biomediche, Palermo, Italy, <sup>2</sup>Università degli Studi di Catania, Dipartimento di Scienze Biomediche, Catania, Italy

Imatinib mesylate, a competitive inhibitor of Bcr-Abl tyrosine kinase, is highly effective for the early stages of chronic myelogenous leukemia (CML). However, frequent relapse have been reported, particularly in patients with advanced-stage disease. In order to overcome this problem, additional immunotherapy may provide an opportunity for improving clinical response. To this aim, we have evaluated the efficacy of human gd T cells against imatinib sensitive or resistant leukemic cell lines (K562S, K562R, KCL22R, LAMA84R). All tested CML cell lines were weakly susceptible to Vg9Vd2 T cell cytotoxicity but a strongly enhanced lysis was found when cell lines were pre-sensitized both with zoledronate and imatinib, resulting in almost 90% lysis. Zoledronate or imatinib treatment alone was less effective in sensitizing tumor lines to cell lysis, suggesting a synergic effect of the combined treatment. Moreover, as it has been previously shown that Vg9Vd2 T lymphocytes may trogocytose B and T cell lymphoma, we tested this capability on CML cell lines. Results shown that: (a) Vg9Vd2 T lymphocytes recognize and conjugate to CML cell lines and trogocytose cell surface membrane fragments; (b) trogocytosis is enhanced by previous treatment of CML cell lines with zoledronate; (c) Imatinib synergize with zoledronate to increase trogocytosis, even with imatinib resistant CML cell lines. To determine the mechanism underlying gd T cell cytotoxicity, we explored the possible contribution of TCR, NKG2D, FasL, perforin and TRAIL-mediated pathways. Cytotoxicity was mainly mediated by perforin, as revealed using concanamycin A vacuolar-ATPase inhibitor, and a strong TCR-mediated response was also demonstrated. NKG2D, FasL and TRAIL seemed to play instead a minor role in gd T cell recognition of targets, with a minor reduction in cytotoxicity using specific blocking antibodies. Promisingly, zoledronate-activated gd T cells also exert their cytotoxic activity against leukaemia cells freshly isolated from patients at onset of CML (not treated yet), with a cell lysis that ranges from 10 to 38%, in 4 tested patients pre-sensitized with zoledronate and imatinib. Thus these findings suggest the clinical utility of intentional activation of gd T cells *in vivo* by zoledronate in combination with imatinib, in those patients refractory to imatinib treatment alone.

**PD09/110 RADIOTHERAPY MAY POTENTIATE IMMUNOTHERAPY THROUGH PRESENTATION OF NOVEL TUMOR ANTIGENS**

A. Sharma<sup>1</sup>, A. Knuth<sup>1</sup>, M. Broek<sup>1</sup>, L. Boehmer<sup>1</sup>, B. Fuchs<sup>2</sup>, M. Pruschy<sup>3</sup>

<sup>1</sup>University Hospital Zurich, Department of Oncology, Zurich, Switzerland, <sup>2</sup>University of Zurich, Department of Orthopedics, Balgrist, Switzerland, <sup>3</sup>University Hospital Zurich, Department of Radiation Oncology, Zurich, Switzerland

Radiotherapy is a standard treatment of many malignancies to date. Recent studies have shown that therapeutic irradiation not only enhances antigen presentation by MHC class I molecules but also results in the presentation of epitopes derived from novel antigens. Furthermore, it was shown in tumor bearing mice (colonic adenocarcinoma) that immunotherapy was only successful if preceded by irradiation. Cancer-testis antigens (CTAs) represent a family of tumor antigens with promising potential for immunotherapy because of their restricted expression in male germ cells and a wide variety of malignant tissues. In addition, the fact that spontaneous humoral and cell-mediated immune responses to CTAs have been demonstrated in cancer patients illustrates the immunogenicity of these antigens. We propose here that irradiation induces the expression of CTAs in various malignancies, which may result in an immune response that is less compromised by immune tolerance.

To test this hypothesis, we irradiated multiple cancer cell lines, which are known not to express CTAs under standard culture conditions, with  $\gamma$ -radiation from a 60Co source. The cells were then harvested at different time points after irradiation and the induction of CTAs and the MHC class I expression was analyzed by RT-PCR, real-time PCR and immunofluorescence. We found that irradiation induced the expression of CTAs. The induction of various CTAs was observed in multiple cancer cell lines in a randomized fashion and this induction was dose and time dependent. In addition,  $\gamma$ -radiation increased the expression of surface MHC class I molecules in a dose and time dependent manner. Our results suggest that a combination of radio- and immunotherapy is a promising and novel approach for the treatment of cancer.

**PD09/111 OPTIMIZER™, A NEW SERUM-FREE CGMP T CELL EXPANSION MEDIUM SPECIFICALLY DESIGNED FOR EXPANSION OF T CELLS FOR USE IN IMMUNOTHERAPY**

M.L. Bonyhadi<sup>1</sup>, Y. Matsuo<sup>2</sup>, Q. Tang<sup>3</sup>, L.M. Humeau<sup>4</sup>, S. Zhang<sup>4</sup>, C.Y. Liu<sup>5</sup>

<sup>1</sup>Invitrogen/Life Technologies Corporation, Cell Therapy Systems, Sammamish, United States, <sup>2</sup>Grandsoul Research Institute for Immunology, Nara, Japan, <sup>3</sup>University of California, San Francisco, Department of Surgery, San Francisco, United States, <sup>4</sup>VIRxSYS Corporation, Gaithersburg, United States, <sup>5</sup>Invitrogen/Life Technologies Corporation, Grand Island, United States

We have developed OpTmizer™, a cGMP serum-free T cell culture medium, which supports the *in-vitro* expansion and culture of various T cell populations under different activation and expansion conditions, including gene modification protocols. As various T cell-based immunotherapy applications are tested in early phase clinical trials, there is a critical need to support the transition of these applications to later phase trials, and eventual commercialization by the development of reagents that meet regulatory requirements and help reduce the cost of goods to manufacture a cell product. Except for HSA content, OpTmizer™ is a protein-free, chemically defined medium, and is made under cGMP. The medium supports high density, high viability, and rapid expansion of T cells in the WAVE™ bioreactor in the absence of human serum (HS) or at low serum concentrations. Moreover, the medium supports different T cell gene-modification protocols, including moloney- and lentiviral-based transduction systems. Data comparing OpTmizer to other culture media will be presented, demonstrating a favorable profile for the culture, expansion, and potent function of different T cell populations, including peripheral blood lymphocytes, regulatory T cells,  $\lambda\delta$  T cells, as well as gene-modified T cells.

**PD09/112 (-)-EPIGALLOICACATECHIN-3-GALLATE COMPENSATES FOR CELECOXIB-MEDIATED UP-REGULATION OF PGHS-2 EXPRESSION IN HUMAN PANCREATIC ADENOCARCINOMA CELLS UNDER TUMOR-ASSOCIATED INFLAMMATORY CONDITIONS**

C. Härdtnert<sup>1</sup>, R. Brandt<sup>1</sup>, G. Multhoff<sup>2</sup>, J. Radons<sup>1</sup>

<sup>1</sup>University of Greifswald, Institute of Medical Biochemistry and Molecular Biology, Greifswald, Germany, <sup>2</sup>Technische Universität München, Department of Radiotherapy and Radio-oncology, Klinikum rechts der Isar, München, Germany

**Aims:** Prostaglandine-H<sub>2</sub> synthase-2 (PGHS-2) inhibitors hold promise for cancer chemoprevention. However, recent toxicity reports suggest that novel strategies are required. One approach to overcome these limitations is the use of lower doses of PGHS-2 inhibitors in combination with other agents with complementary

mechanisms. In this study, the effect of (-)-epigallocatechin-3-gallate (EGCG), a promising chemopreventive agent from green tea, was tested alone and in combination with the PGHS-2-specific inhibitor celecoxib on the expression of IL-1-induced tumorigenic factors in human pancreatic adenocarcinoma cells. This approach acts as a model system for tumor-associated pancreatic inflammation playing a key role in pancreatic malignancy.

**Methods:** IL-1-stimulated Colo357 cells were incubated with EGCG, celecoxib and both substances in a time- and dose-dependent manner. Secretion of IL-6, IL-8, PGE<sub>2</sub> was analyzed by immunoassays. PGHS-2 expression was determined by Western blotting, cell viability by MTS assay whereas activation of caspase 3 and 9 was determined fluorimetrically. Statistical analysis was performed using nonparametric Mann-Whitney test.

**Results:** IL-1-induced production of IL-6 was inhibited synergistically by EGCG and celecoxib, whereas secretion of IL-8 remained unaffected. PGE<sub>2</sub> production was reduced by celecoxib. Co-incubation of Colo357 with EGCG and celecoxib synergistically reduced cell viability via apoptosis induction as evidenced by determination of caspase 3 and caspase 9 activity. Interestingly, EGCG compensates PGHS-2 up-regulation mediated by celecoxib.

**Conclusions:** EGCG and celecoxib are promising candidates for the development of new concepts how such deadly biological activities working in pancreatic cancer may be therapeutically targeted. It has to be seen as an attempt to supplement the hitherto existing conventional methods to fight the poor outcome of the disease. The aim is to improve the existing therapeutical networks by the development of poorly afflicting strategies. Against the background of a high cardiovascular risk associated with PGHS-2-specific pharmacological inhibitors, a combinatory treatment with EGCG and the PGHS-2 inhibitor celecoxib might overcome the limitations associated with PGHS-2 inhibitors since agents at low doses and with complementary mechanisms will be used. Such combined administration should positively affect the balance between risk and benefit in fighting the interplay of tumor-associated pancreatic inflammation and carcinogenesis in high-risk patients with pancreatic neoplasia.

## PD11 – ANTIBODY ENGINEERING AND ANTIBODY THERAPY

### PD11/1 ENGINEERED TRIVALENT ANTIBODY FRAGMENTS, CONTAINING COLLAGEN-DERIVED SEQUENCES, WITH IMPROVED PROPERTIES FOR *IN VIVO* TUMOR TARGETING AND IMAGING

Á.M. Cuesta<sup>1</sup>, D. Sánchez-Martín<sup>1</sup>, L. Sanz<sup>1</sup>, J. Bonet<sup>2</sup>, M. Compte<sup>1</sup>, L. Kremer<sup>3</sup>, F.J. Blanco<sup>4</sup>, B. Oliva<sup>2</sup>, L. Álvarez-Vallina<sup>1</sup>

<sup>1</sup>Hospital Universitario Puerta de Hierro Majadahonda, Molecular Immunology Unit, Madrid, Spain, <sup>2</sup>Parc de Recerca Biomèdica de Barcelona, Structural Bioinformatics Lab, Biomedical Informatics Research Unit, Barcelona, Spain, <sup>3</sup>Centro Nacional de Biotecnología, Consejo Superior de Investigaciones Científicas, Protein Tools Unit, Madrid, Spain, <sup>4</sup>CIC bioGUNE, Parque Tecnológico de Bizkaia, Structural Biology Unit, Bizkaia, Spain

**Objectives:** Antibodies designed for tumor-targeting *in vivo* should fulfill several requirements: high specificity and affinity for the target antigen, low immunogenicity, and adequate pharmacokinetic properties. So, the ideal tumor-targeting antibody should be intermediate-sized multivalent molecules, which provided rapid tissue penetration, high target retention, and rapid blood clearance.

We have developed and characterized a new class of multivalent antibody by using the N-terminal association subdomain of collagen XVIII (NC1), responsible for the non-covalent trimerization of collagen alpha chains, to drive multimerization. This new antibody format (scFv-NC1), comprises a single-chain antibody (scFv) connected to the NC1 domain by a flexible linker.

Three different scFv-NC1 antibodies were generated with specificity for the hapten 4-hydroxy-5-iodo-3-nitrophenyl (NIP), the human tumor-associated cell surface carcinoembryonic antigen (CEA), and an angiogenesis-associated laminin epitope.

**Methods:** In order to characterize the binding affinity, and stability *in vitro* and the *in vivo* properties of the scFv-NC1 antibodies; serum stability, surface plasmon resonance, and tumor targeting assays in tumor-bearing mice were performed.

**Results:** All the scFv-NC1 antibodies are trimeric in solution, exhibit excellent antigen binding capacity, providing them at least a 100-fold increase in functional affinity than the monovalent scFv. Serum stability studies in human and mouse serum indicates that scFv-NC1 antibodies retain 80-90% of its binding activity. Our results also demonstrate the feasibility of producing functional bispecific scFv-NC1 antibodies, which concurrently bind two different ligands. Computer graphic modeling indicates a tripod-shaped structure with three highly flexible scFv heads radially outward oriented.

The control B1.8 scFv-NC1 antibody showed no detectable localization in any of the three tumor types studied, while an anti-CEA scFv-NC1 antibody showed excellent specific tumor targeting in CEA-positive tumors. Most importantly, a L36 scFv-NC1 antibody that recognizes an angiogenesis-associated laminin epitope, showed excellent tumor localization in several cancer types.

**Conclusion:** These results indicate that this new scaffold for multimerizing scFv is potentially generalizable to many, if not all, scFv. They also illustrate the potential of this new antibody format for imaging and therapeutic applications, and suggest that some laminin epitopes might be universal targets for cancer targeting.

### PD11/2 THE IMPACT OF HINGE LENGTH ON THE ABILITY OF IMMUNOGLOBULIN A (IGA) TO MEDIATE BIVALENT BINDING TO WIDELY SPACED ANTIGENIC EPITOPES

M.J. Lewis<sup>1</sup>, T. Kretek<sup>1</sup>, C. Georgiadis<sup>1</sup>, S. Thomson<sup>1</sup>, J.M. Woolf<sup>1</sup>

<sup>1</sup>University of Dundee Medical School, Division of Medical Sciences, Dundee, United Kingdom

**Objectives:** Despite general similarities between immunoglobulins (Igs) in different mammals, each species has evolved its own unique complement of Igs. The array of related but distinct Ig structures that have evolved represent an elegant set of variations upon a theme. In humans, the two subclasses of IgA, IgA1 and IgA2, differ markedly in hinge length – IgA1 possesses an extended hinge, while IgA2 has a much shorter hinge region. IgA1 evolved relatively recently and IgA1 equivalents are only present in chimpanzees, gorillas, orang-utans and gibbons. We sought to determine the impact of these different hinge lengths on the antibody's ability to bind bivalently to closely spaced or more widely separated antigen molecules.

**Methods:** Wildtype IgA1 and IgA2, and hinge mutants of each, all with identical variable regions, were used in antigen-binding ELISA and surface plasmon resonance (SPR) experiments. Antigen molecules were immobilised on either ELISA wells or sensor chips at two different densities such that on one surface the antigen molecules were spaced significantly further apart, on average, than on the other. Chaotropic disruption was used to assess bivalent binding in the ELISA, while dissociation rate was used as an indicator of the level of bivalent binding in the SPR experiments.

**Results:** In comparison to IgA2, IgA1 exhibited more avid binding to, and dissociated more slowly from, surfaces that had widely spaced antigen molecules. Mutants that possessed hinge lengths predicted to be longer than that of IgA2 but less than that of IgA1 exhibited intermediate binding characteristics. Our findings suggest that IgA1 is particularly efficient at bivalent interaction with antigens spaced at relatively large distances apart.

**Conclusions:** The acquisition of IgA1 by humans and apes appears to increase the range of possibilities for high avidity antigen recognition, which should allow for more effective elimination of invading pathogens. The advantages afforded by this capability may, in part, explain why IgA1 has been retained after its evolutionary emergence. Further, inclusion of IgA1-like hinge structures in therapeutic antibodies might offer novel advantages, since the enhanced avidity afforded by the hinge should translate to lower dosage requirements with associated reduction in treatment costs.

### PD11/3 IMPAIRMENT OF B-CELL TRAFFICKING BY ANTI-CD22 THERAPY WITH EPRATUZUMAB

C. Daridon<sup>1,2</sup>, D. Blassfeld<sup>1</sup>, K. Reiter<sup>1</sup>, D. Frölich<sup>1</sup>, A. Hostmann<sup>1</sup>, D.M. Goldenberg<sup>3</sup>, T. Dörner<sup>1,2</sup>

<sup>1</sup>Charité University Hospital, Berlin, Germany, <sup>2</sup>Deutsches Rheumaforschungszentrum, Berlin, Germany, <sup>3</sup>Center for Molecular Medicine and Immunology, Belleville, United States

**Objective:** Epratuzumab, a humanized anti-CD22 monoclonal antibody, targets B cells and is under investigation as a therapeutic in non-Hodgkin's lymphoma and system lupus erythematosus (SLE). Unlike rituximab, epratuzumab has a reduced depletion of circulating B cells. The mechanism of action of epratuzumab on B cells remains unknown, for which reason we are studying the mechanism of action by which it affects B cells. The major point line of study was pursued in this work: The impact of epratuzumab on B cell migration.

**Methods:** To address how epratuzumab is involved in B-cell migration, a transwell migration assay was performed and evaluated by FACS analysis. PBMCs from 9 HD controls and 9 SLE patients were isolated and incubated with or without epratuzumab in the presence of different chemokines, such as CxCL9, CxCL10, CxCL11, CxCL12, or CxCL13. Statistics were performed by the Wilcoxon test.

**Results:** Epratuzumab showed an influence on B cell migration from HD and SLE patients. In fact, the spontaneous migration capacity of B cells from SLE patients and HD was comparable, between 10-15% of B cells are attracted to CxCL12 and CxCL13, and to a lower extent towards CxCL9, CxCL10, and CxCL11. In contrast to spontaneous migration, epratuzumab enhanced the migration of B cells and led to opposite effects on B cells obtained either from HD or SLE patients. Whereas B cells from HD were preferentially attracted by CxCL13 and CxCL9, CxCL10, and CxCL11 after epratuzumab treatment, B cells from SLE patients migrated significantly more towards CxCL12. Of note, epratuzumab induced comparable changes in the migration of naive and memory B cells of HD, whereas only the migration of naive B cells were affected in SLE.

**Conclusions:** Epratuzumab has an impact on the migration of B cells in HD and SLE, showing a different response profile in SLE. This monoclonal antibody seems to alter trafficking of B cells which may be part of its mechanism of action in the treatment of SLE.

Systemic lupus erythematosus: SLE; healthy donors: HD; peripheral blood mononuclear cells: PBMCs.



**PD11/4 ANTI-TUMOR TREATMENT OF TUMOR-BEARING IMMUNOCOMPETENT MICE WITH ANTI-CD20 MAB INDUCES AN ADAPTIVE IMMUNE RESPONSE THAT CAN BE STRENGTHENED BY IL-2 INFUSION**

R. Abes<sup>1,2,3</sup>, E. Bonin-Gélizé<sup>2,3</sup>, J.-L. Teillaud<sup>2,3</sup>

<sup>1</sup>Laboratoire Français du Fractionnement et des Biotechnologies (LFB), Les Ulis, France, <sup>2</sup>INSERM U872/Centre de Recherche des Cordeliers, Team 14, Paris, France, <sup>3</sup>Paris Descartes University & Pierre et Marie Curie (UPMC) University, Paris, France

The long-lasting clinical responses observed in lymphoma patients treated with rituximab, an anti-CD20 mAb, suggests that this antibody can induce an anti-tumor immune response. We have therefore investigated whether anti-CD20 treatment of CD20+ tumor bearing immunocompetent mice can trigger a specific adaptive immune response and whether it is possible to potentiate this response by subsequent IL-2 infusion. C57Bl/6 mice were i.v. injected with EL4 tumor cells expressing human CD20 and treated with repeated i.p. injections of the anti-CD20 mouse mAb CAT-13 or not. Whereas all untreated animals died around Days 25–35, about 60–70% EL4-huCD20 mice treated with CAT-13 survived without showing any health problem. Surviving mice that had been treated with CAT-13 were then challenged at Day 70 by a new i.v. injection of either EL4-huCD20 or EL4 tumor cells without any accompanying mAb treatment. All mice injected with EL4 cells died between Day 19 and 26 after the tumor challenge, while about 50–60% of mice challenged with EL4-huCD20 were still alive at Day 70 after this challenge. A single i.v. injection of spleen cells isolated from these animals into naive recipients subsequently injected with EL4-huCD20 tumor cells 24h later was sufficient enough to protect the latter animals. Thus, all these data indicate that anti-CD20 mAb treatment induces a long-lasting cellular adaptive immune response. Since it has been shown that IL-2 exerts an anti-tumor effect through the activation of T cells in cancer, we then injected IL-2 to the surviving CAT-13 treated mice, just before challenging the animals with an i.v. injection of EL4-huCD20 tumor cells. After 70 days, a significant increase of the survival rate of IL-2 infused animals was observed as compared to animals challenged with EL4-huCD20 cells only. Thus, IL-2 injection at distance from mAb treatment strengthens the immune response against EL4-huCD20 tumor cells induced by this treatment. Interestingly, no tumor-facilitating effect that could have been triggered by the stimulation of CD25+ Treg following IL-2 infusion was observed. In conclusion, our work shows that an anti-CD20 mAb treatment can induce a long-lasting adaptive immune response that can be manipulated with IL-2.

**PD11/5 THE THERAPEUTIC POTENTIAL OF MONOCLONAL ANTIBODY (MAB) AGAINST HUMAN ENDOGENOUS RETROVIRUS W (HERV-W) ENVELOPE PROTEIN IN MULTIPLE SCLEROSIS: RESULTS FROM A NEW EAE-ANIMAL MODEL**

A.B. Lang<sup>1</sup>, C. Bernard<sup>1</sup>, P.N. Marche<sup>2</sup>, H. Perron<sup>3</sup>

<sup>1</sup>Geneuro, Plan-les-Ouates, Switzerland, <sup>2</sup>University Joseph Fourier, INSERM, Grenoble, France, <sup>3</sup>Geneuro-Innovation, Lyon, France

HERV-W encodes an envelope protein (ENV) that is a potent agonist for TLR4 on antigen-presenting cells and activates a pro-inflammatory and autoimmune cascade. In addition, it triggers superantigen-like dysregulation of T-lymphocytes in such primed context. Convincing evidence has been found that HERV-W is associated with the evolution and prognosis of multiple sclerosis (MS) and certain other neurological diseases. We studied levels of ENV in peripheral blood from 103 patients with MS, 14 with clinically isolated syndromes (CIS) prodromal to MS and 88 with other neurological and non-neurological diseases, compared with 76 normal controls. The prevalence of HERV-W/ENV in MS patients was high (about 75%). Antigenaemia also occurred in 5 of 8 cases of chronic inflammatory demyelinating polyneuropathy, but not in other neurological and non-neurological diseases tested. No significant difference in antigenaemia was seen between either possible (CIS) or definite MS, or between different stages of MS. By assessing the effects of ENV in a mouse model similar to classical Experimental Allergic Encephalomyelitis (EAE), but using SCID mice grafted with human peripheral blood mononuclear cells providing a functional human lymphoid immune system, we found that ENV can trigger a CNS inflammatory response reproducing MS features. In contrast to controls, ENV induced paresis, weight loss, and central nervous system inflammation and demyelination visualised on MRI scanning and confirmed by terminal immunohistology. Next, we selected and tested in this model, mAbs capable of inhibiting immunopathogenic effects induced by HERV-W/ENV protein, in the hope that this will open perspectives for targeted serotherapy. A specific anti-ENV mAb selected for its inhibiting effects on ENV interaction with TLR4 in human lymphoid cell culture reversed the clinical, MRI and histological changes in comparison with untreated EAE controls. It had no apparent adverse effects in non-EAE mice. A therapeutic strategy using chimaeric/humanised therapeutic antibody against ENV protein has also been evaluated. In conclusion, the association of HERV-W with MS, its prevalence in affected patients together with evidence that this protein induces MS-like inflammation and demyelination that can be cured by a specific monoclonal antibody in animal models, suggests this is an appropriate therapeutic target.

**PD11/6 GENERATION OF GENE-ENGINEERED CHIMERIC DNA MOLECULES BY RECOMBINANT TECHNOLOGIES FOR SPECIFIC THERAPY OF AUTOIMMUNE DISEASES**

A.I. Tchobanov<sup>1</sup>, N. Mihaylova<sup>1</sup>, K. Nikolova<sup>1</sup>

<sup>1</sup>Institute of Microbiology – Bulgarian Academy of Sciences, Immunology, Sofia, Bulgaria

**Objectives:** Systemic lupus erythematosus (SLE) is a polygenic autoimmune disease characterized by B cell hyperactivity leading to the appearance of autoantibodies to different self antigens. The targeting of a self-epitopes to the available auto-reactive antigen binding B cells has a dramatic negative effect on their response. This targeting could be achieved by constructing a chimeric DNA molecules containing both B-epitopes as well as the single-chain variable fragment (scFv) from an antibody specific to a cell surface receptors delivering an inhibitory intracellular signal. Such a gene-engineered molecule could be used as a naked DNA vaccine. The DWEYSVWLSN peptide has been recently shown to mimic the antigenic epitopes of dsDNA. We hypothesize that it is possible to re-establish tolerance to native DNA in mice with spontaneous lupus-like diseases by administering to them a chimeric DNA molecule, encoding a scFv from a monoclonal antibody against the inhibitory FcγRIIb, coupled to DWEYSVWLSN peptide.

**Methods:** We have constructed a chimeric DNA molecule able to be expressed in eukaryotic cells and after that to cross-link cell surface immunoglobulin with the inhibitory FcγRIIb on DNA-specific B cells in a mouse model of lupus. We have created a gene-engineered DNA construct encoding a scFv from a monoclonal antibody against the inhibitory FcγRIIb, coupled to DNA sequencing coding DWEYSVWLSN peptide. The chimeric DNA molecules were constructed using various methods and protocols of molecular biology such a PCR, DNA recombinant methods and Immunological methods.

**Results:** Groups of lupus-prone MRL/lpr mice were injected intramuscularly (i.m.) with plasmid DNA encoding the chimeric molecule, or with the empty plasmid only and 21 days later they were boosted. The administration of the chimeric DNA molecule to 5-weeks old animals prevented the appearance of IgG anti-DNA antibodies while in the control group they rose dramatically. This result correlated with a low degree of proteinuria in the chimera treated animals and the prevention of disease activity.

**Conclusions:** In the present study we have proven that by using this artificial gene-engineered antibody it is possible to suppress selectively the activity of disease-associated B-lymphocytes and to change the natural course of a spontaneous autoimmune disease.

**PD11/7 MODULATION OF IMMUNE RESPONSE BY COMBINED TARGETING OF COMPLEMENT RECEPTORS AND LOW AFFINITY FCGAMMA RECEPTORS**

Z. Szekeres<sup>1</sup>, M. Herbat<sup>1</sup>, Z. Szittner<sup>1</sup>, A. Erdei<sup>1,2</sup>, J. Prechl<sup>2</sup>

<sup>1</sup>Eotvos Lorand University, Department of Immunology, Budapest, Hungary, <sup>2</sup>Immunology Research Group, Hungarian Academy of Sciences, Budapest, Hungary

**Objectives:** Pathogens became opsonized by immunoglobulins and fragments of complement proteins soon after their entering into the body. These immune complexes (IC) induce effective pathogen specific innate and humoral immune response via immunocomplex-binding receptors, such as murine complement receptor type 1 and 2 (mCR1/2) and murine low affinity Fc receptors for IgG (mFcγRII/III). Though these receptors have been characterized individually, their cooperative role in immune complex-mediated responses has not yet been adequately clarified. Aim of this study was to target mCR1/2 and mFcγRII/III by specific single-chain fragments of antibody (scFv), individually or in combination, thus modelling IC.

**Methods:** We used the well-characterized 7G6 scFv against mCR1/2 and 2.4G2 scFv against mFcγRII/III for targeting. To multimerize or combine these two scFvs, we monobiotinylated them and conjugated to streptavidin or streptavidin-coated microspheres. Such complexes were first investigated with respect to target receptor recognition in vitro and in vivo by flow cytometry. After that, mice were immunized with the complexes. Antibody response against the scFv C-terminal polypeptide sequence containing c-myc tag and hexahistidine tag was measured by ELISA and used for characterizing the induced immunity.

**Results:** Results show that targeting of mFcγRII/III induces higher antigen specific IgG1 antibody response than the control group immunized with non-targeted peptide-streptavidin complexes. Targeting of mCR1/2 does not significantly differ from the control. Combined targeting of both receptors fails to induce a higher antigen specific antibody response than individual targeting of mFcγRII/III. Furthermore, using streptavidin-coated microspheres, rather than streptavidin, increases the immunogenicity of antigen.

**Conclusion:** In summary, in vivo experiment support the observation that mFcγRII/III is more potent a target than CR1/2 and show that combined targeting of CR1/2 and FcγRII/III receptors does not result in synergistic or cumulative enhancement of the antigen specific immune response.

**PD11/8 LYMPHOCYTE DISPLAY: A NOVEL ANTIBODY SELECTION PLATFORM BASED ON T CELL ACTIVATION**

V. Alonso-Camino<sup>1</sup>, D. Sánchez-Martín<sup>1</sup>, M. Compte<sup>1</sup>, L. Sanz<sup>1</sup>, L. Álvarez-Vallina<sup>1</sup>

<sup>1</sup>Hospital Universitario Puerta de Hierro Majadahonda, Molecular Immunology Unit, Madrid, Spain

The display of foreign polypeptides and proteins on the surface of viruses or cells provides an important tool for the engineering of biomolecules and the analysis of their interactions with binding partners. Display technology has made great progress over the last 10 years and covers applications ranking from basic research to diagnosis and therapy. The microbial surface display technologies for screening antibody libraries include phage, yeast and bacteria platforms. Cell-free systems, as ribosomal display, are also available. Although the above mentioned systems have been successfully used for the isolation of antibodies, concerns may rise about

the lack of post-translational modifications and proper folding of selected proteins. In order to allow processing of the displayed antibody in a eukaryotic environment, screening systems based on viruses and mammalian cells have been described. In this report, we describe the design and testing of a mammalian cell surface display platform in T lymphocytes. The display of antibodies on the surface of T lymphocytes, as a part of a chimeric-immune receptor (CIR) mediating signaling, may ideally link the antigen-antibody interaction to a demonstrable change in T cell phenotype, due to subsequent expression of the early T cell activation marker CD69. In this proof-of-concept, an *in vitro* selection was carried out using a human T cell line lentiviral-transduced to express a tumor-specific CIR on the surface, against a human tumor cell line expressing the carcinoembryonic antigen. Based on an effective interaction between the CIR and the tumor antigen, we demonstrated that combining CIR-mediated activation with FACS sorting of CD69<sup>+</sup> T cells, it is possible to isolate binders to tumor specific cell surface antigen, with an enrichment factor of at least 10<sup>3</sup>-fold after two rounds, resulting in a homogeneous population of T cells expressing tumor-specific CIRs. The display of antibody repertoires on the surface of a T cell in the context of activating molecules might allow us to select *in vivo* tumor-specific T cells as consequence of a process of extravasation, mediated by cellular adhesion molecules, and of a process of retention and activation, mediated by the CIR.

# PD11/9 TOWARDS THE CAUSE OF THE INDUCTION OF SERIOUS ADVERSE EFFECTS IN TGN1412 TREATED PROBANDS

L. Sender<sup>1</sup>, Z. Waibler<sup>1</sup>, C. Merten<sup>2</sup>, R. Hartig<sup>2</sup>, M. Gunzer<sup>2</sup>, B. Schraven<sup>2</sup>, U. Kalinke<sup>3</sup>

<sup>1</sup>Paul-Ehrlich-Institut, Langen, Germany, <sup>2</sup>Otto-von-Guericke Universität, Magdeburg, Germany, <sup>3</sup>TWINCORE, Centre for Experimental and Clinical Infection Research, Hannover, Germany

Superagonistic anti-CD28 antibodies such as TGN1412 activate T lymphocytes without triggering the TCR/CD3-complex. In rats and mice these reagents induce preferential expansion of regulatory T cells and can be used for the treatment of autoimmune diseases. In March 2006, six healthy volunteers experienced serious adverse reactions during a first-in-human clinical trial of the superagonistic anti-CD28 monoclonal antibody TGN1412. However, preclinical studies did not show any signs of toxicity neither in *in vitro* studies with human immune cells nor in *in vivo* studies using rodents and non-human primates. We addressed the question why TGN1412 induced serious adverse events in humans but not in non-human primates and other animal models. Sequence analysis revealed that the CD28 extracellular domains of humans and non-human primates, including TGN1412 binding sites, were completely conserved. Developing a flow cytometry-based method for determination of receptor occupancy using primary T cells, we showed that binding of TGN1412 to CD28 on human and non-human primate T cells was similar. Furthermore, FACS analysis indicated a comparable ratio of CD4<sup>+</sup> vs. CD8<sup>+</sup> T cells in blood samples of the two species. Interestingly, TGN1412 as well as a commercially available superagonistic anti-CD28 antibody induced a sustained calcium response in human naïve and memory CD4<sup>+</sup> T cells, whereas Macaca derived T cells showed a reduced calcium flux into the cytosol. The calcium release was associated with the induction of pro-inflammatory cytokines, most notably IFN- $\gamma$  and TNF- $\alpha$ . Thus, our data suggest a molecular basis for the severe side effects caused by TGN1412 and impinge upon the relevance of non-human primates as preclinical models for reagents that are supposed to modify the function of human T cells.

# PD11/10 PHAGE DISPLAY GENERATION OF A NEW CLASS OF HUMAN CYTOTOXIC ANTI-TRANSFERRIN RECEPTOR ANTIBODY FRAGMENTS

R. Crépin<sup>1</sup>, J. Marks<sup>2</sup>, M.-A. Poul<sup>1</sup>

<sup>1</sup>LBPA, UMR CNRS 8113, Ecole Normale Supérieure de Cachan, Cachan, France, <sup>2</sup>UCSF, Departments of Anesthesia and Pharmaceutical Chemistry, San Francisco, United States

High proliferating cells require large amount of iron that is essential for DNA replication as well as proper respiratory chain activity. The binding of diferric charged transferrin (Tf) mediates iron cell uptake through transferrin receptor (TfR) endocytosis. Most cancer cells over express TfR. The inhibition of Tf internalization has proven to be efficient in reducing cancer cells proliferation, making it a good target for monoclonal antibody based immunotherapy.

In this work, we investigated the growth inhibitory potential of human anti-TfR antibody fragments (in the single-chain variable fragment-scFv 28 kD monovalent format) selected by phage display for their ability to internalize into living cancer cells. We hypothesized that the stringency of a functional cell-based antibody phage display selection allows the isolation of functional ligand-like antibodies. We have shown that the six anti-TfR scFvs obtained interfere with Tf binding to TfR and that two antibodies, namely 3GH7 and 3TF12, were capable of inhibiting the growth of various haematopoietic cancer cell lines.

We improved 3GH7 and 3TF12 inhibitory potential by engineering high affinity bivalent 55 kD antibody formats, namely H7CH and F12CH. H7CH and F12CH inhibited B lymphoma cell line Raji and erythroleukemia cell line ERY-1 proliferation with an improved IC50 compared to the monovalent formats (2 nM instead of 40 nM).

We further show that cytotoxicity was associated with an up-regulation of cell surface TfR levels. This is a unique characteristic when comparing with other cytotoxic mouse monoclonal anti-TfR antibodies that were all shown to decrease TfR levels. Cytotoxicity was also associated with the detection of strong apoptotic cell death.

These properties allow us to define a new class of fully human anti-TfR antibody fragments suitable for immunotherapy of tumors that are iron-dependant for sustained proliferation and that express high level of TfR. We are currently testing these antibodies on a mouse xenograft model.

# PD11/11 ELECTROSTATIC ALLOSTERY – A NOVEL MECHANISM FOR NEUTRALIZATION OF PROTEIN ANTIGENS BY ANTIBODIES

J.D. Dimitrov<sup>1</sup>, L.T. Roumenina<sup>1</sup>, B.P. Atanasov<sup>2</sup>, S.V. Kaveri<sup>1</sup>, S. Lacroix-Desmazes<sup>1</sup>

<sup>1</sup>INSERM UMR5 872 Centre de Recherche des Cordeliers, Paris, France, <sup>2</sup>Institute of Organic Chemistry – Bulgarian Academy of Sciences, Sofia, Bulgaria

The enormous diversity in the structural organization of the binding sites of antibodies generates a variety of possible antigen recognition mechanisms. The binding of immunoglobulins usually causes steric hindrance of functional important sites on their target molecules. In the present study by using theoretical and experimental approaches, we demonstrate a unique role for electrostatics in interaction between a human pathogenic antibody – BO2C11 and its target antigen – the coagulation factor VIII (FVIII). Kinetic and thermodynamic analyses of BO2C11 binding to FVIII indicated that this interaction is characterized by an ionic strength dependency that is uncommon for other protein-protein interactions. By using continuum electrostatics calculations and steady-state fluorescence measurements, we further demonstrated that BO2C11 binding to FVIII induces long-distance perturbations in the electrostatic potential and in the local electrostatic parameters (degree of ionization, proton affinity and electrostatic energy) of charged residues in the C2 domain of FVIII. The effects were not consecutive of structural alternations in C2. The distant changes in the electrostatic parameters were not delocalized, but affected predominantly the residues that constitute a binding site for von Willebrand factor (VWF) – a protein essential for FVIII stability and half-life in the circulation. Thus, the allosteric perturbation of surface electrostatics at a VWF binding site on C2 could explain the pathogenic effect of the BO2C11 in preventing FVIII binding to VWF. Our findings suggest that some antibodies modify their targets by alteration of protein surface electrostatics at a long-distance from the binding site. This phenomenon may open novel therapeutic strategies towards disruption of protein-protein interactions.

# PD11/12 MODIFIED IMMUNOGLOBULIN IMPROVES SURVIVAL IN EXPERIMENTAL SEPSIS

I. Djoumerska-Alexieva<sup>1</sup>, J. Dimitrov<sup>1,2</sup>, E. Voynova<sup>1</sup>, Z. Stefanova<sup>1</sup>, T. Vassilev<sup>1</sup>

<sup>1</sup>Stefan Angelov Institute of Microbiology, Sofia, Bulgaria, <sup>2</sup>INSERM Unit 872, Paris, France

**Objectives:** Currently available therapeutic strategies in sepsis are not satisfactory. Its first phase, known as “inflammatory”, is responsible for the early mortality of the patients. Normal pooled therapeutic immunoglobulin (intravenous immunoglobulin, IVIg) preparations are known to have an anti-inflammatory activity, mediated by their interactions with complement components, inhibitory receptors on immune cells, down-regulation of T-, B-cell and dendritic cell functions and effect on cytokine networks. IVIg has had, however, only a moderate beneficial effect in the complementary treatment of septic patients. The aim of the study is to develop and test improved, “next-generation” immunoglobulins for the passive immunotherapy of sepsis and related inflammatory diseases.

**Methods:** Septic shock was induced in outbred ICR mice by the i.p. injection of *E.coli* LPS or by the CLIP technique. Animals were treated with commercial IVIg, exposed to Fe(II) ions (see *J.Biol.Chem.*2006;281;439). Survival between the experimental groups was compared and the serum levels of 28 inflammatory mediators were measured by an antibody array technique. Effects on coagulation and on blood biochemical parameters were evaluated by standard methods. The kinetic and thermodynamic constants of the interaction between a Fe(II)-exposed monoclonal IgG antibody to its target antigen were determined using the BIAcore 2000 surface plasmon resonance-based system.

**Results:** The administration of a Fe(II)-exposed IVIg preparation improved significantly survival in both experimental septic shock models, while the native commercial IVIg was not protective. In a separate series of experiments it was shown that the “improved” IVIg, had not only a prophylactic but also a therapeutic effect as it was active even when administered six hours after the sepsis induction. The continuous presence of ferrous ions was not needed for the seen beneficial outcome. Treatment with the modified IVIg resulted in partial correction of the coagulation abnormality and in down-regulation of serum IL-6, IL-12p40p70, sTNF RII and MIP-1 gamma levels. The BIAcore studies demonstrated that the paratope of some antibodies from the IgG pool acquired an additional structural flexibility upon exposure to ferrous ions (for details see *Autoimm.Rev.* 2008;7;410).

**Conclusion:** The immunoglobulin preparation, modified by the exposure to pro-oxidative ferrous ions, prevents sepsis-related death in two animal models.

**PD11/13 CONSTRUCTION OF A PLASMID FOR THE EXPRESSION OF COMPLETE HUMAN MONOCLONAL IGE ANTIBODIES AND PRODUCTION OF HUMAN MONOCLONAL IGE SPECIFIC FOR THE MAJOR GRASS POLLEN ALLERGEN, PHL P 5**C. Madritsch<sup>1</sup>, S. Flicker<sup>1</sup>, S. Scheiblhofer<sup>2</sup>, J. Thalhammer<sup>2</sup>, R. Valenta<sup>1</sup><sup>1</sup>Div. of Immunopathology, Dept. of Pathophysiology, Center for Physiology and Pathophysiology, Medical University of Vienna, Vienna, Austria, <sup>2</sup>Christian Doppler Laboratory for Allergy Diagnosis and Therapy, Department of Molecular Biology, University of Salzburg, Salzburg, Austria**Objectives:** To construct and evaluate a plasmid suitable for the expression of complete human monoclonal IgE antibodies of any desired specificity.**Methods:** We have removed from the expression vector pLNOH2 the portion coding for the human IgG constant region and replaced it by a PCR-amplified genomic DNA fragment coding for the human epsilon heavy chain constant region to obtain plasmid pLNOH2-IgE. In order to express a complete human IgE antibody specific for the major grass pollen allergen Phl p 5, the variable heavy chain fragment of a Phl p 5-specific IgE Fab was inserted into pLNOH2-IgE. This construct together with plasmid pLNOK expressing the light chain of the Phl p 5-specific IgE Fab was co-expressed in COS 7 cells. The complete Phl p 5-specific human IgE antibody was analyzed with regard to allergen and isotype-specificity by ELISA. Its biological activity was tested by loading RBL-2H3 cells which had been transfected with cDNAs coding for the human high affinity IgE receptor, FcεRI. The cross-reactivity of the Phl p 5-specific IgE with natural group 5 allergens was studied by immunoblotting using grass pollen extracts from various grass and corn species.**Results:** We report the construction of an expression vector pLNOH2-IgE, which allows the expression of complete human monoclonal IgE antibodies and exemplify its usefulness by the production of a human monoclonal IgE antibody specific for the major grass pollen allergen Phl p 5, termed huMabEP5. huMabEP5 was biologically active in RBL-release assays and cross-reacted with natural group five allergens from Timothy grass, Kentucky Blue grass, Rye grass and Rye.**Conclusion:** The expression vector system described by us allows to produce functional human monoclonal IgE antibodies of any specificity. Such monoclonal IgE antibodies will represent useful tools to investigate the immunological mechanisms underlying IgE-mediated allergies and to develop diagnostic and therapeutic strategies for diseases in which IgE antibodies play a role. This study was supported by grant 813003 of the Austrian Research Promotion Agency.**PD11/14 BOTH NEUTRALIZING AND EFFECTOR FUNCTIONS OF ANTIVIRAL MONOCLONAL ANTIBODIES CAN COOPERATE TO INDUCE PROTECTIVE ENDOGENOUS ANTIVIRAL IMMUNITY**R. Nasser<sup>1</sup>, M. Pelegrin<sup>1</sup>, M. Plays<sup>1</sup>, M. Piechaczyk<sup>1</sup>, L. Gros<sup>1</sup><sup>1</sup>IGMM, CNRS-UMR5535, Montpellier, France

Neutralizing monoclonal antibodies (Mabs) are increasingly considered for blunting human viral infections. Most of these Mabs are defined mechanistically by their neutralizing ability. However, Mabs can also bind complements and promote antibody dependent cell mediated cytotoxicity (ADCC) through the formation of immune complexes.

Because in-vivo study of human viruses is inherently difficult, we use the lethal FrCas<sup>E</sup> retroviral infection model of mice to elucidate the mechanisms whereby neutralizing antibodies exert their antiviral actions. We unexpectedly showed that short passive immunization with a neutralizing Mab (667), in addition to an immediate blunting of FrCas<sup>E</sup> propagation, induces a long-term protective immune response including both the humoral and cellular immune responses. A paramount question is now to elucidate the molecular and cellular mechanisms underlying the mounting of this protective immunity.In order to investigate the effect of neutralization by 667 Mab, we used infections with variable inoculum of FrCas<sup>E</sup> and treatments by either 667 or a 667-derived F(ab)<sup>2</sup> fragment. The follow up of both the pathology (erythroleukemia) and immune responses (humoral and cellular) in infected animals point to an essential role of the Fc fragment of 667 in the development of antiviral immunity. Indeed, our results indicate that the 667 Mab, besides its neutralizing effects, can employ effector ADCC functions, which seem the determining factor for the reduction of the viral induced infection in neonatal mice and thus the protection of mice against viral induced diseases.

Thus, our data clearly indicate that the neutralization by 667 Mab is necessary but not sufficient for the development of long-term antiviral protection. Rather, 667 Mab, via its Fc fragment, can form immune complexes with infected cells in-vivo and thus contributes to the development of a strong antiviral immune response. Therefore, our work opens new perspectives for designing future immunotherapies of chronic human infections.

**PD11/15 IN VITRO PREDICTION OF CYTOKINE RELEASE SYNDROME: PREVENTING ANIMAL TESTING ON HIGH RISK DRUG CANDIDATES**A. Roghanian<sup>1</sup>, L. Hepburn<sup>1</sup>, C. Lu<sup>1</sup>, D.L. Lanham<sup>1</sup>, M.G. Wing<sup>2</sup>, C.M. Kirton<sup>1</sup><sup>1</sup>Huntingdon Life Sciences, Huntingdon Research Centre, Department of Experimental Biology, Huntingdon, United Kingdom, <sup>2</sup>Huntingdon Life Sciences, Huntingdon Research Centre, Clinical and Translational Sciences, Huntingdon, United KingdomAcute cytokine release syndrome (CRS)/cytokine storm is associated with some therapeutic antibodies (Ab) in man, leading to a spectrum of clinical signs from nausea, chills and fever to more serious dose limiting hypotension and tachycardia. When anticipated this syndrome is typically manageable. However, in 2006 this adverse reaction became headline news when a massive and unexpected CRS occurred within a few hours of dosing six healthy volunteers with a therapeutic Ab (TGN1412) putting their lives at risk due to multiple organ failure. Preclinical studies in monkeys did not predict this adverse event, thus there is a need to develop accurate and robust human *in vitro* assays to evaluate the safety risks for biotherapeutics to guarantee the safety of clinical subjects. The precise mechanism of this syndrome probably varies between Abs, but it typically involves Fc-receptor stimulation of NK cells, and/or the Fc-receptor dependent cross-linking of cytokine stimulatory molecules such as CD3. *In vitro* isolated human leukocytes and whole blood cultures may be useful for predicting cytokine release by these two distinct mechanisms. The utility of these *in vitro* methods should allow investigators to establish the mechanism of potential CRS for an Ab product. Here we used various blood cell cultures, time-points, coating reagents/conditions for immobilising Abs and reagents to reliably enable us to predict the potential cytokine release of therapeutic Abs *in vitro*. Using the data generated, we have been able to develop two parallel cytokine release assays (CRA), which we refer to as *Type I* (Fab:antigen mediated) and *Type II* (Fab:antigen and Fc-receptor mediated) CRA, and have successfully employed to test known stimulatory monoclonal antibodies (mAbs) and unknown mAbs entering preclinical tests. These rapid and low-budget screening methods can be applied to identify biotherapeutics likely to cause cytokine release in man and evaluate the impact of molecular engineering strategies to reduce the risk of causing this syndrome. In this way biotherapeutics will enter pre-clinical development with a better chance of reaching the market, thereby significantly reducing animal usage at early stages of drug development.**PD11/16 CHARACTERIZATION OF ANTI-LICOS ANTIBODIES**B. Akkaya<sup>1,2</sup>, H.L. Walsh<sup>1,2</sup>, S.J. Davis<sup>1,2</sup>, R.J. Cornall<sup>1</sup><sup>1</sup>University of Oxford, Nuffield Department of Clinical Medicine, Oxford, United Kingdom, <sup>2</sup>University of Oxford, MRC Human Immunology Unit, Oxford, United Kingdom**Objectives:** ICOS is a costimulatory receptor expressed on T cells that is homologous to CD28 and CTLA-4. It is expressed at very high levels on follicular helper T-cells within the light zone of germinal centers. LICOS is a B7-like molecule expressed on antigen presenting cells, including B cells, that is capable of binding to ICOS. The ICOS-LICOS interaction has an important role in T cell-dependent antibody responses. It has been shown that dysregulated follicular helper T-cells assist self-reactive B cells in the context of antibody-mediated autoimmune diseases and that the disease state is ameliorated in animal models of human lupus and rheumatoid arthritis by blocking the ICOS-LICOS interaction. Therefore, the use of monoclonal antibodies generated against LICOS could be a therapeutic option for treating autoimmunity.**Methods:** We have generated three hybridomas that secrete monoclonal anti-LICOS antibodies (clone 23, 52 and 56). To characterize the epitopes of these antibodies, we generated four mutant recombinant LICOS proteins carrying mutations of proposed binding-site residues and transiently expressed the proteins on 293T cells. The cells were then analyzed by fluorescence-activated cell sorting. Further characterization of antibody binding was undertaken using ELISA analysis of soluble, wild type and chimeric mouse/human LICOS, and surface plasmon resonance based assays.**Results:** FACS analysis showed that all four of the mutated proteins bound all three antibodies, implying that none of the antibodies are capable of blocking the ICOS/LICOS interaction. However, by ELISA, we found that two of the antibodies bind to the d1 domain of LICOS whereas the third binds d2. Furthermore, surface plasmon resonance-based assays showed that one of the antibodies, clone 23, does indeed block the ICOS-LICOS interaction.**Conclusions:** Analysis of the antibody affinities, investigation of their epitopes and studies of the *in vitro* activities of the antibodies are in progress, the results of which will be presented. The present findings strongly support the possibility that clone 23 blocks the ICOS-LICOS interaction and might be useful therapeutically.**PD11/17 COMBINING BISPECIFIC ANTIBODIES AND COSTIMULATORY ANTIBODY-CYTOKINE FUSION PROTEINS FOR TARGETED CANCER IMMUNOTHERAPY**P. Diebolder<sup>1</sup>, K. Frey<sup>1</sup>, A. Banaszek<sup>1</sup>, R. Kontermann<sup>1</sup>, D. Müller<sup>1</sup><sup>1</sup>University of Stuttgart, Institute of Cell Biology and Immunology, Stuttgart, Germany

Recombinant bispecific antibodies have shown to be able to retarget cytotoxic T cells to tumor cells in a MHC-independent manner, triggering effector cell activation and consecutive tumor cell death. Considering that costimulation is an essential requirement not only to initiate T cell activation, but also for the regulation of a proper T cell response, we propose the combination of recombinant bispecific antibodies with selective costimulation for cancer immunotherapy. For targeted costimulation we generated antibody-cytokine fusion proteins, composed of a tumor cell-specific recombinant antibody moiety fused to the extracellular domain of the costimulatory ligand B7.2 and 4-1BBL, respectively. These recombinant fusion proteins exhibited costimulatory properties, which were found to be ligand-specific and substantially constrained to antigen-mediated cell binding. In view of combining properly the bispecific antibody with the costimulatory antibody-cytokine fusion proteins, avoiding competition between both molecules, construct variants differing in the tumor-specific antibody moiety were generated, target-



ing different epitopes on the same antigen or different antigens on the same tumor cell. Combinatorial effects were analyzed incubating the antibody constructs with antigen positive tumor cells in presence of PBMCs and determining the stimulation of T cells by measuring IL-2 and IFN-gamma release in ELISA. Both kind of costimulatory antibody-cytokine fusion proteins (B7.2 and 4-1BBL constructs) enhanced the stimulatory effect induced by the recombinant bispecific antibody. Signal increase was achieved by constructs in combinatorial settings targeting different antigens on the same tumor cell. On the other hand costimulatory activity was also observed combining constructs targeting different epitopes on the same antigen on the tumor cell, even though in this case competition by sterical hindrance could not be circumvented. In summary, the combination of recombinant bispecific antibodies with antibody-cytokine fusion proteins activating specific costimulatory receptors is a promising approach to potentiate antitumor T cell response in cancer immunotherapy.

#### PD11/18 ISOLATION, CLONING AND CHARACTERISATION OF THE LLAMA GLAMA HEAVY-CHAIN IMMUNOGLOBULIN GENE LOCUS

J. Wesolowski<sup>1</sup>, F. Haag<sup>1</sup>, F. Koch-Nolte<sup>1</sup>

<sup>1</sup>University Medical Center Hamburg, Germany, Institute of Immunology, Hamburg, Germany

In addition to conventional heterotetrameric immunoglobulins, llamas produce heavy-chain antibodies (hcAbs) that exhibit a propensity to bind to and block clefts on protein surfaces. These hcAbs lack the light chain and the first constant domain (CH1), and carry IgG Fc domains. Unique features of the antigen-binding domain of hcAbs, designated VHHs, include very long CDR3s and a set of hydrophilic residues in FR2 (i.e. in a region corresponding to the interface of VH with VL domains). The organization of the heavy-chain locus in vertebrates usually consists of upstream variable (VH) segments followed by diversity (DH) and joining (JH) mini-genes, the enhancer, constant regions (CH) of different subtypes and, finally, the locus control region. The CH region usually begins with C $\mu$  followed by C $\delta$ , different numbers of C $\gamma$  isotypes, C $\epsilon$  and C $\alpha$ , additional copies of which are found in some species within the C $\gamma$  cluster. The number of VH, DH, and JH differs between species.

In order to determine whether the heavy chain and conventional immunoglobulins are encoded within the same or different loci, we constructed a germline BAC genomic library from liver DNA of a llama. Using VH and CH probes we isolated 17 clones containing insert sizes ranging from 90-220kb. The inserts were sequenced using the Roche 454 titanic sequencing technology. The results reveal a variable region containing interspersed VH and VHH domains. The constant region resembles the basic overall structure of other mammalian heavy-chain loci, but contains interspersed conventional and heavy-chain only IgG isotypes (which carry a splice site mutation in the CH1 domain). The heavy-chain only isotypes can be transcribed as soluble or membrane bound antibodies. It remains to be determined how the recombination machinery manages to pair VHH and heavy-chain IgG isotypes and how the very long CDR3s are generated. Moreover, it is conceivable that several developmental stages, i.e. pairing with surrogate light chain and expression of cell surface IgM are circumvented in B cells producing hcAbs.

#### PD11/19 VERY EFFICIENT FUNCTIONAL INHIBITION OF CELL SURFACE AND INTRACELLULAR RECEPTORS BY SCFV FRAGMENT MEDIATED ER RETENTION

T. Böldicke<sup>1</sup>, M. Bernal<sup>1</sup>, S. Somplatzki<sup>1</sup>, G. Sergeev<sup>1</sup>, S. Bauer<sup>2</sup>, C.J. Kirschning<sup>3</sup>

<sup>1</sup>Helmholtz Centre for Infection Research, Molecular Biotechnology, Braunschweig, Germany, <sup>2</sup>Philipps University, Immunology, Marburg, Germany, <sup>3</sup>Institut of Medical Microbiology, Technical University of Munich, Immunology and Hygiene, Munich, Germany

We generated different very effective intracellular scFv fragments (intrabodies) fused with a ER retention sequence starting from the cDNA of high specific hybridoma clones recognizing the oncogenic receptor VEGFR-2, the pattern recognition receptors TLR2, TLR9 and the adhesion molecule NCAM to inhibit the function of these proteins.

The method includes gene amplification of the variable domains VH and VL by using the method of linker-ligated PCR, assembly PCR of VH, VL and a short synthetic linker to construct the scFv-fragment and cloning of the scFv fragment into the ER intrabody expression vector pCMV/myc/ER. Rapid functional analysis of the generated ER intrabodies can be easily performed after transiently co-transfection of the intrabody genes, the target gene and reporter gene eGFP into HEK293 cells, extracellular FACS analysis of eGFP gated cells and co-immunoprecipitation of intrabody and target protein. In the next step the function of the intrabody will be tested in the specific target cell (e.g. immune cells or tumor cells) and evaluation of the intrabody in a appropriate mouse model will follow using local or systemic adenoviral intrabody gene transfer.

All five ER intrabodies generated against four different proteins completely and specifically block the translocation of these proteins from the ER to the cell surface/endosomal compartment. Furthermore the anti-TLR2 intrabody inhibited TLR2 specific signaltransduction *in vivo*. Recently we showed that intracellular binding of the anti-TLR2 intrabody to the target TLR2 led to very efficient and specific accumulation of TLR2 inside the ER compartment. The group of S. Burgdorf started now to analyze if the complex of TLR2/anti-TLR2 intrabody will be degraded inside the ER or in the proteasome. Recombinant adenoviruses with the anti-TLR2 and anti-TLR9 intrabody were generated and will be tested in a collagen II induced rheumatic arthritis mouse model.

ER intrabodies are a very specific and efficient tool to inhibit the function of cell surface and intracellular localized receptors *in vitro* and *in vivo*.

#### PD11/20 DESIGN OF RECOMBINANT ANTIBODY-BASED VACCINES

L.S. Høydahl<sup>1,2</sup>, I.B. Rasmussen<sup>1,2</sup>, T. Frigstad<sup>2,3</sup>, E. Lunde<sup>2</sup>, B. Bogen<sup>2,3</sup>, I. Sandlie<sup>1,2</sup>

<sup>1</sup>University of Oslo, Department of Molecular Biosciences, Oslo, Norway, <sup>2</sup>Centre for Immune Regulation, University of Oslo, Oslo, Norway, <sup>3</sup>Institute of Immunology, Rikshospitalet University Hospital, Oslo, Norway

**Objectives:** To compare different modes of antigen (Ag) fusion to antibodies (Abs) to achieve antigen presenting cell (APC) targeting of Ag, recombinant Abs (rAbs) were constructed with either of two variants of a T cell epitope grafted into the constant domain structure (Troybodies), the same epitopes attached as C-terminal fusions, or a globular Ag as a C-terminal fusion.

**Methods:** We have previously shown that targeting of Ags to APCs enhances CD4<sup>+</sup> T cell responses. This can be achieved with rAbs with specificity for APC surface molecules and constant regions with an integrated Ag. Target binding, followed by internalization and lysosomal degradation, releases the Ag for presentation on MHC class II molecules to specific T cells.

The antigen used originates from the IgA myeloma protein, M315, in which the variable region of the  $\lambda 2$  light chain ( $\lambda 2^{315}$ ) contain a T cell epitope recognized by specific T cells in context of I-E<sup>d</sup> (Eisen, Simms et al. 1968; Bogen, Malissen et al. 1986). Two peptides from  $\lambda 2^{315}$  were used (aa 91-101 and aa 89-105), as well as scFv derived from M315.

**Results:** We found that all rAbs retained target binding and were secreted from producing cells as complete Ab molecules. C-terminal epitope fusions were secreted at levels comparable to wild type (wt) Ab, whereas loop grafting or scFv C-terminal fusion resulted in reduced levels. The rAbs retained binding to soluble human Fc $\gamma$ RI, Fc $\gamma$ RIIA, and Fc $\gamma$ RIII, except for a C $\mu$ 2-Troybody.

All targeted rAbs induced primary T cell responses *in vitro*, with both proliferation and cytokine secretion being 100-10.000 fold more efficient than untargeted controls. Their potency varied, with a C $\mu$ 2-Troybody inducing by far the most potent T cell responses followed by a rAb with C-terminal epitope fusion, and lastly a C $\mu$ 1-Troybody and rAb with C-terminal scFv fusion. Furthermore, rAbs were able to prime APCs *in vivo* after intravenous (i.v.) injection, as determined by ability of isolated APCs to stimulate specific T cells *in vitro*. The same hierarchy between C $\mu$ 2, scFv and C $\mu$ 1 rAbs as in the *in vitro* T cell proliferation assay was seen.

**Conclusion:** These results are important for design of Ab-based vaccines.

#### PD11/21 DETECTION OF ALLERGENIC PARVALBUMINS WITH RECOMBINANT SINGLE-CHAIN ANTIBODIES IN FISH PROTEIN EXTRACTS AND FISH CONTAINING PRODUCTS

M. Kostadinova<sup>1</sup>, M. Bublin<sup>1</sup>, N. Balazs<sup>1</sup>, S. Wagner<sup>1</sup>, C. Radauer<sup>1</sup>, H. Breiteneder<sup>1</sup>

<sup>1</sup>Medical University of Vienna, Department of Pathophysiology, Vienna, Austria

**Objectives:** To date, the only proven therapy of fish allergy is avoidance of responsible fish. Fish allergy is mainly associated with a single type of allergen in fish, namely parvalbumin. It is the major allergen for 95% of patients suffering from IgE-mediated hypersensitivity to fish. The aim of this study was to produce recombinant parvalbumin-specific human antibodies as single chain variable fragments (scFv) by phage display technology.

**Methods:** The human synthetic single-chain variable fragment (scFv) phage antibody library ETH-2 was used to isolate the scFvs by three rounds of biopanning. The biopanning was carried out against purified cod, carp and trout parvalbumins. Afterwards, individual phage clones were tested for their binding to the allergens by ELISA. Twenty five positive clones were selected for DNA sequence analysis and one of them was selected on the basis of their high reactivity to all three tested parvalbumins for expression as scFv-alkaline phosphatase fusion antibody in *E. coli*. The produced recombinant antibodies were tested for their ability to recognize parvalbumin in different fish protein extracts as well as in products like canned, smoked or breaded fish by ELISA and immunoblotting.

**Results:** The selected parvalbumin specific scFv-alkaline phosphatase fusion antibody was successfully expressed in *E. coli* with a yield of 2.3 mg/L bacteria culture. The scFv recognized parvalbumin in cod, carp and trout protein extract without any background. The detection limit of the 1 $\mu$ g/ml scFv was found to be 0.5  $\mu$ g/ml of purified cod, carp and trout parvalbumin tested by ELISA. Further, the scFv was able to recognize parvalbumin in processed fish products of fried carp, smoked trout and breaded cod sticks, but not in products from herring, sardine and mackerel.

**Conclusion:** The recombinant parvalbumin-specific scFv as alkaline phosphatase fusion proteins make possible one-step detection of parvalbumin in different fish protein extracts and fish containing products. This can be useful for standardization of protein extracts used for allergy diagnosis and for the detection of allergens in different fish containing products.

This work was supported by grant SFB-F01802 from the Austrian Science Fund.

- PD11/22 IDENTIFICATION OF A HEPATITIS C VIRUS (HCV) E2 HUMAN B-CELL NEUTRALIZING EPIOTOPE NOT INVOLVED IN THE CD81 BINDING**  
 R. A. Diotti<sup>1</sup>, M. Perotti<sup>1</sup>, G. Sautto<sup>1</sup>, N. Mancini<sup>1</sup>, D. De Marco<sup>1</sup>, N. Clementi<sup>1</sup>, A. H. Patel<sup>2</sup>, A. W. Tarr<sup>3</sup>, M. Clementi<sup>1</sup>, R. Burioni<sup>1</sup>  
<sup>1</sup>Università Vita-Salute-San Raffaele Scientific Institute, Microbiology and Virology Department, Milano, Italy, <sup>2</sup>University of Glasgow, MRC Virology Unit, Glasgow, United Kingdom, <sup>3</sup>University of Nottingham, Institute of Infection, Immunity and Inflammation and Division of Microbiology, Nottingham, United Kingdom  
**Objective:** The hepatitis C virus (HCV) E2 glycoprotein represents a key antigenic structure for an effective vaccine against the virus. However the variability rate of this protein represents a challenge in the HCV vaccine development. Precise definition and mapping of conserved epitopes of E2 targeted by the human neutralizing response is crucial for the design of novel molecules able to elicit an immune response with low changes for virus escape. In the present study, the epitope of a human cross-reactive Fab was characterized.  
**Methods:** The epitope bound by neutralizing human cross-reactive antibody (designed Fab e8) was evaluated using three complementary approaches: (i) cross-competition assay with mouse monoclonal antibodies directed against known E2 epitopes, (ii) alanine scanning mutagenesis experiments, and (iii) computational analysis of peptides selected from phage display random peptide library.  
**Results:** Fab e8 was able to bind E2 derived from HCV genotypes 1a, 1b, 2a, 2b and 3. While inactive in inhibiting E2 binding to CD81, Fab e8 was able to neutralize HCV pseudoparticles bearing E1-E2 of genotype 1a, suggesting that the epitope recognized is located outside the CD81 binding region of HCV/E2. Cross-competition assays, alanine scanning mutagenesis experiments and computational epitope prediction located the epitope of Fab e8 outside the CD81 binding region.  
**Conclusion:** The present study supplies the first evidence and characterization of a human neutralizing HCV/E2 epitope on conserved E2 region outside CD81 binding site.
- PD11/23 INTRAVENOUS IMMUNOGLOBULIN UP-REGULATES THE EXPRESSION OF THE INHIBITORY FCγRIIB B CELL RECEPTORS**  
 K. Nikolova<sup>1</sup>, A. Tchobanov<sup>1</sup>, I. Djoumerska-Aleksieva<sup>1</sup>, M. Nikolova<sup>2</sup>, T. Vassilev<sup>1</sup>  
<sup>1</sup>The Stephan Angelov Institute of Microbiology, Department of Immunology, Sofia, Bulgaria, <sup>2</sup>National Center of Infectious and Parasitic Diseases, Central Laboratory of Immunology, Sofia, Bulgaria  
**Objectives:** Pooled human intravenous immunoglobulin (IVIg) preparations are known to modulate autoimmune/inflammatory diseases via several F(ab')<sub>2</sub>- and Fc-dependent mechanisms. We show that the *in vitro* and the *in vivo* exposure to IVIg of B lymphocytes from lupus-prone MRL/lpr and from healthy Balb/c mice results in an increased expression of their surface inhibitory FCγRIIB receptors.  
**Methods:** 12 weeks-old female Balb/c and MRL/lpr mice were injected with IVIg and 12 hours later the expression of FCγRIIB on CD19+ spleen cells was determined by flow cytometry. A recent study by our group has shown that it is possible to silence targeted dsDNA-specific B cells in lupus mice by administering an antibody chimera able to cross-link their surface FCγRIIB with the dsDNA-binding BCRs (Eur J Immunol. 2007;37:3587). B lymphocytes from MRL/lpr mice were exposed to IVIg and further to the same antibody chimera. The numbers of anti dsDNA IgG antibody-producing cells were determined by ELISpot. The phosphorylation state of the FCγRIIB ITIM motifs was evaluated using an ECL-based immunoblot technique.  
**Results:** The culturing of the mouse splenocytes in the presence of the antibody chimera resulted in a dose-dependent increase in the tyrosine phosphorylation of the studied inhibitory receptor. The subsequent exposure of the same cells to IVIg and to the chimera resulted in an additive effect on the phosphorylation levels. The biological consequence of the enhanced signaling through FCγRIIB was proven by the increased suppressive effect of the chimeric antibody on the number of B lymphocytes differentiating into IgG anti-DNA antibody-producing plasma cells. F(ab')<sub>2</sub> fragments of IVIg had a similar activity to the intact preparation, whereas Fc fragments had no effect.  
**Conclusion:** This study describes a novel approach with clinical relevance for the selective silencing of the activity of autoreactive disease-associated B lymphocytes.
- PD11/24 IGG1 HEAVY CHAIN-CODING GENE POLYMORPHISM (G1M ALLOTYPES) AND DEVELOPMENT OF ANTIBODIES-TO-INFLIXIMAB**  
 C. Magdelaine-Beuzelin<sup>1,2,3</sup>, S. Vermeire<sup>4</sup>, M. Goodall<sup>5</sup>, F. Baert<sup>6</sup>, M. Noman<sup>4</sup>, G. Van Assche<sup>4</sup>, M. Ohresser<sup>2,7</sup>, D. Degenne<sup>2,3</sup>, J. M. Dugoujon<sup>8</sup>, R. Jefferis<sup>5</sup>, P. Rutgeerts<sup>4</sup>, M.-P. Lefranc<sup>9</sup>, H. Watier<sup>1,2,3</sup>  
<sup>1</sup>Université François Rabelais, Tours, France, <sup>2</sup>CNRS UMR 6239, Tours, France, <sup>3</sup>Centre Hospitalier et Universitaire, Immunologie et Centre Pilote de Suivi Biologique des Anticorps Thérapeutiques, Tours, France, <sup>4</sup>University Hospital Gasthuisberg, Gastroenterology, Leuven, Belgium, <sup>5</sup>University of Birmingham, Birmingham, United Kingdom, <sup>6</sup>Hartziekenhuis Hospital, Gastroenterology, Roeselare, Belgium, <sup>7</sup>Université François Rabelais, Tours, France, <sup>8</sup>Université Paul Sabatier, Anthropologie Moléculaire et Imagerie de Synthèse, FRE 2960, CNRS, Toulouse, France, <sup>9</sup>Université Montpellier 2, IMGT, Institut de Génétique Humaine UPR CNRS 1142, Montpellier, France  
**Objective:** The chimeric anti-TNF-α antibody infliximab is known to induce antibodies-to-infliximab (ATI) in some treated patients. Immunogenicity in murine variable domains is expected; however, constant domains of its human heavy γ1 chain may also be implicated since it expresses G1m1 and G1m17 allotypes. This allelic form may be immunogenic in patients that are homozygous for the G1m3 allotype commonly expressed in Caucasian populations.  
**Methods:** Because G1m allotypic divergence may explain the presence of ATI or may influence their concentration, a genotyping method was developed and validated to determine antithetical (*i.e.* mutually exclusive) G1m3 and G1m17 allotypes (amino acid 120 of CH1 according to the IMGT unique numbering) at theIGHG1 gene level (CH1 359g/a nucleotide polymorphism). 245 blood donors and 118 previously described patients suffering from Crohn's disease, treated with infliximab, and having developed ATI in 73 of them, were genotyped.  
**Results:** TheIGHG1 CH1 359g/a polymorphism does not depart from Hardy-Weinberg equilibrium in the control population, and allele frequencies were similar in controls and patients. No association was found between the patient G1m allotypes and the presence of ATI or their concentration. It remains possible that anti-Gm1 antibodies are not well detected by the ELISA used for ATI detection and/or that the G1m allotypes are minor antigens on IgG1.  
**Conclusions:** TheIGHG1 polymorphism does not seem to play a major role in the induction of ATI. Further analyses will be required to determine if it is also the case for humanized or fully human antibodies bearing the same G1m allotypes.
- PD11/25 TARGETING THE LYMPHOCYTE ECTO-ENZYME CD38 WITH SINGLE DOMAIN ANTIBODIES FROM SHARK AND LLAMA**  
 K. Juárez<sup>1</sup>, M. Unger<sup>1</sup>, A. Licea<sup>2</sup>, F. Lund<sup>3</sup>, H.-C. Lee<sup>4</sup>, F. Goldbaum<sup>5</sup>, F. Haag<sup>1</sup>, F. Koch-Nolte<sup>1</sup>  
<sup>1</sup>Universitätsklinikum Hamburg-Eppendorf, Immunologie, Hamburg, Germany, <sup>2</sup>CICESE, Marine Biotechnology, Ensenada, Mexico, <sup>3</sup>Trudeau Institute, New York, United States, <sup>4</sup>University of Hong Kong, Hong Kong, China, <sup>5</sup>Fundacion Instituto Leloir, Buenos Aires, Argentina  
 Besides conventional antibodies, camelids and sharks have independently evolved exceptional antibodies composed only of heavy chains. The variable domains of these antibodies, designated VHH and VNAR, respectively, exhibit a high degree of chemical and thermal stability and are easily produced as recombinant single domain antibodies (sdAbs) (1). VHHs and VNARs possess an unusually long CDR3 able to penetrate into cavities or crevices on proteins, e.g. the active site of enzymes. Such sdAbs can serve as novel tools to specifically inhibit lymphocyte ecto-enzymes (2).  
 The ecto-NADase CD38 is the predominant hydrolase of extracellular NAD<sup>+</sup> and regulates lymphocyte effector functions by producing second messengers that mobilize intracellular calcium reservoirs. CD38 also acts as an antagonist of NAD-dependent ecto-ADP-ribosyltransferases (ARTs, CD296, CD297) by restricting their access to the substrate NAD<sup>+</sup>. Clinically, CD38 is a prognostic marker for Chronic Lymphocytic Leukemia.  
 Our goal is to isolate CD38-specific sdAbs from phage-display libraries derived from immunized sharks and llamas immune libraries. High diversity phage display libraries were obtained from immunized shark and llamas against murine and human CD38, respectively. Several rounds of selection were performed on immobilized CD38 and/or CD38-expressing cell lines. CD38-specificity of enriched phages was verified by ELISA and FACS. CD38 specific sdAbs were produced and purified from *E. coli* periplasm. We are currently testing the capacity of the purified sdAbs to inhibit CD38 activity.  
 1) Holliger P, Hudson PJ. 2005. Engineered antibody fragments and the rise of single domains. Nat Biotechnol. 23:1126-1136.  
 2) Koch-Nolte F, Reyelt J, Schössow B, Schwarz N, Scheuplein F, Rothenburg S, Haag F, Alzogaray V, Cauerhff A, Goldbaum FA. 2007. Single domain antibodies from llama effectively and specifically block T cell ecto-ADP-ribosyltransferase ART2.2 in vivo. FASEB J. 21:3490-3498.
- PD11/26 INDUCTION OF TOLERANCE TO THERAPEUTIC FVIII BY MATERNO-FETAL TRANSFER IN A HEMOPHILIA A MOUSE MODEL**  
 Y. Meslier<sup>1</sup>, S. André<sup>2</sup>, M. Teyssandier<sup>2</sup>, S. Kaveri<sup>2</sup>, S. Lacroix-Desmazes<sup>2</sup>  
<sup>1</sup>INSERM, U872, Paris; Centre de Recherche des Cordeliers, Université Pierre et Marie Curie – Paris 6, UMR S 872, Immunolgy, Paris, France, <sup>2</sup>INSERM, U872, Paris; Centre de Recherche des Cordeliers, Université Pierre et Marie Curie – Paris 6, UMR S 872; Université Paris Descartes, UMR S 872, Immunology, Paris, France  
 Hemophilia A is an X chromosome-linked recessive hemorrhagic disorder that is characterized by impaired factor VIII (FVIII) production. Bleeding episodes can be prevented or treated by replacement therapy using plasma-derived or recombinant FVIII. However, administration of exogenous FVIII induces, in up to 30% of the patients, an alloimmune response and the occurrence of FVIII-inhibiting immunoglobulin G. These anti-FVIII IgG inhibit the pro-coagulant activity of FVIII, and thus preclude the further use of therapeutic FVIII. Since patients with alloantibodies become resistant to conventional replacement therapy, the development of inhibitors is a major therapeutic challenge. The aim of our work is to induce tolerance to therapeutic FVIII by the materno-fetal transfer of FVIII-IgG1 fusion proteins. In mice, this transfer is effected by transcytosis during pregnancy through the placenta, and after birth through the intestinal epithelium during lactation. Transcytosis across the placenta and the intestinal epithelium is performed by the MHC class I-related receptor Fc neonatal receptor (FcRn). The approach includes i) the generation of chimeric molecules that include different domains of FVIII and the Fc fragment of mouse IgG1, ii) the injection of the chimeric proteins into

pregnant and lactating FVIII-deficient mice, iii) the determination of the ability of the offspring to tolerate FVIII at adulthood. We will characterize the mechanisms of tolerance to FVIII: central and/or peripheral tolerance, T and/or B cell tolerance, and persistence of the tolerance in the absence of antigen challenge or during prophylactic FVIII administration.

# PD11/27 SUBSTANDARD IMMUNOGLOBULIN AS A SOURCE OF PASSIVE ANAPHYLACTIC REACTION

P. Zdziański<sup>1</sup>, J. Majda<sup>2</sup>

<sup>1</sup>Lower Silesian Center for Cellular Transplantation & National Bone Marrow Donor Registry, <sup>2</sup>Silesian Center for Cellular Transplantation, Institute of Immunology & Experimental Therapy, Clin. Immunol, Wrocław, Poland, <sup>3</sup>4th Military Hospital, Laboratory, Wrocław, Poland

Drugs save lives and prevent diseases only if they are safe, efficacious, of good quality and are used rationally. It is therefore essential to identify pharmaceutical products, which, by virtue of pharmacological properties, are likely to provoke allergy.

In this report the serious allergic reaction (local and generalized) after subcutaneous infusion of immunoglobulin was described.

46-years old woman with common variable immunodeficiency was qualified to immunoglobulin replacement therapy. Unexpectedly oedema, confluent wheals and erythema in the place of infusion subcutaneous immunoglobulin (ScIg) was observed. Early and late phase reaction occurs (20 min and 6–12 h after infusion). Serum regain level was undetectable before and 18 IU/ml after ScIg infusion. So we test the IgE concentration in a series of drug samples. It was between 138 and 232 IU/ml. Specific IgE (UNICAP 100 PHARMACIA) was within wide range from 198 (mix of food) to 2809 kUA/l (mix of grass), but much of allergen-specific IgE tested was class 2 or 3 (i.e. 0.71–17.5 kUA/l). Further, allergic rhinoconjunctivitis and acute urticaria developed later, after allergen exposition (when she goes to the country). The history resembles classical passive cutaneous anaphylaxis and Prausnitz-Küstner (PK) reaction.

This observation indicates that the anaphylactic reactions during immunoglobulin replacement therapy may be due to the IgE impurity of the substandard commercial immunoglobulin product. Until now the anaphylactic reaction during immunoglobulin administration has been misinterpreted as a drug allergy (i.e. specific immune reaction to component(s) of pharmaceutical product). These reactions, especially IgA anaphylactoid reactions, are probably overdiagnosed.

This report indicates that subcutaneous immunoglobulin administration as an option to treat at home is controversial.

# PD11/28 NK CELL-BASED ASSAY FOR EVALUATING INTERACTION OF THERAPEUTIC MONOCLONAL ANTIBODIES WITH FcγRIIIa

A. Bolzec<sup>1</sup>, N. Congy-Jolivet<sup>1</sup>, C. Javaud<sup>2</sup>, V. Carre<sup>2</sup>, H. Watier<sup>1</sup>, G.J. Thibault<sup>1,3</sup>

<sup>1</sup>University of Tours, UMR CNRS 6239, Faculty of Medicine, Lab of Immunology, Tours, France, <sup>2</sup>Glycode SA, Uzerche, France, <sup>3</sup>University of Auvergne-Clermont1, Clermont-Ferrand, France

**Objectives:** Pharmacogenetic studies have demonstrated that Fc/FcγRIIIa interactions are highly involved in the clinical responses to several therapeutic monoclonal antibodies (tmAbs). Engineering the Fc portion and/or selecting the patients with the higher effector cell responses may be proposed to improve tmAbs efficacy. The first objective was to evaluate the Fc-dependent functions of tmAbs in order to propose a tool to be used in their development and quality control. The second objective was to evaluate the inter-individual variability of NK cell responses to tmAbs.

**Methods:** purified NK cell CD107 expression, IFN-γ synthesis and CD16 down-modulation induced by plate bound tmAbs have been evaluated by flow cytometry.

**Results:** CD107 expression, IFN-γ synthesis and FcγRIIIa down-modulation induced by ten commercially available tmAbs were highly variable. The differences observed between the tmAbs were partially dependent on their affinity for FcγRIIIa expressed on NK cells. CD107/IFN-γ and FcγRIIIa down-modulation revealed the very high inter-individual variation of NK cell responses from eighteen healthy donors. This variation depends on the FcγRIIIa-V158F polymorphism of the donor but it is still important within each genotype.

**Conclusion:** These results warrant further studies to evaluate the ability of this method to predict the clinical response in tmAbs-treated patients (theranostic test).

# PD11/29 CXCR5 AS THERAPEUTIC TARGET IN NON-HODGKIN'S B CELL LYMPHOMA AND AUTOIMMUNE DISEASES

M. Koch<sup>1</sup>, F. Oden<sup>1</sup>, H. Panjideh<sup>1</sup>, F. Wilde<sup>1</sup>, J. Rossbacher<sup>1</sup>, M. Lipp<sup>1</sup>

<sup>1</sup>Max-Delbrück-Center for Molecular Medicine, Department of Molecular Tumor Genetics and Immunogenetics, Berlin, Germany

At least 22 antibodies for therapeutic use are currently on the market and many more are under clinical study. While the majority is based on unmodified IgG molecules, numerous mono-, bi-, tri- and tetraspecific antibody constructs are under current development.

This work is focusing on the homeostatic chemokine receptor CXCR5. Being expressed on mature recirculating B cells, follicular B helper T cells (TFH) and a subset of memory T cells, CXCR5 is essentially involved in the development and maintenance of tissue microarchitecture of secondary lymphoid organs. In the germinal center, CXCR5 expressing antigen-specific TFH cells initiate proliferation of B cells specific for the same antigen and therefore are suspected to play a central role in the formation of autoreactive lymphoid structures and the continuous synthesis of (auto) antibodies in these structures. Elimination or functional inactivation of TFH cells may especially be suited for the therapy of chronic inflammatory and autoimmune diseases. Furthermore, CXCR5 is highly expressed on 90% of Non-Hodgkin's B cell lymphoma (NHL). In both cases the chemokine receptor CXCR5 might serve as a suitable therapeutic target.

For this purpose the quadroma technique offers the possibility to generate hybrid hybridomas producing bispecific antibodies (bspAb). With the attraction of effector cells, destruction of the linked target cells can be enforced when bspAb is applied. In addition, its intact Fc portion is capable of recruiting FcγRI bearing accessory cells such as dendritic cells, macrophages and natural killer cells releasing activating costimulatory signals.

After purification of the bspAb, cytotoxicity assays are carried out *in vitro*. Further investigation of therapeutic effects *in vivo* in murine tumor and autoimmune models will follow. Eventually, chimerization or humanization of the Ab constructs will reduce immunogenicity and therefore be of high interest for clinical applications.

# PD11/30 ON THE ROLE OF MONOCLONAL ANTIBODY AFFINITY IN MEDIATING PROTECTION AGAINST AUTOIMMUNE INFLAMMATORY DISEASES

K. Dallenbach<sup>1</sup>, R.R. Beerli<sup>1</sup>, T.A. Röhn<sup>1</sup>, M.F. Bachmann<sup>1</sup>

<sup>1</sup>Cytos Biotechnology AG, Schlieren, Switzerland

Monoclonal antibodies (mAbs) have recently emerged as new drug modalities for the treatment of chronic inflammation. Indeed, blocking cytokines, such as TNF, using mAbs has been established as disease-modifying therapy for inflammatory diseases including rheumatoid arthritis and psoriasis.

It is generally assumed that mAbs need to have a high affinity for the target cytokine in order to show efficacy. However, no study has ever directly addressed this issue. To elucidate this question, we have generated a panel of mouse IL-17-specific mAbs. Variable regions of selected hypermutated high affinity anti-IL-17 antibodies will be mutated back to germline sequence, which is expected to lower their affinity for IL-17. The ability of these antibodies, which recognize the same epitope with different affinities, to block chronic inflammation will subsequently be tested in two murine models of autoimmunity: collagen-induced arthritis (CIA) and experimental autoimmune encephalomyelitis (EAE). To estimate the importance of antibody-induced immune complex formation for removal of the cytokine from the circulation, cocktails of antibodies recognizing different epitopes on IL-17 will be compared to single antibody treatment. These experiments will reveal the role of antibody affinity versus their ability to induce immune complex formation for the treatment of autoimmune diseases.

# PD11/31 PRECLINICAL CHARACTERIZATION AND VIRAL SAFETY OF A NEW HUMAN PLASMA- DERIVED LIQUID 20 PERCENT IMMUNOGLOBULIN CONCENTRATE FOR SUBCUTANEOUS ADMINISTRATION

W. Teschner<sup>1</sup>, H.A. Butterweck<sup>1</sup>, G. Pölsler<sup>1</sup>, T.R. Kreil<sup>1</sup>, A. Weber<sup>1</sup>, E.-M. Muchitsch<sup>1</sup>, H.J. Ehrlich<sup>1</sup>, H.-P. Schwarz<sup>1</sup>

<sup>1</sup>Baxter Innovations GmbH, Wien, Austria

**Objective:** Preclinical data of a new plasma-derived 20 percent liquid human immunoglobulin preparation for subcutaneous administration are presented.

**Methods:** For manufacturing the process scheme of KIOVIG, Baxter's 10 percent intravenous liquid immunoglobulin product was followed except for the final concentration step. The purity of the product was determined by cellulose acetate electrophoresis and enzyme-linked immunosorbent assays (ELISAs), and the molecular size distribution by high-performance size-exclusion chromatography (HP-SEC). Antibody titers against viruses were tested by ELISA and haemagglutination. The guinea pig protection test was used to measure the *Corynebacterium diphtheriae* antibody titer, a model for antibacterial antibodies.

In down-scaled models, process intermediates were used to determine the viral reduction capacity of cold ethanol fractionation, solvent-detergent (SD) treatment, nanofiltration and incubation at low pH and elevated temperature.

Local tolerability was assessed in rabbits after subcutaneous administration. Pharmacokinetic parameters were evaluated in dogs after a single subcutaneous injection.

**Results:** The manufacturing process consistently removed non-IgG proteins as shown by a gammaglobulin content of  $\geq 99\%$  and very low IgA or IgM levels in the final product. A broad spectrum of antibodies in high titers was found. HP-SEC showed that the IgG monomer and dimer content is greater than 99 percent. Cold ethanol fractionation effectively removed all viruses except bovine viral diarrhoea virus; lipid-enveloped viruses were inactivated by Baxter's SD treatment; the nanofiltration step effectively removed viruses which were greater than the filter pore size but also small viruses when specific antibodies were present in the antibody solution. The virus inactivation capacity of low pH treatment at elevated temperature was shown to be virtually independent of the protein concentration in a range of 10 to 21 percent.

The new 20 percent product and KIOVIG show similar tolerability in rabbits. Subcutaneous application in dogs was excellently tolerated and resulted in a lower maximum concentration, slower release and an apparently longer elimination half-life than with intravenous administration.

**Conclusion:** The new 20 percent liquid subcutaneous immunoglobulin has an excellent viral safety profile, an unprecedented high purity and a nonclinical safety profile similar to the marketed product KIOVIG.



**PD11/32 THERAPEUTIC PROTEINS : ASSESSING THE RISK OF “UNWANTED” IMMUNOGENICITY**S. Cornet<sup>1</sup>, K. Heynink<sup>1</sup>, S. Pattijn<sup>1</sup>, Y. Gansemans<sup>1</sup>, S. De Pauw<sup>1</sup>, D. D'Halluin<sup>1</sup>, K. Moens<sup>1</sup>, V. Goossens<sup>1</sup>  
<sup>1</sup>Algonomics, Epibase IV, Gent, Belgium

Therapeutic proteins are, to a variable extent, immunogenic. Formation of antidrug antibodies poses a risk, as it possibly compromises drug safety and alters pharmacokinetics. The generation of these antibodies is driven by T helper cell epitopes.

Epibase<sup>®</sup> is a structure-based *in silico* method to identify T cell epitopes. The correlation between experimental and predicted values is higher for the structure-based Epibase<sup>®</sup> than for sequence-based methods. *In silico* T cell epitope prediction is a valuable tool during lead selection.

Epibase IV performs T-cell immunogenicity screenings *in vitro*, providing high content read outs, using multi-parameter flow cytometry and ELISPOT technology. Immune responses (expression of activation markers, proliferation, cytokine secretion) to antigens in PBMC of healthy, naïve donors are measured in order to document the immunogenicity of a product before its first clinical dose in humans.

Two levels of heterogeneity for the evaluation of the immunogenicity of an antigen (protein or peptide) in a human donor population is taken into account:

1. The HLA distribution of the target population and thus the probability that a peptide-HLA interaction can take place and results in presentation to T-cells.
2. The probability of a peptide/HLA complex to be recognized by antigen-specific T-cells, defined by the T-cell precursor frequency in the donor sample.

The Epibase IV immunogenicity screening strategy consists of selecting a minimum of 50 donor samples representing the HLA-distribution of the target population, optimization of product-specific assay parameters (dose, formulation,...) and subsequent full population screening. Statistical data analysis on the multiple parameter data set results in the identification of responsive donors using pre-defined thresholds and a comparison of the average immune responses over the population. Immunogenicity of the product of interest is described in reference to control proteins (Tetanus Toxoid and KLH) or peptides (Recall peptides and Pan DR sequences) and/or product-specific benchmarks.

**PD11/33 FIRST EFFECTIVE NEUTRALISATION OF WHOLE SCORPION VENOM BY A BISPECIFIC NANOBODY**I. Hmila<sup>1</sup>, D. Saerens<sup>2</sup>, R. Ben Abderrazek<sup>1</sup>, C. Vincke<sup>2</sup>, N. Abidi<sup>1</sup>, Z. Benlasfar<sup>3</sup>, H. Dabbek<sup>4</sup>, M. El Ayeb<sup>1</sup>, S. Muyldermans<sup>2</sup>, B. Bouhaouala-Zahar<sup>1</sup>

<sup>1</sup>Institute Pasteur of Tunis, Laboratory of Venoms and Toxines, Tunis, Tunisia, <sup>2</sup>Vrije Universiteit Brussel, Laboratory of Cellular and Molecular Immunology, Brussels, Belgium, <sup>3</sup>Institute Pasteur of Tunis, Unité des services animaliers, Tunis, Tunisia, <sup>4</sup>Centre Régional de Production Animale de Kébili, Kebili, Tunisia

**Objectives:** Our aim is to ameliorate the unsatisfactory current horse serum anti scorpion venom therapy with a novel format of antibody fragments. The introduction of Nanobodies, the variable fragments derived from the heavy chain only antibodies of dromedary, constitute a new therapeutic compound with a reproducible toxin neutralizing capacity, avoiding the repeated immunisations of horses and the inter batch variations. Nanobodies with MW of 15 kDa will also penetrate in tissues more readily than the horse Fab'2 (100 kDa) to reach more rapidly the highly diffusible scorpion toxins (7kDa).

**Methods:** We constructed two toxin-specific Nanobody libraries from two immunized dromedaries. The binders to the two most toxic compounds within the scorpion venom were retrieved from these libraries by phage display technology. The neutralisation capacity of the various Nanobodies was monitored in mice. The best performing Nanobody for each of the two antigenic groups of toxins were used to generate a bispecific Nanobody construct (30 kDa) separated by the structural upper hinge of a natural llama antibody. The neutralizing capacity of this bispecific Nb was assessed in mice for its ability to neutralize the whole venom and its capacity to protect mice when the venom and the antidote are injected separately via distinct routes.

**Results:** Three consecutive rounds of panning were performed for each library to select specific binders against toxin I. A panel of 13 Nanobodies was obtained that recognize this toxin. Remarkably, the neutralising capacity of NbAahIF12 was exceptional strong as it neutralises more than 100 LD50 when administered by intracerebroventricular route. This NbAahIF12 was tethered to NbAahII10, selected previously and raised against the toxin II group. This NbF12-10 bispecific construct displays a significant neutralising capacity of the crude venom (containing both toxins) that exceeds the current Fab'2 based horse antivenom. Importantly, the bispecific construct injected intravenously 15 minutes after a subcutaneously administered toxic dose of the venom protects the mouse.

**Conclusion:** The neutralising and the protective capacity of our bispecific NbF12-10 demonstrate that this next-generation immunotherapeutic agent for scorpion envenoming in humans could replace the current serotherapeutic Fab'2 derived horse sera as it is safer and better.

**PD11/34 DEVELOPMENT OF SARCOIDOSIS DURING ETANERCEPT THERAPY; A REPORT OF 3 CASES**I.M. Skoie<sup>1</sup>, K. Wildhagen<sup>1</sup>, R. Omdal<sup>1</sup>

<sup>1</sup>Stavanger University Hospital, Internal Medicine, Clinical Immunology Unit, Stavanger, Norway

After over 10 years use of TNF- $\alpha$  inhibitors, their side effects and complications are reasonably well-documented. Recently, however, occurrences of granulomatous reactions have been reported and the TNF- $\alpha$  receptor protein, etanercept has been the culprit in most of these cases. This is intriguing because the TNF- $\alpha$  antibody drug infliximab have shown promising results in treatment of sarcoidosis whereas etanercept has failed in this matter. Because of different mechanisms of action, questions have been raised regarding etanercept's possible unique role in promoting sarcoidosis or whether this is a class effect of all TNF- $\alpha$  inhibitors. We present 3 patients who developed sarcoidosis while on etanercept treatment and discuss if possible differences in cytokine profiles and T regulatory (T<sub>reg</sub>) cell function in patients taking different TNF- $\alpha$  inhibitors may explain this paradox. The granulomas in sarcoidosis consist of epitheloid cells like macrophages and giant cells surrounded by mostly CD4+ T cells. Cytokines such as TNF- $\alpha$  and IFN- $\gamma$  appear to be of crucial importance for the formation and continuation of granulomas. T<sub>reg</sub> cells surround the T-cells and suppress their proliferation and cytokine production. Recent studies in RA patients suggest impaired T<sub>reg</sub> cells function, and treatment with infliximab appears to restore the number and function of these cells. T<sub>reg</sub> cells are also shown to be down regulated in sarcoidosis. It is known that TNF- $\alpha$  inhibits the suppressive function of T<sub>reg</sub> cells. Suppression of TNF- $\alpha$  is greater and more prolonged with infliximab and adalimumab. Etanercept treatment maintains low levels of TNF- $\alpha$  and therefore T<sub>reg</sub> cells remain down-regulated to some extent compared with infliximab which more or less completely inhibits TNF- $\alpha$  and IFN- $\gamma$ . It has been documented that infliximab and adalimumab suppress IFN- $\gamma$  production while etanercept does not. If the presence of IFN- $\gamma$  and TNF- $\alpha$  is essential for granuloma formation and these cytokines are preserved with etanercept treatment, the patient may be more susceptible to develop sarcoidosis compared with treatment with the monoclonal antibodies. The effects of TNF- $\alpha$  antagonists in the inhibition or induction of granulomatous process are complex. Understanding these interactions may shed light on the pathophysiology of sarcoidosis and other inflammatory disorders.

**PD15 – LYMPHOPROLIFERATIVE AND MYELOPROLIFERATIVE DISORDERS****PD15/1 IDENTIFICATION OF MALT1 PROTEASE ACTIVITY AS A POTENTIAL THERAPEUTIC TARGET FOR ACTIVATED B CELL-LIKE SUBTYPE OF DIFFUSE LARGE B CELL LYMPHOMA**U. Ferch<sup>1</sup>, B. Kloos<sup>1</sup>, V. Pfander<sup>1</sup>, D. Krappmann<sup>2</sup>, J. Ruland<sup>1</sup>

<sup>1</sup>Technical University Munich; Klinikum Rechts der Isar, III Medical Department, Munich, Germany, <sup>2</sup>German Research Center for Environment and Health, Institute of Toxicology, Neuherberg, Germany

**Objectives:** Diffuse large B cell lymphoma (DLBCL) is the most common type of non-Hodgkin's lymphoma. Two important subtypes are the “germinal center B cell-like” (GCB) and the “activated B cell-like” (ABC) subtype. Following standard immunochemotherapy, the ABC DLBCL subtype shows a more aggressive clinical behavior compared to GCB DLBCL. ABC DLBCL cells depend on aberrant signalling to nuclear factor- $\kappa$ B (NF- $\kappa$ B) for survival, which is mediated by constitutive activity of the CARMA1/BCL10/MALT1 (CBM) complex. Recently, MALT1 has been demonstrated to exhibit proteolytic activity, which is required for full antigen receptor mediated NF- $\kappa$ B activation in T cells. Here we tested, whether MALT1 exhibits proteolytic activity in DLBCL and whether MALT1 protease inhibition could present a potential therapeutic target.

**Methods:** Using well characterized cell lines of the ABC and the GCB subtype, we analyzed the cleavage of substrates of MALT1 by immunoblot. MALT1 protease activity was inhibited by treatment with the tetrapeptide inhibitor zVRPR-fmk. The impact on NF- $\kappa$ B activity was investigated by gelshift assay and subcellular fractionation experiments. Moreover, the effect of MALT1 inhibition on DLBCL proliferation and survival was addressed by flow cytometry.

**Results:** Here we provide evidence that MALT1 protease is constitutively active in ABC DLBCL cells, as all tested ABC DLBCL cell lines exhibit cleavage of the known substrates of MALT1, namely BCL10 and the NF- $\kappa$ B inhibitor A20. Moreover, treatment of ABC-DLBCL cell lines with the MALT1 inhibitor zVRPR-fmk led to decreased NF- $\kappa$ B activity and downregulated expression of the NF- $\kappa$ B-dependent prosurvival gene Bcl-x<sub>L</sub>. In addition, zVRPR-fmk impaired survival and proliferation of all tested ABC DLBCL lines, resulting in growth inhibition. Importantly, these effects were specific for ABC DLBCL, as cell lines of the NF- $\kappa$ B-independent GCB DLBCL subtype showed only a moderate reduction in total cell numbers after prolonged treatment with zVRPR-fmk.

**Conclusion:** These results indicate that pharmacological inhibition of MALT1 protease activity could become a potential therapeutic approach for the specific treatment of ABC DLBCL, which is optimally adjusted to the molecular pathogenic mechanisms.

**PD15/2 IL-21 AND IL-15 DIFFERENTIALLY REGULATE GENE EXPRESSION IN B-CHRONIC LYMPHOCYTIC LEUKEMIA (CLL) CELLS**L. De Cecco<sup>1</sup>, M. Capaia<sup>2</sup>, S. Zupo<sup>2</sup>, G. Cutrona<sup>2</sup>, S. Matis<sup>2</sup>, A. Brizzolara<sup>2</sup>, A. M. Orenzo<sup>2</sup>, S. Fabbri<sup>2</sup>, M. Ferrarini<sup>2</sup>, S. Canevari<sup>1</sup>, S. Ferrini<sup>2</sup>

<sup>1</sup>Fondazione IRCCS “Istituto Nazionale dei Tumori”, Milan, Italy, <sup>2</sup>Istituto Nazionale per la Ricerca sul Cancro, Genoa, Italy

**Objectives:** IL-21 and IL-15 are members of the IL-2 cytokine family, displaying opposite functions on CLL-B cells. IL-15 mediates proliferation and survival, whereas IL-21 induces apoptosis with a slow kinetics, suggesting involvement of gene regulation. Therefore we studied their ability to modulate gene expression in CLL cells.

**Methods:** CLL-B cells from 13 patients were pre-activated on adherent CD40L-transduced L cells and then stimulated with IL-15, IL-21 or medium only. Total RNA was *in vitro* amplified, labeled with biotin and hybridized on Sentrix Bead Chip HumanRef-8-v2 (Illumina, San Diego, CA). The array contains over 22,000 probes

types, representing 18,196 unique genes derived from UniGene database. Intensity values were quality checked and the data set was normalized using the quantile algorithm and BeadStudio Version 3 software. For each gene, a detection p value of < 0.05 was set and 50% of missing values were allowed as a cutoff to filter the reliable data, yielding an expression matrix containing 12,070 probe types.

**Results:** IL-15 significantly ( $p < 0.001$ ) modulated 102 genes (69 up and 33 down) whereas IL-21 modulated a greater number of genes (2095, among which 124 at a fold change > 2). Thirty-eight genes showed a concordant modulation by IL-21 and IL-15. Among these genes some encode for cytokines receptors (IL2RA, IL2RB, IL15RA up and IL13RA1 down) or cytokines (TNFSF10 and IFNG up). Also GMZB, which has been previously implicated in IL21-mediated apoptosis, was up-regulated by both IL-15 and IL-21. Twenty-seven genes showed an opposite modulation, in particular the chemokines CCL3 and CCL4 and the cytokine LTB (all up in IL-15 and down in IL-21-treated cells). GO clustering showed that IL-21 modulates several genes involved in cell cycle and in mitochondrial processes, which will be further studied to identify IL-21-apoptotic pathways.

**Conclusions:** The differential modulation of some genes by IL-21 and IL-15 may reflect their activation of different signaling pathways (JAK3/STAT3 and JAK1/STAT1 for IL-21 and JAK1/STAT5 for IL-15). IL-21 and IL-15 modify the expression of several chemokine genes that are thought to be relevant for CLL biology. GMZB is not a likely candidate for IL-21-mediated apoptosis in CLL.

PD15/3

#### EXPRESSION AND FUNCTION OF TOLL-LIKE RECEPTORS IN CHRONIC LYMPHOCYTIC LEUKEMIA CELLS

M. Muzio<sup>1</sup>, C. Scielzo<sup>1</sup>, S. Bertilaccio<sup>1</sup>, M. Frenquelli<sup>1</sup>, G. Simonetti<sup>1</sup>, B. Apollonio<sup>1,2</sup>, P. Ghia<sup>1,2</sup>, F. Caligaris-Cappio<sup>1,2</sup>  
<sup>1</sup>San Raffaele Scientific Institute, Molecular Oncology, Milano, Italy, <sup>2</sup>Vita-Salute San Raffaele University, Milano, Italy

Mature B-lymphocytes can recognize microbial antigens via B-cell-receptor (BCR) in a specific way and via Toll-like receptors (TLR) in a costimulatory manner. A wealth of information is gathering on the possible role of antigenic stimulation in the natural history of Chronic Lymphocytic Leukemia (CLL), a chronic lymphoid malignancy characterized by the accumulation of CD5+ monoclonal B lymphocytes in primary and secondary lymphoid tissues. However little is known regarding the repertoire and function of TLR in CLL cells. The TLR family includes 10 different transmembrane proteins devoted to recognize specific pathogen-associated molecular patterns and to alarm immunocompetent cells to trigger an immune response. Here, we studied fresh leukemic cells for the expression pattern of TLR1 to TLR10, NOD, RP105 and SIGIRR (also known as TIR8). CLL cells were found to express several pattern recognition receptors including TLR1, TLR2 and TLR6. The specific TLR expressed by CLL cells were functional. Leukemic cells, upon stimulation with TLR1/2/6 ligands, such as bacterial lipopeptides, activated the nuclear factor-kappaB signalling pathway, expressed CD86 and CD25 activation molecules, and were protected from spontaneous apoptosis. These findings further support the hypothesis that CLL cells resemble antigen-activated B-cells and suggest a potential role of TLR in modulating CLL cell response in the context of specific antigen recognition.

PD15/4

#### HUMAN RED BLOOD CELLS CARRY T CELL GROWTH AND SURVIVAL BIOACTIVITIES THAT ARE DIFFERENTLY MODULATED BY CYCLOSPORINE A AND RAPAMYCIN

R.F. Antunes<sup>1,2</sup>, F.A. Arosa<sup>2,3</sup>

<sup>1</sup>IBMC – Instituto de Biologia Molecular e Celular, Lymphocyte Biology, Porto, Portugal, <sup>2</sup>ICBAS, University of Porto, Porto, Portugal, <sup>3</sup>Instituto de Biologia Molecular e Celular, Lymphocyte Biology, Porto, Portugal

Red blood cells (RBC) have emerged as a novel regulatory cell type endowed with bioactivities towards human peripheral blood T cells activated in vitro with TCR-dependent stimuli. Here, we show that the RBC bioactivities act on intracellular pathways initiated by TCR-dependent and TCR-independent stimuli capable of driving T cells into the cell cycle, including IL-15 and the mixture of phorbol dibutyrate and calcium ionophore A23187. RBC bioactivities specifically inhibit apoptosis pathways related with oxidative stress while enhancing cell cycle progression. The RBC bioactivities are not mediated by accessory cells and are able to rescue normal and leukemic T cells from cell death induced by serum deprivation. By using cyclosporine A (CsA) and rapamycin (Rapa), two known immunosuppressors, we show that the bioactivities carried by RBC appear to deliver signals to activated T cells that converge on the calcineurin and, to a lesser extent, mTOR pathways. Thus, preincubation of RBC with CsA, but not with Rapa, almost completely abolished their capacity to enhance T cell division. Although treatment of RBC with Rapa slightly decreased their capacity to enhance T cell proliferation, their capacity to inhibit T cell apoptosis was unexpectedly augmented. These results show for the first time that RBC are carriers of T cell growth and survival bioactivities that appear to be modulated differently by the action of CsA and Rapa, which may have implications in clinical situations where these immunosuppressors are administered.

PD15/5

#### THE LOCAL MICROENVIRONMENT PLAYS A DIFFERENT ROLE IN SUPPORTING CHRONIC LYMPHOCYTIC LEUKEMIA (CLL) CELLS SURVIVAL ACCORDING TO THE MUTATIONAL STATUS OF THE TUMOR IMMUNOGLOBULIN HEAVY CHAIN VARIABLE REGION (IGVH)

F. Pantaleoni<sup>1</sup>, M. Coscia<sup>1</sup>, C. Riganti<sup>1</sup>, M. Rigoni<sup>1</sup>, S. Peola<sup>1</sup>, C. Vitale<sup>1</sup>, D. Drandi<sup>1</sup>, M. Ladetto<sup>1</sup>, A. Bosia<sup>1</sup>, M. Boccadoro<sup>1</sup>, M. Massaia<sup>1</sup>

<sup>1</sup>University of Torino, Torino, Italy

**Objectives:** Chronic lymphocytic leukemia (CLL) is a clinically heterogeneous disease. The mutational status of the tumor immunoglobulin heavy chain variable region (IgVH) is a well established prognostic marker: patients with unmutated (UM) IgVH have a bad prognosis, whereas patients with mutated (M) IgVH have a good prognosis. It is currently unknown whether the tumor microenvironment has a different weight in supporting the survival of M and UM cells.

**Methods:** Purified M and UM CLL cells were cultured in the presence or in the absence of IL-4, CD40L, murine stromal cells (M2-10B4), CLL derived bone marrow stromal cells (BMSC) and autologous T lymphocytes. Apoptotic and necrotic cells were quantified by annexin V and propidium iodide staining. The expression of Bcl-2 was evaluated by flowcytometry and the nuclear translocation of NF- $\kappa$ B by EMSA.

**Results:** Purified UM CLL cells showed significantly higher apoptotic rates than leukemic cells of M patients. Spontaneous *in vitro* apoptosis of UM cells was associated with a progressive time dependent downregulation of the intracellular expression of Bcl-2 and with a complete loss of the active form of NF- $\kappa$ B. On the contrary, the higher long term viability of M CLL cells was paralleled by the maintenance of Bcl-2 and NF- $\kappa$ B expression.

We next investigated the effect of extrinsic survival factors on the apoptotic rate of UM cells. IL-4 and CD40L, alone or in combination, as well as murine and human BMSC were capable of rescuing UM tumor cells from apoptosis. The pro-survival effect of these stimuli was exerted through the upregulation of Bcl-2 and was totally independent from the recovery of NF- $\kappa$ B expression. A pro-survival effect on UM CLL cells was also exerted by autologous T cells. Indeed, in coculture experiments, the presence of sufficient numbers of T lymphocytes could prevent UM CLL cells apoptotic death by restoring the expression of NF- $\kappa$ B and Bcl-2.

**Conclusions:** These data indicate that the survival of tumor cells from UM CLL patients is dependent on the local microenvironment. On the contrary, tumor cells from M CLL patients are intrinsically more resistant to apoptosis and minimally influenced by the local microenvironment.

PD15/6

#### DOXYCYCLINE-INDUCIBLE, REVERSIBLE EXPRESSION OF THE PROTO-ONCOGENES C-MYC AND PIM-1 IN MURINE PREB AND B CELLS

C. Bouquet<sup>1</sup>, F. Melchers<sup>1</sup>

<sup>1</sup>MPI for Infection Biology, Berlin, Germany

**Objectives:** B-cell lymphomas are thought to emerge from multiple, successive translocations, deletions or mutations of different proto-oncogenes. The resulting oncogenic cell population is often clonal and shows a specific B cell maturation stage.

Two proto-oncogenes, c-myc and pim-1, known to be upregulated in certain B cell lymphomas (e.g. Burkitt's Lymphoma, diffuse large B cell lymphoma, plasmacytoma) were chosen to test their transformation-inducing capacities during B cell development *in-vitro* and *in-vivo*.

**Methods:** Self-inactivating retroviral vectors carrying doxycycline-inducible forms of c-myc and pim-1 were integrated into the genome of murine preB cells by retroviral infection. The *in-vitro* proliferation behaviour of these cells at different maturation stages was assessed.

In addition, transgenic preB cells carrying c-myc and pim-1 were transplanted in B cell deficient host mice. Influence of overexpression of these two proto-oncogenes at different timepoints of reconstitution of the B cell compartment was assessed by ELISA and FACS analysis and re-cultivation *in-vitro*.

**Results:** Fetal liver derived preB cells only proliferate *in-vitro* in the presence of Interleukin-7 on stromal cells (OP-9). The transgenic preB cells, however, could be grown in the absence of IL-7 and stroma when the two proto-oncogenes were overexpressed in the same cell. Overexpression of only one proto-oncogene in these preB cells did not result in growth-factor independent growth.

Immature IgM+ cells developed *in-vitro* from these transgenic preB cells proliferated in the presence of supporting growth factors and doxycycline for at least two weeks, whereas untreated cells died within days.

*In-vivo*, transgenic B cells expanded in doxycycline-induced host mice compared to untreated mice. Cells isolated from doxycycline-treated mice could be cultivated *in-vitro* in the presence of doxycycline, whereas in the absence of doxycycline the cells died. Hence, doxycycline-induced, pim-1/c-myc dependent proliferation is reversible.

In marked contrast, mature naive transgenic B cells isolated from untreated transplanted mice could not be induced to proliferate or survive long-term *in-vitro* after proto-oncogene induction.

We conclude that the proliferation-inducing, transforming activity of c-myc plus pim-1 is B-cell differentiation state-specific and works in preB and immature, but not in naive mature B cells.

**PD15/7 IL-12 INHIBITS DIRECTLY THE GROWTH OF HUMAN PRIMARY ACUTE MYELOID LEUKEMIA CELLS**E. Ferretti<sup>1</sup>, C. Cocco<sup>2</sup>, E. Di Carlo<sup>3</sup>, D. Montagna<sup>4</sup>, E. Ognio<sup>5</sup>, F. Locatelli<sup>6</sup>, V. Pistoia<sup>1</sup>, I. Airolidi<sup>2</sup><sup>1</sup>G. Gaslini Institute, Laboratory of Oncology, Genoa, Italy, <sup>2</sup>G. Gaslini Institute, A.I.R.C. Tumor Immunology Unit, Department of Experimental and Laboratory Medicine, Genoa, Italy, <sup>3</sup>G. d'Annunzio University Foundation, Department of Oncology and Neurosciences, <sup>4</sup>G. d'Annunzio University and Ce.S.I. Aging Research Center, Chieti, Italy, <sup>5</sup>IRCCS Policlinico San Matteo, Pediatric Haematology/Oncology Fondazione, Pavia, Italy, <sup>6</sup>Istituto Nazionale per la Ricerca sul Cancro, Animal Model Facility, Genoa, Italy**Objectives:** Acute myeloid leukemia (AML) is a myeloid haematopoietic malignancy characterized by rapid proliferation of tumor cells and accumulation in the bone marrow. In pediatric patients with AML, the 5-year survival rates range from 40% to 50%, highlighting the need for novel therapies. In this study, we have investigated i) the expression and function of IL-12R in AML cells, ii) the direct anti-tumor activity of the cytokine on AML cells in vitro and in vivo and iii) the mechanisms involved.**Methods:** IL-12R expression in four human AML cell lines and in neoplastic cells from 14 AML patients was studied by flow cytometry. Primary AML cells and cell lines were treated with hrIL-12 and tested for apoptosis (propidium iodide/AnnexinV double staining and flow cytometric analysis), proliferation (Ki67 staining and flow cytometric analysis) and angiogenesis (CAM assay). Angiogenic genes modulated by IL-12 in primary AML cells were studied by PCR array technique. SCID-NOD mice were injected intra-peritoneum with the U937 AML cell line and treated with hrIL-12 or medium. Tumor masses from SCID-NOD mice were explanted two weeks after U937 inoculation and analyzed by immuno-histochemistry, flow cytometry and PCR array.**Results:** Neoplastic cells isolated from AML patients and AML cell lines expressed constitutively complete IL-12R. IL-12 treatment of both primary AML cells and cell lines inhibited significantly proliferation and angiogenesis in vitro, while unaffacting survival. The inhibition of angiogenesis was related to down-regulation of a wide panel of pro-angiogenic genes including CCL2, VEGF-D, VEGFR2 and neuropilin 2. Tumors formed by U937 cells in SCID/NOD mice were significantly smaller following IL-12 vs PBS treatment due to inhibition of angiogenesis and induction of apoptosis.**Conclusions:** This study demonstrates that IL-12 inhibits directly the growth of human primary AML cells and may provide a rational basis for the development of a clinical trial.**PD15/8 ACTIVE RAP1, A SMALL GTPASE THAT INDUCES MALIGNANT TRANSFORMATION OF HEMATOPOIETIC PROGENITORS, REGULATES PROTEIN EXPRESSION**M. E. Lafuente Duarte<sup>1</sup>, Y. Iwamoto<sup>2</sup>, C. V. Carman<sup>3</sup>, A. A. F. L. Van Puijenbroek<sup>4</sup>, E. Constantine<sup>1</sup>, L. Li<sup>2</sup>, V. A. Boussiotis<sup>2</sup><sup>1</sup>Universidad Complutense de Madrid. F. Medicina, Microbiología I, Area de Inmunología, Madrid, Spain, <sup>2</sup>Massachusetts General Hospital and Harvard Medical School, Transplantation Biology Research Center, Boston, United States, <sup>3</sup>Center for Blood Research, Harvard Medical School, Pathology, Boston, United States, <sup>4</sup>Dana Farber Cancer Institute, Harvard Medical School, Adult Oncology, Boston, United States

Rap1 a member of the Ras superfamily, regulates cytoskeletal changes in lower eukaryotes and integrin-mediated adhesion in hematopoietic cells. Sustained activation of Rap1 in mouse hematopoietic stem cells causes expansion of hematopoietic progenitors, followed by a myeloproliferative disorder mimicking chronic myeloid leukemia. Moreover, these mice develop a B-cell lymphoproliferative disorder resembling chronic lymphocytic leukemia. Rap1 activation has been correlated to enhanced adhesion of hematopoietic progenitors to stroma cell in the hematopoietic microenvironment. However little is known about the relation between Rap1 sustained activation and the capability to regulate gene expression.

**Objectives:** In the present study we have attempted to elucidate the potential molecular mechanisms via which active Rap1 may mediate its functional outcomes. We are interested in determining the potential role of Rap1-GTP as a regulator of the gene expression profile in the cell.**Methods:** We used HEK 293 cells as a tool to examine the molecular effects of Rap1 activation. We overexpressed the Rap1 constitutively active mutant Rap1E63 and determined the induction of potential morphological changes, the location of the activated Rap1, and the changes in the protein expression pattern of the cell.**Results:** Overexpression of a Rap1 constitutively active mutant induced morphological changes in the cell. The active form of Rap1 was localized predominantly in the nucleus and nuclear localization of endogenous Rap1-GTP was also detected upon physiologic activation. A potential consequence of nuclear localization of Rap1-GTP is the regulation of gene expression. To determine this we used a high throughput proteomic approach to identify gene products potentially modulated by Rap1-GTP. Out of 1000 proteins examined, 64 proteins were upregulated and 66 proteins were downregulated. The differentially expressed gene products belong to cytoskeletal regulator proteins, signaling molecules, transcription factors, viability regulators, and protein transporters.**Conclusions:** Sustained activation of Rap1 can induce changes in the protein expression pattern of the cells. The analysis presented provides the first fingerprint of gene product expression regulated by Rap1 and may contribute to our understanding of malignant transformation mechanisms regulated by this small GTPase.**PD15/9 FOXP3<sup>+</sup>CD25<sup>-</sup> TUMOR CELLS WITH REGULATORY FUNCTION IN SÉZARY SYNDROME**J.B. Heid<sup>1</sup>, A. Schmidt<sup>1</sup>, N. Oberle<sup>1</sup>, S. Goerd<sup>2</sup>, P.H. Krammer<sup>1</sup>, E. Suri-Payer<sup>1</sup>, C.-D. Klemke<sup>1,2</sup><sup>1</sup>German Cancer Research Center (DKFZ), Tumor Immunology Program, Heidelberg, Germany, <sup>2</sup>University Medical Center Mannheim, Ruprecht-Karls-University of Heidelberg, Department of Dermatology, Venereology and Allergy, Mannheim, GermanyCutaneous T cell lymphoma (CTCL) has been suggested by in vitro experiments to represent a malignant CD4<sup>+</sup> T cell proliferation with an inducible regulatory T cell (Treg) phenotype (CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup>). We investigated percentages of FOXP3<sup>+</sup> and CD25<sup>+</sup> cells in the blood in 15 Sézary, 14 mycosis fungoides (MF), 10 psoriasis patients, and 20 normal healthy donors (NHD). We found similar numbers of FOXP3<sup>+</sup> cells in MF (10.4% of blood CD4<sup>+</sup> cells), psoriasis (11.1%) and NHD (9.8%). In 8/15 (53%) Sézary patients significantly reduced percentages of FOXP3<sup>+</sup> cells were seen in the blood (2.9%) and skin (10.4%). Interestingly, 6/15 (40%) Sézary patients showed significantly increased percentages of FOXP3<sup>+</sup> cells (39.7% [blood], 20.3% [skin]), however, these cells did not express CD25. In these latter patients clone-specific T cell receptor-(TCR)-Vβ-chain antibodies were used to demonstrate that these FOXP3<sup>+</sup>CD25<sup>-</sup> cells were the monoclonal CTCL tumor cells. FOXP3<sup>+</sup>CD25<sup>-</sup> CTCL tumor cells showed a highly demethylated status of the foxp3 gene locus similar to Treg, and they were functionally able to suppress IL-2 mRNA induction in TCR-stimulated conventional T cells. Thus, FOXP3<sup>+</sup>CD25<sup>-</sup> CTCL tumor cells with functional features of Treg define a sub-group of Sézary patients which might carry a different prognosis and might require different treatment.**PD15/10 DIFFERENTIAL EXPRESSION OF WNT GENES IN LEUKEMIC CELLS FROM VARIOUS SUBTYPES OF ACUTE LYMPHOBLASTIC LEUKEMIA**A. Memarian<sup>1</sup>, H. Asgarian-Omran<sup>1</sup>, M. Shabani<sup>1</sup>, P. Vosough<sup>2</sup>, J. Khoshnoodi<sup>1</sup>, M. Jeddi-Tehrani<sup>3,4</sup>, H. Rabbani<sup>3,4</sup>, F. Shokri<sup>1,4,5</sup><sup>1</sup>Department of Immunology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran, Islamic Republic of, <sup>2</sup>Clinic of Hematology, Ali-Asghar Hospital, Faculty of Medicine, Iran University of Medical Sciences, Tehran, Iran, Islamic Republic of, <sup>3</sup>Immune and Gene Therapy Lab, Cancer Center Karolinska, Karolinska Hospital, Stockholm, Sweden, <sup>4</sup>Monoclonal Antibody Research Center, Avicenna Research Institute, Tehran, Iran, Islamic Republic of, <sup>5</sup>National Cell Bank of Iran, Pasteur Institute of Iran, Tehran, Iran, Islamic Republic of**Objectives:** Dysregulation of WNT signaling has been reported in many malignancies. The present study was conducted to investigate the expression pattern of 14 members of the WNT gene family in leukemic cells from different immunophenotypic subtypes of acute lymphoblastic leukemia (ALL).**Methods:** Leukemic cells from 71 newly-diagnosed ALL patients were immunophenotyped by flow cytometry. Semi-quantitative RT-PCR was performed on leukemic cells of all patients. Peripheral blood mononuclear cells (PBMC) from 36 age-matched normal healthy individuals were employed to determine baseline expression levels of these genes.**Results:** The ALL patients were categorized into B-ALL (76%), T-ALL (22.6%) and mixed lineage (1.4%), based on immunophenotypic results. The B-ALL cases were further classified into pro-B (11%), pre-B I (56%), pre-B II (28%) and immature/mature-B (5%). Among the WNT genes, WNT-7B (p=0.026), WNT-9A (p=0.02) and WNT-16B (p=0.023) were significantly over-expressed and WNT-2B (p=0.033), WNT-5A (p=0.016), WNT-7A (p< 0.0001) and WNT-10A (p< 0.0001) were significantly down-regulated in B-ALL patients compared to normal subjects. Among the T-ALL patients, however, down-regulation of WNT-2B (p=0.004), WNT-5B (p=0.047), WNT-7A (p< 0.0001), WNT-10A (p< 0.0001) and WNT-11 (p=0.001) was evident. Comparing the B-ALL and the T-ALL subtypes revealed no difference with the exception of WNT-11 which was over-expressed (p=0.041) in the B-ALL patients. Comparison between different B-ALL subtypes showed significant over-expression of WNT-7B (p=0.003) and WNT-9A (p=0.044) in pre-B I and WNT-5B (p=0.046) in immature/mature-B leukemic cells.**Conclusion:** Our results indicate differential expression of the WNT genes in different subtypes of ALL and suggest contribution of certain members of the WNT family in leukemogenesis of ALL.**Keywords:** WNT, Acute lymphoblastic leukemia, Immunophenotype, RT-PCR**PD15/11 AUTOIMMUNE LYMPHOPROLIFERATIVE SYNDROME (ALPS) COMPLICATED BY POLYARTERITIS NODOSA-LIKE MANIFESTATIONS AND TREATED BY ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION**N. Naumann-Bartsch<sup>1</sup>, D. Stachel<sup>1</sup>, P. Morhart<sup>1</sup>, G. Staatz<sup>2</sup>, J. Jüngert<sup>1</sup>, W. Holter<sup>1</sup><sup>1</sup>University Children Erlangen, Clinic for Children and Adolescents, Erlangen, Germany, <sup>2</sup>University Clinic Erlangen, Institute of Radiology, Erlangen, Germany**Introduction:** Autoimmune lymphoproliferative syndrome (ALPS) is caused by defects in the Fas-induced apoptotic pathway resulting in a dysregulation of lymphocyte homeostasis. Besides presentation with lymphadenopathy and splenomegaly, ALPS patients have a higher incidence of autoimmune phenomena. However, to our knowledge, polyarteritis nodosa (PAN)-like disease with numerous arterial aneurysms has not been previously reported in ALPS.**Case:** A 15-month-old boy was diagnosed with ALPS when he presented with persistent lymphadenopathy, hepatosplenomegaly, hypergammaglobulinemia, and expansion of double-negative TCRαβ<sup>+</sup>CD4<sup>+</sup>CD8<sup>-</sup> T lymphocytes. Treatment comprised of repeated pulses of high dose methylprednisolone, intravenous immunoglobulin, and mycophenolate mofetil to control lymphoproliferation and recurring autoimmune cytopenias. Nonetheless, at 3 years of age, disease progressed with



massive lymphadenopathy, hypertension, maculopapular skin lesions, abdominal pain, right arm paresis, and persistent cytopenia. Sonography revealed round structures with an arterial flow profile in the pelvis suggestive of aneurysms. MR angiography detected numerous arterial aneurysms in a PAN-like distribution, mainly located in iliac, mesenteric, renal, femoral, brachial, and middle meningeal arteries. Coronary arteries were also affected. Additionally, segmental renal infarcts were noticed. A pulse therapy with cyclophosphamide was commenced but resulted in only moderate benefit. As the clinical course of the patient was worsening, allogeneic hematopoietic stem cell transplantation (HSCT) was indicated. After reduced-intensity conditioning (Flu/Mel/ATG), the patient received bone marrow of an 9/10 HLA-matched unrelated donor but did not engraft. Following fully myeloablative conditioning (Bu/Cy/Eto/ATG) and retransplantation with peripheral blood stem cells of the same donor, graft failure occurred again. After a third conditioning (Flu/TT/Campath/TBI with 4 Gy), HSCT with cells from a second donor was performed, at which one half of the cells was infused intravenously and the other half was directly intra-bone injected. Subsequent to successful engraftment, lymphadenopathy and hepatosplenomegaly regressed, and no additional arterial aneurysms appeared. The patient remains in stable clinical condition.

**Conclusion:** This report demonstrates for the first time that PAN-like disease can occur in ALPS and supports previous studies in mouse models suggesting that the Fas/Fas-ligand system is essential for inhibition of vascular inflammation. In severe ALPS cases, HSCT might be considered and myeloablative rather than reduced-intensity conditioning may be required.

#### PD15/12 A CLONOTYPIC ANTIBODY RECOGNIZING A CDR3 REGION OF AN ID-SPECIFIC T-CELL IN A MURINE MODEL OF MULTIPLE MYELOMA

T. Frigstad<sup>1</sup>, B. Bogen<sup>1</sup>

<sup>1</sup>Centre for Immune Regulation, Institute of Immunology, University of Oslo and Oslo University Hospital, Rikshospitalet, Oslo, Norway

**Objectives:** B cells process their BCR and present V-region derived peptides, called Idiotype (Id)-peptides, on their MHC class II molecules. Id-peptide/class II complexes can be recognized by Id-specific CD4<sup>+</sup> T-cells that help the Id<sup>+</sup> B-cells. The MOPC315 myeloma produces a monoclonal Ig with  $\lambda 2^{315}$  L chains (Eisen, Simms et al. 1968). When processed, an Id-peptide comprising residues 91-101 of the  $\lambda 2^{315}$  chain is presented by MHC class II (I-E<sup>d</sup>) to Id-specific CD4<sup>+</sup> T-cells (Bogen, Malissen et al. 1986; Bogen and Lambiris 1989). We have established several such Id-specific T-cell clones, which can be divided into seven groups according to the amino acid sequences of their CDR3 loops (Snodgrass, Fisher et al. 1992). We wanted to investigate and identify the epitope of a mAb, AB10, which specifically stains a T-cell, 7A10B2, having a TCR only differing a few amino acids from several other Id-specific T-cell clones.

**Methods:** The specificity of the AB10 mAb was analyzed using immunoprecipitation and flowcytometry, and its binding characteristics were compared with sequence analysis of TCRs within the seven groups of Id-specific T-cell clones.

**Results:** Immunoprecipitation of whole cell lysate followed by Western blotting confirmed that the AB10 mAb indeed bound and precipitated a TCR. Binding to structurally similar TCRs was analyzed by surface staining of T-cells followed by flow cytometry. We found that AB10 only bound T-cells expressing the 7A10B2 TCR, but not several other T-cell clones carrying structurally very similar TCRs. Analyzing the VDJ regions of the TCRs we found that five out of seven clones had TCRs utilizing the same V $\alpha$  and six out of seven the same V $\beta$  segment. Two of these clones only differ in their aa sequence at two aminoacids encoded by the D $\beta$  region.

**Conclusion:** The AB10 mAb stained and precipitated TCRs from the 7A10B2 T-cell clone, but not TCRs from a clone with a nearly identical TCR. Both clones express the same VDJ regions with the only difference being two amino acids in the D $\beta$  region. We therefore conclude that the AB10 mAb is specific for the CDR3 loop in the  $\beta$ -chain of the 7A10B2 T-cell receptor.

#### PD15/13 NKT PERCENTAGES CORRELATE WITH CLINICAL STAGE AND ZAP-70 EXPRESSION IN PATIENTS WITH B-CLL

J. Rolinski<sup>1</sup>, A. Bojarska-Junak<sup>1</sup>, I. Hus<sup>2</sup>, P. Wdowiak<sup>1</sup>, M. Wasiak<sup>1</sup>, M. Lewandowska<sup>1</sup>, A. Dmoszynska<sup>2</sup>

<sup>1</sup>Medical University of Lublin, Dept. of Clinical Immunology, Lublin, Poland, <sup>2</sup>Medical University of Lublin, Department of Hematooncology, Lublin, Poland

Natural killer T (NKT) cells are members of both the innate and adaptive immune systems, they bridge the gap between them. They are T cells that recognize lipid antigens presented by CD1d, a nonclassical MHC molecule. NKT cells play both effector and regulatory roles in infectious and autoimmune diseases. Also it has been shown that they have both positive and negative function in tumor immunosurveillance. There is, so far, a very little information on the amounts and the role of NKT in B-cell chronic lymphocytic leukemia (B-CLL). In the presented study, we evaluated NKT cells percentages in peripheral blood of 285 with B-CLL patients as well as correlation with the stage of the disease and known prognostic factors: ZAP-70, CD38, Rai stages,  $\beta 2$  microglobulin, LDH. Patients were considered positive for ZAP-70 or CD38 when the expression was found in 20% or more leukaemic cells. ZAP-70 expression was positive in 47,37% of B-CLL patients and 52,63% were ZAP-70 negative. In ZAP-70 positive patients, the percentage of NKT cells was significantly lower than in ZAP-70 negative ones ( $p=0,0197$ ). We have also found that ZAP-70 expression negatively correlated with the percentages of NKT cells ( $P=0,0037$ ). A significant difference was noted in percentages of NKT cells for the two groups in terms of Rai staging ( $P=0,024$ ): patients who were in Rai stages 0-2 had higher percentages of NKT cells (0,89%) as compared to those in Rai stages 3-4 (0,64%). We have also found a significantly lower percentage of NKT cells in peripheral blood of patients requiring therapy during the follow-up period than in patients who did not ( $P=0,0088$ ). We conclude that NKT cells may play a role in anti-tumour immunity in B-CLL. Higher percentages of NKT cells might be a good prognostic factor, however further studies in this subject are required including NKT subpopulations.

#### PD15/14 ELIMINATION OF MYELOMA CELLS WITH A NEW BORTEZOMIB-BASED COMBINATION THERAPY

S. Meister<sup>1</sup>, V. Lang<sup>1</sup>, B. Frey<sup>2</sup>, U. Schloetzer-Schrehardt<sup>3</sup>, R.E. Voll<sup>4</sup>

<sup>1</sup>Nikolaus-Fiebiger-Center for Molecular Medicine, University of Erlangen-Nuremberg, IZKF N2, Erlangen, Germany, <sup>2</sup>University Hospital Erlangen, Department of Radiation Oncology, Erlangen, Germany, <sup>3</sup>University Hospital Erlangen, Department of Ophthalmology, Erlangen, Germany, <sup>4</sup>University Hospital Erlangen, Department for Internal Medicine 3 and Institute for Clinical Immunology, Erlangen, Germany

Multiple myeloma (MM), an incurable plasma cell neoplasia, is characterized by overproduction of monoclonal immunoglobulins (Ig). The clinically approved proteasome inhibitor bortezomib (Bz) represents a promising agent for the therapy of relapsed multiple myeloma. However, long-term remissions are difficult to achieve, in fact myeloma cells often develop secondary resistance to proteasome inhibition. We recently demonstrated that myeloma cells are highly sensitive towards proteasome inhibitors due to their extensive rate of immunoglobulin synthesis, thereby triggering the terminal unfolded protein response (UPR) resulting in cell death. Here, we want to identify synergistic agents sensitizing myeloma cells towards Bz. For these studies we used the calcium channel blocker verapamil that has been shown to inhibit proliferation of leukemia cells and to interfere with multidrug resistance-based drug elimination. Hence, we analysed the effect of Bz together with verapamil on the viability and ER-stress in human myeloma cell lines. The combination of Bz and verapamil synergistically decreased cell viability of myeloma cell lines by inducing apoptotic and necrotic cell death. Importantly, Bz-mediated activation of major UPR components was enhanced by verapamil, also evidenced by a vast expansion of the ER. Further, Bz/verapamil treatment resulted in caspase activation followed by PARP cleavage. NF- $\kappa$ B DNA-binding activity markedly declined in myeloma cells treated with both inhibitors. Compared to Bz alone, we found increased amount of ubiquitinated proteins in detergent-insoluble fractions in the presence of Bz/verapamil. Interestingly, secreted IgG was reduced in the supernatant of myeloma cells by adding verapamil. In conclusion, verapamil increased the pro-apoptotic effect of Bz by inducing additional ER-stress signals along with inhibiting the NF- $\kappa$ B activity and modulating drug transport mechanism. Thus, the combination therapy, Bz together with verapamil, provide a more effective treatment-strategy for multiple myeloma than the Bz-monotherapy and may overcome drug resistance.

#### PD15/15 COMPARATIVE EXPRESSION OF ORPHAN RECEPTOR TYROSINE KINASE (ROR1) IN LYMPHOID AND MYELOID LEUKEMIAS

M. Shabani<sup>1</sup>, H. Asgarian-Omran<sup>1</sup>, M. Hojjat-Farsangi<sup>1</sup>, P. Vossoughi<sup>2</sup>, R.A. Sharifian<sup>3</sup>, J. Khoshnoodi<sup>1</sup>, M. Jeddi-Tehrani<sup>4,5</sup>, H. Mellstedt<sup>6</sup>, H. Rabbani<sup>1</sup>, F. Shokri<sup>1,6</sup>

<sup>1</sup>Department of Immunology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran, Islamic Republic of, <sup>2</sup>Clinic of Hematology, Ali-Asghar Hospital, Faculty of Medicine, Iran University of Medical Sciences, Tehran, Iran, Islamic Republic of, <sup>3</sup>Clinic of Hematology and Oncology, Vali-Asr Hospital, Faculty of Medicine, Tehran University of Medical Sciences, Tehran, Iran, Islamic Republic of, <sup>4</sup>Immune and Gene Therapy Lab, Cancer Center Karolinska, Karolinska Hospital, Stockholm, Sweden, <sup>5</sup>Monoclonal Antibody Research Center, Avesina Research Institute, Tehran, Iran, Islamic Republic of, <sup>6</sup>National Cell Bank of Iran, Pasteur Institute of Iran, Tehran, Iran, Islamic Republic of

**Objectives:** It has recently been shown that ROR1 – a member of the receptor tyrosine kinase family – is overexpressed in B-cell chronic lymphocytic leukemia (B-CLL). In this study the expression profile of ROR1 mRNA was investigated in Iranian patients with B-CLL, acute lymphoblastic leukemia (ALL) and acute myelogenous leukemia (AML).

**Methods:** RT-PCR was performed on bone marrow and/or peripheral blood samples of 57 ALL, 84 CLL, 12 AML patients and 33 normal individuals. The ratio of ROR1 to  $\beta$ -actin band densities was compared between patient and normal samples to identify ROR1 overexpression. Leukemic CLL cells were classified into immunoglobulin variable region heavy chain (IgVH) gene mutated (n=55) and unmutated (n=29) subtypes and also indolent (n=42) and progressive (n=39) subtypes. Leukemic ALL cells were immunophenotypically categorized into four subgroups.

**Results:** While ROR1 was expressed in 96% of B-CLL and 40% of ALL, all AML patients were ROR1 negative. No significant differences were observed between different B-CLL subtypes or ALL subgroups for ROR1 expression.

**Conclusion:** Our findings propose ROR1 as a new tumor-associated antigen overexpressed in chronic and acute lymphoid (CLL and ALL), but not acute myeloid (AML) leukemias. Expression of ROR1 seems to be associated to lineage and differentiation stages of leukemic cells with a potential implication for immunotherapy.

# PD15/16 IMMUNOGLOBULIN VARIABLE REGION HEAVY CHAIN GENES USAGE AND MUTATIONAL STATUS OF THE LEUKEMIC B-CELLS IN IRANIAN PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA

M. Hoojati-Farsangi<sup>1,2</sup>, M. Jeddi-Tehrani<sup>3,4</sup>, S.M. Razavi<sup>5</sup>, R.A. Sharifian<sup>6</sup>, H. Mellstedt<sup>7</sup>, F. Shokri<sup>2,3,7</sup>, H. Rabbani<sup>3,4</sup>

<sup>1</sup>School of Medicine, Bushehr University of Medical Sciences, Department of Immunology, Bushehr, Iran, Islamic Republic of, <sup>2</sup>School of Public Health, Tehran University of Medical Sciences, Department of Immunology, Tehran, Iran, Islamic Republic of, <sup>3</sup>Avicenna Research Institute, ACECR, Monoclonal Antibody Research Center, Tehran, Iran, Islamic Republic of, <sup>4</sup>Cancer Center Karolinska, Karolinska Hospital, Immune and Gene Therapy Lab, Stockholm, Sweden, <sup>5</sup>Firozgar Hospital, Faculty of Medicine, Iran University of Medical Sciences, Clinic of Hematology and Oncology, Tehran, Iran, Islamic Republic of, <sup>6</sup>Vali-Asr Hospital, Faculty of Medicine, Tehran University of Medical Sciences, Clinic of Hematology and Oncology, Tehran, Iran, Islamic Republic of, <sup>7</sup>Pasteur Institute of Iran, National Cell Bank of Iran, Tehran, Iran, Islamic Republic of

**Objectives:** The mutational status of the immunoglobulin variable region heavy chain genes (IgVH) is an important prognostic marker in chronic lymphocytic leukemia (CLL). Little is known about the Asian CLL patients. In this study the IgVH genes usage and somatic hypermutation status have been investigated in 87 Iranian CLL patients.

**Methods:** Peripheral blood mononuclear cells (PBMC) were obtained from 87 CLL patients, clinically classified into indolent (n=44), progressive (n=40) and newly-diagnosed (n=3) groups. Genomic DNA and total cellular RNA were extracted from PBMCs of patients and PCR amplification was performed using VH family specific degenerative primers. Sequencing of IgVH region was performed after cloning of PCR products. Sequences were analysed by IMGT Database and for each sample, VH segment was identified by matching to the closest known human germline gene.

**Results:** Based on a cut-off of 98% nucleotide sequence homology with the closest known human germline IgVH gene, 64.4% and 35.6% of the patients expressed mutated and unmutated VH genes, respectively, with most of non-progressive patients being in the mutated group (35/44 vs. 19/40; p=0.009). Progression free survival (PFS) and time to first treatment (TTFT) were significantly higher in our mutated and non-progressive patients compared to unmutated and progressive subtypes. The most frequently used VH gene was IGVH3-07 (12.6%) followed by IGVH3-30 (11.4%), IGVH3-48 (9.2%), IGVH4-39 (6.9%) and IGVH1-08 (6.9%) genes, which taken together comprised nearly half of VH genes expressed in CLL patients. Of the IgVH genes, IGVH3-07 was significantly over-represented in non-progressive compared to progressive CLL patients (p=0.036), whereas IGVH1-69 and IGVH1-02 were expressed at a higher frequency in unmutated compared to mutated CLL patients (p<0.03). Comparison of VH gene usage in our patients with that of Western CLL patients revealed significant differences in expression of IGVH1-69, IGVH3-07, IGVH3-21 and IGVH4-34 genes. Analysis of the IgVH CDR3 sequences revealed a high frequency use of certain CDR3 motifs, such as YYGMDV in our samples.

**Conclusion:** Our results show differential expression pattern of IgVH genes in leukemic B-cells of Iranian and Western CLL patients and imply contribution of antigen selection and regional (ethnic/geographic) parameters in leukemogenesis of CLL.

# PD15/17 EXPRESSION PROFILE OF GALECTIN-1 AND GALECTIN-3 GENES IN DIFFERENT SUBTYPES OF CHRONIC LYMPHOCYTIC LEUKEMIA

H. Asgarian-Omran<sup>1</sup>, P. Forghani<sup>1</sup>, M. Hoojati-Farsangi<sup>1</sup>, A. Roohi<sup>1</sup>, R.A. Sharifian<sup>2</sup>, S.M. Razavi<sup>3</sup>, M. Jeddi-Tehrani<sup>4,5</sup>, H. Rabbani<sup>4</sup>, F. Shokri<sup>1,6</sup>

<sup>1</sup>School of Public Health, Tehran University of Medical Sciences, Department of Immunology, Tehran, Iran, Islamic Republic of, <sup>2</sup>Clinic of Hematology and Oncology, Vali-Asr Hospital, Faculty of Medicine, Tehran University of Medical Sciences, Tehran, Iran, Islamic Republic of, <sup>3</sup>Clinic of Hematology and Oncology, Firozgar Hospital, Faculty of Medicine, Iran University of Medical Sciences, Tehran, Iran, Islamic Republic of, <sup>4</sup>Immune and Gene Therapy Lab, Cancer Center Karolinska, Karolinska Hospital, Stockholm, Sweden, <sup>5</sup>Monoclonal Antibody Research Center, Avesina Research Institute, Tehran, Iran, Islamic Republic of, <sup>6</sup>National Cell Bank of Iran, Pasteur Institute of Iran, Tehran, Iran, Islamic Republic of

**Objectives:** Galectin-1 (Gal-1) and Gal-3 molecules are two members of the Galectin family which are involved in many vital biological and pathological processes. This study was undertaken to investigate the expression pattern of Gal-1 and -3 at mRNA and protein levels in leukemic cells from different subtypes of Iranian patients with chronic lymphocytic leukemia (CLL).

**Methods:** Peripheral blood mononuclear cells (PBMC) were obtained from 85 Iranian CLL patients, clinically classified into indolent (n=42), progressive (n=40) and newly-diagnosed (n=3) groups. Nucleotide sequence analysis of the immunoglobulin variable region heavy chain (IgVH) genes of the leukemic cells has allowed classification of the patients into mutated (n=55) and unmutated (n=30) groups. PBMC isolated from 12 elderly normal subjects served as controls to determine baseline expression levels. A semi-quantitative RT-PCR was applied to determine mRNA expression levels of Gal-1 and -3 in the samples. The protein expression of Gal-1 and -3 was analyzed in 8 CLL samples at surface and intra-cytoplasmic levels by flow cytometry.

**Results:** Our results showed significant down-regulation of Gal-3, but not Gal-1 in CLL patients in comparison to elderly normal subjects (p<0.001). Comparison between the clinical subtypes of our CLL patients revealed significantly higher representation of Gal-3 mRNA in indolent patients compared to the progressive group (p<0.05). Mutated and unmutated IgVH groups did not show any significant differences for Gal-1 and -3 mRNA expression. Investigation of protein expression in leukemic cells of 8 patients failed to reveal Gal-3 expression, but Gal-1 was expressed at intra-cytoplasmic level in most of the cases studied.

**Conclusion:** Down-regulation of Gal-3, but not Gal-1 gene expression in our CLL patients, and particularly in patients with progressive disease suggests a regulatory role for this gene in initiation and progression of CLL.

**Keywords:** Galectin, Chronic lymphocytic leukemia, Indolent, Progressive, RT-PCR, Flow cytometry.

# PD15/18 EFFECT OF ISOSORBIDE DINITRATE ON VASCULAR ENDOTHELIAL GROWTH FACTOR PRODUCTION BY HUMAN LEUKEMIC CELL LINES IN VITRO

F. Hajighasemi<sup>1</sup>, A. Mirshafiei<sup>2,3</sup>

<sup>1</sup>Faculty of Medicine, Shahed University, Department of Immunology, Tehran, Iran, Islamic Republic of, <sup>2</sup>School of Public Health, Department of Pathobiology, Tehran, Iran, Islamic Republic of, <sup>3</sup>Immunology, Asthma and Allergy Research Institute, Tehran University of Medical Sciences, Tehran, Iran, Islamic Republic of

**Background:** Vascular endothelial growth factor (VEGF) has mitogenic effect for endothelial cells and is an important mediator of tumor expansion, metastasis and angiogenesis in vivo. Isosorbide dinitrate, as a nitric oxide donor, has been widely used in treatment of many cardiovascular diseases such as congestive heart failure and acute coronary syndromes. Furthermore this drug was found to have inhibitory effect on angiogenesis, tumor growth and metastasis in vivo. In the present study we evaluated the isosorbide effect on the VEGF production using some human leukemic cell lines.

**Methods:** Human leukemic MOLT-4, JURKAT and U937 cells were cultured in complete RPMI medium. The cells at the exponential growth phase were then incubated with different concentrations of Isosorbide (0.0004–0.4 mM) in the presence or absence of PMA (25ng/ml) for 24 hours. The VEGF concentrations in the culture supernatants were measured by enzyme immunoassay kits (R&D systems) according to the manufacturer's instructions.

**Results:** The level of VEGF produced by the human leukemic cell lines which was treated with different concentrations of isosorbide, did not show any significant difference with untreated control cells.

**Conclusions:** The results of this study showed that isosorbide had no significant effect on VEGF production. Our findings suggest that anti-angiogenesis effect of isosorbide could be mediated through VEGF-independent mechanism(s). Further studies are warranted to determine definite isosorbide effect on VEGF and other angiogenic factors production in patients as well as animal models.

# PD15/19 PRIMARY EFFUSION LYMPHOMA POSITIVE FOR HHV-8 AND NEGATIVE FOR EBV ORIGINATED FROM PRE-GERMINAL CENTRE B-CELLS

J. Modrego Ruiz<sup>1</sup>, C. Rodríguez-Sáinz<sup>1</sup>, S. Sánchez-Ramón<sup>1</sup>, J. Gil<sup>1</sup>, M. Rodríguez Mahou<sup>1</sup>, J.J. Rodríguez Molina<sup>1</sup>, E. Fernández-Cruz<sup>1</sup>

<sup>1</sup>Hospital General Universitario Gregorio Marañón, Immunology, Madrid, Spain

**Objective:** Primary effusion lymphoma (PEL) is a rare type of B-cell lymphoproliferative disorder involving body cavities which is mainly observed in immunocompromised patients. Lymphomatous cells show features of immunoblastic and anaplastic cells with a non-B non-T phenotype and are characterized by the presence of the human herpesvirus 8 (HHV-8) genome and commonly also Epstein Bar virus (EBV).

The cellular origin of PEL is controversial. A previous report indicates that EBV positive PELs are derived from germinal centre or post-germinal centre B-cells bearing hypermutating IgH rearrangements. However, EBV negative PELs may originate from either germinal/post-germinal centre or naïve B-cells. The pre-germinal centre naïve B-cells origin has been established on the basis of two cases of HHV-8+ EBV- PELs displaying no somatic mutation in their rearranged Ig genes. The aim of this study was to characterize a new case of PEL, HHV-8 positive and EBV negative, determining its cellular origin by the study of the hypermutating IgH status.

**Patient and methods:** A patient of 69-year-old male, with a previous history of chronic hepatopathy and biliary cirrhosis associated to antinuclear and anti-mitochondrial-M2 autoantibodies and hypergammaglobulinemia, showed in the ascitic fluid atypical lymphoid cells with expression of CD138, positive for HHV-8 and negative for EBV. **Methods:** DNA obtained from the ascitic liquid sample was analysed for the IgH rearrangement clonality by PCR-heteroduplex analysis and for hypermutation status by PCR of FR1-JH IgH rearrangement and sequencing. The sequence was aligned to NCBI/GenBank using the IgBLAST software (National Center for Biotechnology Information, National Institutes of Health, Bethesda, MD) and the IMGT/V-QUEST tool (International ImMungGeneTics information system, Marie-Paule Lefranc, University of Montpellier, CNRS, France).

**Results:** DNA from the primary effusion resulted monoclonal for immunoglobulin heavy chain gene rearrangement. The region IgH rearranged expressed the family VH3-21, showing germline configuration with an unmutated status, lacking somatic mutations.

**Conclusion:** This characterization of a new case of PEL positive for HHV-8 and negative for EBV confirms the pre-germinal centre naïve B-cell origin in this lymphoproliferative disorder.

# PD15/20 IN VITRO CYTOTOXIC EFFECT OF SEMEN CUSCUTA (CUSCUTA CHINENSIS LAM) WHOLE EXTRACT ON HUMAN ACUTE LYMPHOBLASTIC LEUKEMIA (ALL) CELL LINE

F. Zeraati<sup>1</sup>, A. Zamani<sup>2</sup>, M. T. Goodarzi<sup>3</sup>, S. M. Malakouti Hashjin<sup>4</sup>, K. Razaghi<sup>5</sup>

<sup>1</sup>Hamadan University of Medical Sciences, Dept. of Pharmacology, Hamadan, Iran, Islamic Republic of, <sup>2</sup>Hamadan University of Medical Sciences, Dept. of Immunology, Hamadan, Iran, Islamic Republic of, <sup>3</sup>Hamadan University of Medical Sciences, Dept. of Biochemistry & Nutrition, Hamadan, Iran, Islamic Republic of, <sup>4</sup>Hamadan University of Medical Sciences, Dept. of Physiology, Hamadan, Iran, Islamic Republic of, <sup>5</sup>Agricultural Center, Hamadan, Iran, Islamic Republic of

Cancer is the second leading cause of death behind heart disease. Plant natural products are valuable sources of novel chemotherapeutic agents with wide range of mechanism of action. Anti-tumoral effect of whole extract from Semen Cuscuta was investigated in vitro on human Caucasian acute Lymphoblastic leukemia (CCRF-CEM) and a normal human lymphocyte (JM) cell lines using Methyl tetrazolium bromide test. After 24 and 48 hours incubation of the cells with the extract, morphological changes and granularity of the cells were examined using inverted microscope. Aqueous extract of Semen Cuscuta showed a cytotoxic effect on CCRF-CEM cell line. Incubation of these cell lines with Semen Cuscuta extract indicated that the number of dead cells and granularity of cells were higher in tumor cells as compared to normal cells. It can be concluded that this plant can not affect the normal cells.

# PD15/21 STAT-5 AND IRF-8 ARE IMPLICATED IN THE IMPAIRMENT OF HUMAN PDC DIFFERENTIATION AND FUNCTION IN CML PATIENTS IN COMPLETE CYTOGENETIC REMISSION

A. Barra<sup>1,2</sup>, L. Brault<sup>1,2</sup>, A. Rossignol<sup>3</sup>, S. Noel<sup>1</sup>, C. Giraud<sup>1,4,5</sup>, A. Turhan<sup>1</sup>, L. Roy<sup>1,5</sup>, F. Guilhot<sup>1,5,6</sup>, J.-M. Gombert<sup>1,2</sup>

<sup>1</sup>Université de Poitiers, EA 3805, Poitiers, France, <sup>2</sup>CHU de Poitiers, Laboratoire d'immunologie, Poitiers, France, <sup>3</sup>Université François Rabelais Tours, UMR CNRS 6239, Tours, France, <sup>4</sup>EFS Centre-Atlantique site de Poitiers, Laboratoire de thérapie cellulaire, Poitiers, France, <sup>5</sup>CHU de Poitiers, Service d'oncologie hématologique et thérapie cellulaire, Poitiers, France, <sup>6</sup>CIC Inserm 802, Poitiers, France

**Objectives:** The immune system plays a critical role in the control of chronic myelogenous leukemia (CML). We have previously shown that plasmacytoid dendritic cells (pDc) differentiation and IFN- $\alpha$  production were impaired along the chronic phase of CML. We have observed in complete cytogenetic remission (CCyR) patients after imatinib mesylate (IM) or IFN- $\alpha$  therapy a partial restoration of the pDc compartment and IFN- $\alpha$  production. Recent results indicate that, in mice, STAT-5 blocks the expression of IRF-8, a transcription factor required for the differentiation of pDc.

**Methods:** The status of p-STAT-3, p-STAT-5 and IRF8 was investigated *ex vivo* by flow cytometric approaches in mononuclear cells (MNC) and pDc of patients who have achieved sustained CCyR and major molecular response after IFN- $\alpha$  therapy (referred as IFN patients, n=8) or after Imatinib mesylate therapy (referred as IM patients n=6) and compared to healthy donors (HD, n=5). pDc's IFN- $\alpha$  production was assessed by intracellular detection after stimulation by *Influenza* virus.

**Results:** Our results showed that the homeostasis of pDc's IFN- $\alpha$  production was altered both in IFN and IM CCyR patients. IRF-8 expression was also deregulated, with a low expression level (based on mean fluorescence analysis of IRF-8 staining) detected in pDc from IFN patients by comparison with the pDc of HD and IM patients. Moreover, higher levels of p-STAT5 were observed in MNC and pDc from IFN patients than from HD and IM patients. Lastly, pDc from IM patients show a clear lack of maturation (low DR and CD86 MFI) in comparison with the pDc from IFN patients and HD.

**Conclusion:** Altogether, our results suggest that the homeostasis of IFN- $\alpha$  production remains abnormal after CCyR induced either by IFN- $\alpha$  or IM therapy, involving independent mechanisms. Indeed, IM seems to block the final maturation of pDc, whereas the pDc of IFN patients show a high degree of terminal maturation (exhaustion), with a decreasing IFN- $\alpha$  production, a low level of IRF-8 expression and an important level of p-STAT5.

Lastly, our results suggest that, in Human, p-STAT5 interferes with the IRF-8 expression and emphasises the importance of maintaining the IFN- $\alpha$  production homeostasis in CCyR patients.

# PD15/22 LEUKEMIA SPECIFIC CD8+ T CELL CLONES: TOWARDS THE IDENTIFICATION OF NEW TUMOR ANTIGENS

A.R. Elia<sup>1</sup>, P. Circosta<sup>1</sup>, S. Stella<sup>1</sup>, L. Mele<sup>2</sup>, B. Allione<sup>2</sup>, A. Cignetti<sup>1</sup>

<sup>1</sup>Laboratory of Cancer Immunology, Institute of Cancer Research and Treatment, Candiolo (TO), Italy, <sup>2</sup>Division of Hematology, Azienda Ospedaliera SS. Antonio e Biagio, Alessandria, Italy

The aim of our study is to generate and expand CTL (cytotoxic T-lymphocytes) clones directed against acute myeloid leukemia (AML) cells and to identify new leukemic antigens. We and others have previously shown that AML cells can differentiate into leukemic dendritic cells (L-DC), which harbor the full range of still unidentified potential tumor antigens. We used L-DC as antigen-presenting cells (APC) to generate autologous leukemia-specific CTL originating from the immune repertoire of AML patients in complete remission. We cloned bulk CTL cultures with specific activity and obtained 4 clones that recognize both L-DC and naïve leukemic cells to the same extent. These clones did not recognize normal autologous targets (unfractionated bone marrow and lymphocytes, purified monocytes). One clone specifically recognized other AML targets expressing HLA-A2 and, to a much lesser extent, also HLA-A2+ normal activated monocytes but not activated lymphocytes. We are now starting to identify the HLA-A2 restricted, shared myeloid antigen targeted by this clone.

Unfortunately, in some patients we could not obtain peripheral lymphocytes during remission. We therefore established a different protocol of antigen presentation, in which normal DC from healthy donors were loaded with apoptotic bodies from leukemic cells and used to stimulate autologous lymphocytes, which were selected to be partially HLA-matched with the leukemic patient. With this strategy, CTL clones could be isolated that recognized both loaded DC and AML cells used for loading. Two clones have been characterized that recognize 5/5 HLA-matched primary AML and 0/8 mismatched AML. These clones do not recognize both resting and activated normal lymphoid, myeloid and CD34+ cells expressing the proper HLA-restriction allele (HLA-B7 and HLA-B44), but they recognize HLA-B7+ or HLA-B44+ melanoma and colon carcinoma cell lines. Considering the allogeneic model used for antigen presentation, we hypothesize that they recognize minor histocompatibility antigens (i.e. polymorphic peptides) from proteins that are selectively expressed by malignant cells and not by normal tissues.

We demonstrated that, using different priming techniques, AML cells can become immunogenic and stimulate potent T-cell responses in vitro that can be studied at clonal level. We are now using these clones for identification of new tumor antigens.

# PD15/23 EVALUATION OF KIT AS A POTENTIAL TARGET OF THERAPEUTIC MONOCLONAL ANTIBODIES

H. Bougherara<sup>1</sup>, F. Subra<sup>1</sup>, M.-A. Poul<sup>1</sup>

<sup>1</sup>LBPA/UMR CNRS 8113/ENS Cachan, Cachan, France

Kit is a tyrosine kinase (TK) receptor involved in cell transformation through overexpression or oncogenic mutations. Two categories of mutants displaying mutations either in the juxtamembrane intracellular domain (regulatory mutants) or in the catalytic domain (catalytic mutants) have been described. Oncogenic Kit is implicated in several malignancies including acute myeloid leukemia and mastocytoma and could potentially be targeted with monoclonal antibodies (mAbs) in a therapeutic approach paralleling the inactivation of cell surface ErbB2 in breast cancer using the anti-ErbB2 mAb Trastuzumab.

Our lab develops fully human mAbs against cell surface antigenic targets by phage display using cell models as selection system. In order to obtain human therapeutic anti-Kit mAbs, we established cells models expressing EGFP tagged human Kit chimeras including wild type Kit and Kit mutants representative of regulatory (mutation V560G) or of catalytic (mutation D816V) mutant types.

We observed that constitutively activated Kit mutants exhibited intracellular localization and altered post-translational modifications suggesting that efficient mutated Kit targeting by mAbs would be impaired by the low cell surface expression of the receptor.

We checked the effects of known Kit TK inhibitors including Imatinib (active on V560G mutant only) and Dasatinib (active on D816V and V560G mutants). Inhibition of constitutive Kit TK activity obtained with Dasatinib induced the redistribution of both mutants to the plasma membrane and post-translational modifications identical to unstimulated wild type Kit. The relocation was correlated with the inhibition of TK activity since Imatinib induced the V560G Kit mutant redistribution at the cell surface but had no effect on the resistant D816V Kit mutant.

Scaltril et al., recently demonstrated that Lapatinib, an ErbB2 TK inhibitor, potentiated anti-ErbB2 trastuzumab-dependent cell cytotoxicity by inducing ErbB2 accumulation and stabilization at the cell surface. Our data demonstrate that TK inhibitors, in addition to their direct effect on Kit TK activity, reverse the aberrant intracellular localization of Kit mutants enabling its exportation and/or stabilization at the cell surface. These observations allowed us to consider oncogenic Kit as a potential cell surface target using Kit specific mAbs in combination with TK inhibitors.

# PD15/24 CONSTITUTIVE ACTIVATION OF NF-KB IN CUTANEOUS T CELL LYMPHOMA (CTCL) : INVOLVEMENT OF TCR PATHWAY

J. Baraut<sup>1</sup>, F. Jean-Louis<sup>1</sup>, H. Bachelez<sup>2</sup>, G. Courtois<sup>3</sup>, L. Michel<sup>1</sup>

<sup>1</sup>INSERM, U976, Paris, France, <sup>2</sup>AP-HP Hopital Saint Louis, Paris, France, <sup>3</sup>INSERM, U781, Paris, France

Primary cutaneous T cell lymphomas (CTCLs) are a heterogeneous group of non-Hodgkin extra-nodal T cell lymphomas, with mycosis fungoides (MF) and Sézary syndrome (SS) as the two major clinical variants. Our recent work demonstrates that tumor cells from CTCL exhibit constitutive activation of the classical NF- $\kappa$ B pathway that is involved in resistance to apoptosis [Sors et al, Blood, 2006]. However, the origin of NF- $\kappa$ B constitutive activation in CTCL remains unknown. One hypothesis is deregulation of the TCR pathway and the aim of our work is to target altered molecular events in TCR-induced NF- $\kappa$ B pathway. CTCL cell lines (HuT-78, SeAx, MyLa) and CD4+ tumor lymphocytes isolated from peripheral blood mononuclear cells (PBMC) of patients with SS were used. CTCL cell lines were co-transfected with negative dominant molecules, specific for the TCR-linked NF- $\kappa$ B pathway, and a plasmid reporter Igk-luc which measures the NF- $\kappa$ B activation level. Transfection either with negative dominants for Bcl10 and PKC $\theta$  (PKC $\theta$ ) or with specific PKC $\theta$  siRNA, inhibited NF- $\kappa$ B transcriptional activity in a dose-dependent manner. This clearly demonstrates that the pathway is deregulated at the level of CARMA1-Bcl10-MALT1 complex and/or PKC $\theta$ . Addition of a specific inhibitor of PKC $\theta$ , rottlerin, after Igk-luc transfection, decreased luciferase activity. Rottlerin [1.56 to 50  $\mu$ M] also inhibited constitutive NF- $\kappa$ B transcription activity, as shown by EMSA and immunocytochemistry (dose-dependent inhibition of p65 NF- $\kappa$ B nuclear staining). Moreover, this inhibitor induced a greater apoptosis of CTCL cell lines and tumor cells from patients with Sézary syndrome, as compared with Jurkat cell line and PBMC from healthy donors. Rottlerin also inhibits NF- $\kappa$ B



(p65) and phosphorylated Bcl10 expression. Alteration of CBM status in CTCL tumor cells was established by immunoprecipitation assays. In conclusion, NF- $\kappa$ B constitutive activation in CTCL is associated with a molecular deregulation at CARMA1-Bcl10-MALT1 and PKC $\theta$  level in TCR pathway, offering new potential therapeutic targets for CTCL.

#### PD15/25 MIGRATORY AND PROSURVIVAL CASCADES INDUCED BY THE HOMEOSTATIC CHEMOKINES CCL19 AND CCL21 IN B-CHRONIC LYMPHOCYTIC LEUKEMIA

C. Cuesta-Mateos<sup>1</sup>, S. López-Giral<sup>1</sup>, M. Alfonso-Pérez<sup>1</sup>, V. Gómez García de Soria<sup>2</sup>, J. Loscertales<sup>2</sup>, S. Guasch-Vidal<sup>1</sup>, A. Beltrán-Núñez<sup>1</sup>, C. Muñoz-Calleja<sup>1</sup>  
<sup>1</sup>Hospital Universitario de la Princesa, Immunology, Madrid, Spain, <sup>2</sup>Hospital Universitario de la Princesa, Hematology, Madrid, Spain

The CCR7 chemokine receptor has been reported to promote the localization of chronic lymphocytic leukemia (CLL) cells into lymph nodes (LNs) where they may gain survival signals, but the mechanisms mediating these effects are largely unknown. We investigated the role of different signaling pathways in the migratory and pro-survival effects exerted by the chemokines CCL19 and CCL21, the CCR7 ligands, in CLL cells. Their chemotactic activity was markedly reduced in the presence of inhibitors of PI3K and the Rho effector molecule ROCK. Moreover, expression of dominant negative forms of PI3K and RhoA blocked the chemotactic effect induced through CCR7, whereas their constitutively activated mutants exerted an opposite effect. Finally, MAPKs were not clearly involved in CLL migration to CCL19/CCL21. Conversely, ERK and JNK, along with PI3K, were responsible for the pro-survival effects mediated by CCR7 in CLL cells. Biochemical experiments confirmed that CCL19/21 promote the PI3K-dependent phosphorylation of the Akt/PKB kinase, the activation of the Rho/ROCK/MLC pathway and the phosphorylation of MAPKs. The important role of PI3K, Rho GTPases and MAPKs in the migration and survival of CLL cells in response to CCL19/21 provides a rationale to explore these signal pathways as promising targets for the therapy of this condition.

#### PD15/26 ANALYSIS OF TRANSCRIPTION FACTORS INVOLVED IN T-PLASTIN SYNTHESIS IN CUTANEOUS T CELL LYMPHOMA

E. Bégué<sup>1</sup>, F. Jean-Louis<sup>1</sup>, H. Bachelez<sup>1</sup>, G. Courtois<sup>2</sup>, M. Laurence<sup>1</sup>  
<sup>1</sup>INSERM, U976, Paris, France, <sup>2</sup>INSERM, U781, Paris, France

Primary cutaneous T cell lymphomas (CTCLs) are a heterogeneous group of non-Hodgkin extra-nodal T cell lymphomas presenting two major clinical variants: mycosis fungoides (MF) and Sézary syndrome (SS). Transcriptome analysis allowed the identification of T-plastin as an upregulated gene in CTCL cells from SS patients not expressed in normal T lymphocytes (Kari et al, J Exp Med, 2003; van Doorn et al, Cancer Res 2004; Nebozhyn et al, Blood 2006). Herein, we studied T-plastin expression in a large cohort of CTCL patients, compared with a wide range of T-cell lymphoproliferative disorders, and searched for involved transcriptional factors.

Peripheral blood lymphocytes (PBL) were isolated from circulating blood of patients with CTCL (n=36), MF (n=12), unidentified erythroderma (n=10), hypereosinophilic syndrome (n=8), other lymphoproliferative disorders (n=10) and healthy donors (n=56). The expression levels of T-plastin and L-plastin was quantified by quantitative real-time polymerase chain reaction assay and CTCL cell lines (SeAx, MyLa) as well as in control leukemic Jurkat cells. Our results showed that T-plastin mRNA were detected in 80% of SS patients, while no expression was detectable in patients with MF, idiopathic erythroderma, hypereosinophilic syndrome with clonal T-cell subset, and healthy donors. Hut-78 cells constitutively express T-plastin whereas SeAx and MyLa cells (derived from one SS and one MF patient respectively) express T-plastin only after PMA+ionomycin (Io) stimulation. T-plastin transcription was induced in PMA+Io stimulated T-cells of 4/5 T-plastin negative-SS patients, but interestingly also in PMA+Io stimulated-control healthy PBLs. Calcineurin inhibitors reduced constitutive and PMA+Io-induced transcription of T-plastin by 30-45% and 50-100% respectively. EMSA and gene reporter assays demonstrated that activation of nuclear factor of activated T cells (NFAT) was responsible, at least partially, for T-plastin expression in tumor T cells. Our experiments strongly suggest the accuracy of T-plastin as a specific marker for Sézary syndrome diagnosis, and bring evidence of NFAT involvement in T-plastin expression, leading to new perspective for the prognosis and treatment of CTCLs.

#### PD15/27 DEMONSTRATION OF SERUM MONOCLONAL IMMUNOGLOBULIN LIGHT CHAIN IN A CASE OF NON-SECRETORY MULTIPLE MYELOMA

F. Sánchez-Jiménez<sup>1</sup>, C. Bermudo-Guitarte<sup>1</sup>, G. Cisneros-Barrera<sup>1</sup>, R. Goberna<sup>1</sup>  
<sup>1</sup>Virgen Macarena University Hospital, Clinical Biochemistry, Seville, Spain

**Introduction:** Non-secretory myeloma (NSM) is a rare variant of multiple myeloma (MM) in which conventional laboratory techniques, electrophoresis and immunofixation, fail to show a paraprotein in either serum or urine. We describe a NSM case that shows a high concentration of serum lambda free light chain and a weak monoclonal band by serum immunofixation during a routine follow-up.

**Methods:** A 64 year old man previously diagnosed as NSM IIIA in June 2004 receives in November of the same year 6 cycles of VAD as therapy achieving a partial remission. In June 2005 receives bone marrow transplant achieving a very good partial response. Periodic controls to spinal column were made in the follow-up persisting osteolytic lesions but without signs of progression. In March 2008 presents 4% of plasmatic cells in bone marrow and no osteolytic lesion in December 2008. However, in October 2008 patient presented a small increase in total kappa (K) and lambda (λ) light chain which coincides with small elevation of gamma fraction, with a bad definition peak that was not informed. In December 2008 serum free light chains (sFLC) were determined indicating high level for λ sFLC (K=7.04 mg/L; λ=186 mg/L; ratio K/λ=0.038). In March 2009 a new serum immunofixation (sIFE) was performed identifying a very weak monoclonal peak for λ. Due to that, in May 2009 a new determination of sFLC and a new sIFE was performed. At this time point sIFE is clearly positive and λ sFLC increased to 752 mg/L.

**Conclusion:** Due to the low secretion that characterizes these patients and to the low sensitivity of the existing methods at the time of diagnosis, probably this patient was identified as NSM instead of oligosecretory MM. Traditional techniques for the identification of MP are not enough for the follow up and consequently for the correct diagnosis. Because of that, when a patient has all the suspicious symptoms for a MM and the traditional techniques as electrophoresis/immunofixation in serum/urine are negative we recommend the use of sFLC due to higher sensitivity.

### PD17 – GENE THERAPY

#### PD17/1 EFFICIENT RECOVERY OF HLA CLASS I EXPRESSION IN HUMAN TUMOR CELLS AFTER BETA2-MICROGLOBULIN GENE TRANSFER USING ADENOVIRAL VECTOR: IMPLICATIONS FOR CANCER IMMUNOTHERAPY

A.B. del Campo<sup>1</sup>, N. Aptsiauri<sup>2</sup>, G. Gonzalez-Aseguinolaza<sup>3</sup>, R.M. Mendez<sup>2</sup>, S. Zinchenko<sup>2</sup>, J. Carretero<sup>2</sup>, F. Ruiz-Cabello<sup>2</sup>, F. Garrido<sup>2</sup>

<sup>1</sup>Hospital Universitario Virgen de las Nieves, Analisis Clínicos, Granada, Spain, <sup>2</sup>Hospital Universitario Virgen de las Nieves, Granada, Spain, <sup>3</sup>Center for Applied Medical Research, Division of Hepatology and Gene Therapy, Laboratory of Gene Therapy of Viral Hepatitis, Pamplona, Spain

**Introduction:** Normal HLA class I expression on the tumor cell surface is critical for the successful outcome of cancer immunotherapy, as T cells can only recognize tumor-derived peptides in a complex with self-HLA class I molecules. Many cancer cells evade immune surveillance via low expression or a total loss of HLA class I antigen, the latter is frequently caused by irreversible structural defects, including mutations in the beta 2-microglobulin gene and loss of heterozygosity (LOH) in chromosome 15. In this context, we describe our attempts to recover the normal HLA class I expression on b2m-deficient cancer cells using a non-replicating adenovirus expressing the wild type human b2m gene.

**Materials and Methods:** Recombinant adenovirus carrying human b2m gene, under the control of CMV promoter, was constructed using Cre-lox recombination system. The concentrated virus was added to the cells at a MOI of 25-80. HLA class I surface expression was evaluated by FACS analysis with a panel of monoclonal antibodies. Quantitative real-time PCR was used to analyze the expression level of the APM. Expression of HLA class I in cell lines and in tumors after adenoviral b2m gene transfer was evaluated by Western blotting and immunohistochemistry.

**Results:** We show that the newly generated virus AdCMV b2m mediated an efficient b2m gene transfer in tumor cell lines of different histological origin, leading to the recovery of normal cell surface expression of HLA class I antigens on human melanoma, prostate, and colorectal carcinoma as was showed by FACS analysis, immunocytochemistry, Western blotting and quantitative PCR. To check the effectiveness of the designed adenoviral vector in *in vivo* system we used a human mouse xenograft model in which HLA I negative human tumor cells were injected into NUDE-SWISS and NOD-SCID mice. Normal HLA class I expression was recovered after treatment with AdCMV b2m vector as demonstrated by immunohistochemical analysis and Western blotting.

**Conclusions:** We propose that the adenoviral-mediated recovery or even increase of HLA class I expression on tumor cells in combination vaccination or adoptive T cell therapy can provide a successful approach to improve the clinical efficacy of cancer immunotherapy.

**PD17/2 IMMUNOCOMPATIBILITY OF GELATIN-BASED HYDROGELS SUPPORTING EX VIVO GENE THERAPY**M. Sirova<sup>1</sup>, V. Pakanova<sup>1</sup>, P. Rossmann<sup>1</sup>, L. Kovar<sup>1</sup>, S. Van Vlierberge<sup>2</sup>, P. Dubrue<sup>2</sup>, E. Schacht<sup>2</sup>, B. Rihova<sup>1</sup><sup>1</sup>Institute of Microbiology ASCR v.v.i., Prague, Czech Republic, <sup>2</sup>Ghent University, Ghent, Belgium

**Objective:** Gene delivery based on cationic polymers is being thoroughly studied as an alternative to viral gene delivery. An *ex vivo* strategy for potential treatment of ocular (such as age-related macular degeneration) and cardiovascular (e.g. restenosis) diseases is proposed, capitalizing on implantable membranes, covered with hydrogel ensuring their biocompatibility, and seeded with *ex vivo* transfected cells releasing the relevant gene product. To achieve a clinically valuable strategy, all the implanted materials must be non-toxic, eliciting no adverse responses at the application site. Immunoacceptability of methacrylamide modified gelatin hydrogel to be used to cover the implants to enhance its cell-interaction efficiency has been studied.

**Methods:** Methacrylamide modified gelatin B was crosslinked to form hydrogel using Irragure<sup>®</sup> and UV treatment. *In vitro* screening of the gelatin hydrogel was based upon quantitative multiplex cytokine assay (FlowCytomix, Bender MedSystems) in supernatants of mouse spleen (SC) and peritoneal (PEC) cells cultivated on hydrogel-covered surfaces with/without mitogens. PEC and macrophage cell line RAW 264.7 were used to trace nitric oxide (NO) production, and various cell lines to probe growth-supporting ability of the hydrogel. Histological examination following subcutaneous implantation of hydrogel-coated implants to mice was performed as the final proof of immunoacceptability of the material.

**Results:** The spectrum of cytokines released from the cells cultivated on the hydrogel-coated surfaces conformed well to that of controls, and to the viability/proliferation of the cells. No inflammatory activity, measured by NO and pro-inflammatory cytokines (IL-1 $\alpha$ , IL-6, TNF $\alpha$ ) production was seen in PEC and monocytes/macrophages RAW 264.7. The hydrogel supported well the growth of fibroblasts, endothelial, epithelial (retinal) cells and monocytes/macrophages. Binding of RGD peptide to the methacrylamide modified gelatin hydrogel moderately increased adhesion and growth of the cells. Histological examination revealed a normal repair process induced by the operation, and no adverse tissue responses induced by the implants. The histological results were further substantiated by detection of Th1/Th2 cytokines in the sera, showing no apparent acute inflammation and no skewing of the cytokine repertoire towards the Th1 or Th2 spectrum.

**Conclusions:** Methacrylamide modified gelatin B hydrogel exhibits good cell growth-supporting characteristics, perfect immunoacceptability, and can support the local gene delivery.

**PD17/3 TREATMENT OF CHRONIC VIRAL HEPATITIS IN WOODCHUCKS BY PROLONGED INTRAHEPATIC EXPRESSION OF INTERLEUKIN-12**G. Gonzalez-Aseguinolaza<sup>1</sup>, J. Crettaz<sup>1</sup>, I. Otano<sup>1</sup>, A. Benito<sup>2</sup>, A. Paneda<sup>1</sup>, I. Aurrekoetxea<sup>1</sup>, P. Berraondo<sup>1</sup>, J.R. Rodriguez-Madoz<sup>3</sup>, F. Kreppel<sup>4</sup>, S. Kochanek<sup>4</sup>, J. Ruiz<sup>5</sup>, S. Menne<sup>6</sup>, J. Prieto<sup>1,7</sup><sup>1</sup>FIMA, Gene Therapy and Hepatology, Pamplona, Spain, <sup>2</sup>Clinica Universitaria de Navarra, UNAV, Department of Radiology, Pamplona, Spain, <sup>3</sup>Mount Sinai School of Medicine, Department of Microbiology, New York, United States, <sup>4</sup>University of Ulm, Division of Gene Therapy, Ulm, Germany, <sup>5</sup>DIGNA Biotech, Madrid, Spain, <sup>6</sup>Cornell University, Department of Clinical Sciences, Ithaca, United States, <sup>7</sup>University Clinic and CIBERhd, Liver Unit, Pamplona, Spain

Hepatitis B virus (HBV) infection is estimated to cause about 1 million deaths per year. Current therapies against chronic HBV are effective but needs to be continued for many years resulting in high cost, drug resistant variants and frequent occurrence of relapse after discontinuation of therapy. Patients with chronic HBV infection show no protective humoral immunity and a weak or undetectable virus-specific T-cell response. The precise mechanism responsible for this immunotolerance is still unknown. Interleukin-12 (IL-12) is a cytokine which is essential in the induction of effective cell-mediated immunity against viruses and other pathogens. IL-12 promotes Th1 type responses, enhances cytotoxic T cell activity and stimulates T lymphocytes and NK cells to produce IFN- $\gamma$ . In the present study we have tested the antiviral potential of IL-12-mediated gene therapy using a high-capacity adenovirus (HC-Ad) encoding murine IL-12 (mIL-12) under the control of a liver-specific inducible promoter responsive to the progesterone antagonist RU486. As an animal model of chronic HBV infection, we have used woodchucks chronically infected with woodchuck hepatitis virus (WHV). WHV is a hepadnavirus with genomic organization, biological properties, and replicative strategy essentially identical to HBV. We demonstrate that prolonged intrahepatic expression of IL-12 overcomes immunological tolerance to WHV antigens and induces sustained antiviral effects in woodchucks with chronic WHV infection and viral load below 10<sup>10</sup> vg/ml. These observations point to IL-12 gene therapy as an alternative approach for the treatment of chronic HBV infection.

**PD17/4 INTENSIVE PHARMACOLOGICAL IMMUNOSUPPRESSION ALLOWS FOR REPETITIVE LIVER GENE TRANSFER WITH RECOMBINANT ADENOVIRUS IN NON-HUMAN PRIMATES**A. Fontanellas<sup>1</sup>, S. Hervás-Stubb<sup>1</sup>, I. Mauleon<sup>2</sup>, J. Dubrot<sup>1</sup>, C. Unzu<sup>1</sup>, A. Palazón<sup>1</sup>, U. Mancheño<sup>2</sup>, A. Sampedro<sup>1</sup>, M. Collantes<sup>2</sup>, J. Prieto<sup>2</sup>, I. Peñuelas<sup>3</sup>, I. Melero<sup>1</sup><sup>1</sup>University of Navarra, FIMA, Pamplona, Spain, <sup>2</sup>University of Navarra, Pamplona, Spain, <sup>3</sup>FIMA, Pamplona, Spain

Repeated administration of gene therapies is hampered by host immunity towards vectors and transgenes. Attempts to circumvent anti-vector immunity include pharmacological immunosuppression or alternating different vectors with the same transgene. Our studies show that B cell depletion with anti-CD20 mAb and concomitant T cell inhibition with clinically available drugs permits repeated liver gene transfer to non-human primates with recombinant adenovirus. Adenoviral vector-mediated transfer of the HSV-1 Thymidine kinase reporter gene was visualized *in vivo* with a semiquantitative transgene-specific positron emission tomography (PET) technique, liver immunohistochemistry and immunoblot for the reporter transgene in needle biopsies. Neutralizing antibody and T cell mediated responses towards the viral capsids were sequentially monitored and found to be repressed by the tested drug combinations.

When anti-adenovirus antibodies (yet at a low titer) along with a weak T cell anti-adenoviral responsiveness were present (indicating previous exposure to adenovirus), the immunosuppressive drug regimen was not effective in achieving gene transfer upon readministration one month after the first exposure to the viral vector, even if the first gene-transfer procedure had been as efficacious as in naïve macaques.

Notably, repeated liver transfer of the HSV1-tk reporter gene with the same recombinant adenoviral vector was achieved in the liver two macaques undergoing a clinically feasible treatment that ablated humoral and cellular immune responses six months following the first exposure to a recombinant adenovirus.

**PD17/5 NKG2A SILENCING IN EFFECTOR CELLS TO IMPROVE EFFECTIVITY OF CELL-BASED THERAPEUTICS**C. Figueiredo<sup>1</sup>, J. Zenk<sup>1</sup>, B. Eiz-Vesper<sup>1</sup>, A. Seltsam<sup>2</sup>, R. Blasczyk<sup>1</sup><sup>1</sup>Hannover Medical School, Institute for Transfusion Medicine, Hannover, Germany, <sup>2</sup>German Red Cross, Blood Services NSTOB, Institute Springe, Springe, Germany

The heterodimeric NKG2A/CD94 receptor delivers an inhibitory signal upon recognition of HLA-E molecules. In several studies it has been demonstrated that T or NK cell cytotoxic activity and cytokine production are significantly reduced by signalling via NKG2A/CD94 receptor. In hematopoietic stem cell transplantation, expression of NKG2A on NK and T cells has been shown to compromise the graft-versus-leukemia effect. In addition, NKG2A was shown to inhibit tumor-specific T cell responses. In this study, we developed a RNAi-based approach to permanently silence the expression of NKG2A molecules on NK and T-cells. The functional relevance of NKG2A silencing for the cytotoxic potential of genetically engineered NK and T-cells was evaluated.

NKG2A+ cells were isolated from fresh PBMCs. Lentiviral vectors were designed to express short hairpin RNA sequences (shRNA) targeting NKG2A transcripts. The level of NKG2A suppression was measured by flow cytometry and real-time RT-PCR. The effect of NKG2A receptor silencing on the cytolytic potential of NK and T cells was evaluated in cytotoxicity assays using K562 and B-LCL cells as targets. In addition, granzyme B mRNA transcript levels were detected by real-time RT-PCR.

The transduction of inducible RNAi cassettes containing the sequences for shRNAs targeting NKG2A caused a reduction of protein expression by up to 80% in NK and T cells. In cytotoxicity assays, it was demonstrated that NKG2A silencing was effective to enhance NK and CD8<sup>+</sup> T cell lysis by up to 40%. In comparison with unmodified cells, granzyme B transcript levels were upregulated by up to 12-fold in NKG2A silenced cells after target exposure. Expression of NKG2A-specific shRNA did not affect the expression of the activating markers NKp44 on NK cells and CD25 on T cells.

Our data suggest that RNAi-mediated silencing of NKG2A in effector cells could improve the effectivity of cell-based immunotherapeutics and might be of particular interest in an autologous approach.

**PD17/6 IMMUNE MODULATION THROUGH RNA INTERFERENCE-MEDIATED SILENCING OF CD40 IN DENDRITIC CELLS**A. A. Pourfathollah<sup>1</sup>, M. H. Karimi<sup>1</sup>, S. M. Moazzeni<sup>1</sup>, Z. S. Soheili<sup>2</sup>, S. Samiee<sup>3</sup>, P. Ebadi<sup>4</sup><sup>1</sup>Tarbiat Modares University, Immunology, Tehran, Iran, Islamic Republic of, <sup>2</sup>Institute of Genetic Engineering and Biotechnology, Tehran, Iran, Islamic Republic of, <sup>3</sup>Iranian Blood Transfusion Organization Research Center, Tehran, Iran, Islamic Republic of, <sup>4</sup>Tarbiat Modares University, Tehran, Iran, Islamic Republic of

RNA interference (RNAi) is an exciting mechanism for knocking down any target gene in transcriptional level. It is now clear that small interfering RNA (siRNA), a 19-21 nt long ds RNA, can trigger a degradation process (RNAi) that specifically silences the expression of a cognate mRNA. Our finding in the present study showed that down regulation of CD40 gene expression in dendritic cells (DCs) by RNAi culminated to immune modulation. Effective delivery of siRNA into dendritic cells would be a reasonable method for blocking of CD40 expression at the cell surface without any effect on other genes and cell cytotoxicity. In the present study, the effects of siRNA against CD40 mRNA on the function and phenotype of dendritic cells were investigated. Dendritic cells were separated from mice spleen and then cultured *in vitro*. By the means of Lipofectamine2000, siRNA were delivered to the cells and the efficacy of transfection was estimated by flow cytometry. By Annexine V and Propidium Iodide staining, we could evaluate the transfected cell viability. Also the mRNA expression and protein synthesis were assessed by real-time PCR and flow cytometry respectively. Knocking down the CD40 gene in DCs caused increase in IL-4 production, decrease in IL-12 production and allostimulation activity. All together, these effects would stimulate Th2 cytokines production from allogeneic T cells *in vitro*.

**PD17/7 IL-10 RNAI APPROACHES AGAINST IMMUNE CELLS AND ALLEVIATE DISEASE SEVERITY IN LUPUS PRONE NZB/W F1 MICE**Y.-H. Chang<sup>1</sup>, K.-H. Lu<sup>2</sup>, B.-L. Chiang<sup>1</sup><sup>1</sup>National Taiwan University Hospital, Department of Pediatrics, Taipei City, Taiwan, Republic of China, <sup>2</sup>Graduate Institute of Immunology, College of Medicine, National Taiwan University, Taipei City, Taiwan, Republic of China

**Background:** SLE is an autoimmune disease caused by multiple pathogenic factors, including genetic, epigenetic, hormone, environmental, and diverse immune components. In previous studies, people demonstrated that removal of IL-10 by antibody treatment was able to suppress the population of B-1 cell, an auto-antibody producing cell, and alleviate the SLE disease intensity. However, the administration of antibody against the critical immune regulatory molecule, IL-10, might cause subsequent ominous side effects. Thus, alternative approaches might be required.

**Methods:** We hope to develop an alternative approach to deplete IL-10 with lentivirus-based RNAi, which is capable to be delivered more specifically and locally. Now we are going on our assays upon an SLE murine model, NZB/W F1, and hope to see certain promising therapeutic effects.

**Results:** We have screened 2 sufficient siRNA segments against IL-10 effectively decrease both mRNA and protein levels in vitro. They are also able to down-regulate the IL-10 level in Balb/c murine model. We now try to investigate the effect of RNAi against IL-10 for the depletion of B-1 cells and subsequent autoantibody production in lupus prone NZB/W F1 mice.

**Conclusion:** The development of RNAi technique provides a new hope of therapeutic design for the target therapy. We believe the study here might shed light on further development of novel treatment for lupus.

**PD17/8 COMPARISON OF LIPOFECTAMINE 2000 AND FUGENE6 EFFICIENCY IN THE TRANSFECTION OF SPLEEN DERIVED DENDRITIC CELLS WITH SIRNA AGAINST CD40 MRNA**P. Ebadi<sup>1</sup>, A. A. Pourfathollah<sup>2</sup>, M. H. Karimi<sup>3</sup>, S. M. Moazzeni<sup>2</sup><sup>1</sup>Faculty Member of Islamic Azad University, Kazeroun Branch, Biology Department, Kazeroun, Iran, Islamic Republic of, <sup>2</sup>Tarbiat Modares University, Faculty of Medical Sciences, Immunology, Tehran, Iran, Islamic Republic of, <sup>3</sup>Shiraz University of Medical Sciences, Transplant Research Center, Shiraz, Iran, Islamic Republic of

In the recent years, different gene-silencing strategies have been evolved to target the specific gene function. Among these methods, RNA interference (RNAi), is one of the most useful tools to inhibit the gene function in the post transcriptional level. Although siRNA is accepted as a research and therapeutic agent, but delivery challenges are still remain. In this field, an efficient and functional reagent with out any toxicity would be attractive. The use of chemical transfection reagents siRNA duplex delivery into mammalian cells is strongly recommended.

So, In this study we compare the efficacy of two chemical reagents, Lipofectamine2000 and FuGENE6 in the delivery of siRNA into mice dendritic cell derived spleen. By the means of fluorescein-labeled non-silencing siRNA duplex as a model system, we could evaluate the efficacy of lipofectamine2000 and FuGENE6 in siRNA transfection, also we could investigate the potential of these reagents to be an efficient siRNA carrier and their effects on CD40 gene knockdown and DC viability. In conclusion, our findings indicate that Lipofectamine2000 in comparison with FuGENE6 has better efficacy in siRNA delivery into dendritic cells.

**PD17/9 ESTABLISHMENT OF A P53 HUMANIZED MOUSE TUMOR MODEL TO EVALUATE IN VIVO SAFETY AND THERAPEUTIC EFFICIENCY OF P53 TCR GENE TRANSFER**H. Echchannaoui<sup>1</sup>, E. Antunes<sup>1</sup>, C. Lotz<sup>1</sup>, M. Theobald<sup>1</sup><sup>1</sup>University Medical Center Utrecht (UMC), Utrecht, Netherlands

A major limitation to the generation of tumor-associated antigens (TAA)-reactive T cells is due to self-tolerance that results in a peripheral T cell repertoire devoid of high-avidity TAA-specific, tumor-reactive cytotoxic T cells (CTL).

We have reported that HLA-A\*0201 (A2.1) transgenic mice can be used to circumvent self-tolerance to universal human TAA, such as p53 and to generate efficient tumor-reactive CTL. We have shown that T cell antigen specificity can be reliably redirected by introducing T cell receptor (TCR) genes. We used A2.1 transgenic mice, in which the mouse CD8 molecule cannot efficiently interact with A2.1 to generate a high-affinity, CD8-independent TCR specific for a commonly expressed, tumor-associated CTL epitope (264-272) derived from the human p53 protein. Retroviral expression of CD8-independent p53-specific TCR into T cells, allowed CD8+ T lymphocytes to acquire a broad tumor-specific CTL activity but also redirected CD4+ T cells into potent tumor-reactive, p53-specific T helper cells. However, implementing TCR-based gene therapy of malignant diseases requires further molecular optimizations.

To enhance the expression of the transduced TCR in T lymphocytes, we codon-optimized the TCR gene sequence. In addition, cysteines were introduced into the constant region of the  $\alpha$  and  $\beta$  chains to promote preferential pairing and prevent/reduce the formation of mixed TCR dimers with undefined specificity. Finally, to entirely prevent interchain pairing between transduced and endogenous TCR chains, we used a single chain p53-specific TCR construct. We will evaluate the safety and therapeutic efficiency of p53 TCR engineered T cells in a humanized Hupki-A2.1 (Human p53 knock in) mouse model. p53-/A2.1 mouse embryonic fibroblasts transformed and transduced with recombinant retroviral vectors encoding different p53 mutants were shown to overexpress p53 and to be effectively recognized by p53 TCR-transduced T cells. Tumor cells will be transferred to preconditioned Hupki-A2.1 recipients that will receive p53 TCR-redirection syngeneic T lymphocytes. In addition a bioluminescent imaging system will be used to monitor tumor growth and to track the infused p53-TCR transduced T cells in live animals.

We will assess p53 TCR-transduced T cells not only for their in vivo effectiveness for tumor eradication but also for their potential risk of inducing autoimmunity.

**PD17/10 CORRECTION OF WISKOTT-ALDRICH SYNDROME BY HEMATOPOIETIC STEM CELL GENE THERAPY**K. Boztug<sup>1</sup>, M. Schmidt<sup>2</sup>, A. Schwarzer<sup>3</sup>, I. Avedillo Díez<sup>1</sup>, R. Dewey<sup>1</sup>, S. Naundorf<sup>4</sup>, K. Kühlcke<sup>4</sup>, I. Kondratenko<sup>5</sup>, L. Maródi<sup>6</sup>, C. von Kalle<sup>2</sup>, C. Klein<sup>1</sup><sup>1</sup>Hannover Medical School, Department of Pediatric Hematology/Oncology, Hannover, Germany, <sup>2</sup>National Center for Tumor Diseases, Department of Translational Oncology, Heidelberg, Germany, <sup>3</sup>Hannover Medical School, Department of Experimental Hematology, Hannover, Germany, <sup>4</sup>EUETS AG, Idar-Oberstein, Germany, <sup>5</sup>Russian Clinical Children's Hospital, Department of Clinical Immunology, Moscow, Russian Federation, <sup>6</sup>Medical and Health Science Center, University of Debrecen, Department of Infectious and Pediatric Immunology, Debrecen, Hungary

Wiskott Aldrich Syndrome is a life-threatening immune-disorder characterized by bleeding secondary to microthrombocytopenia, immunodeficiency, autoimmunity, and susceptibility to lymphoma. Based on extensive preclinical studies, a clinical gene therapy protocol was developed at Hannover Medical School. We here present a preliminary analysis of the first two patients treated in 2006. Patients showed evidence of long-term gene marking and WAS protein expression in myeloid and lymphoid cells as well as CD34+ hematopoietic stem cells, respectively. Furthermore, the majority of peripheral thrombocytes showed evidence of WASP expression. Functional reconstitution was documented in dendritic cells (podosome formation), T cells (proliferation in response to CD3-signaling), and NK cells (formation of immunological synapse). TCR Vb spectratyping analyses showed an improvement of receptor skewing upon gene therapy. Clinically, the patients are in excellent condition approximately 2.5 years after gene therapy. Eczema, autoimmunity, bleeding diathesis and immunodeficiency has resolved completely in these patients. Molecular insertion site analysis yielded a polyclonal pattern of hematopoiesis. In sum, hematopoietic stem cell gene therapy may be of benefit for patients with WASP deficiency. More extensive studies and longer follow-up are needed to determine the safety profile of this experimental approach.

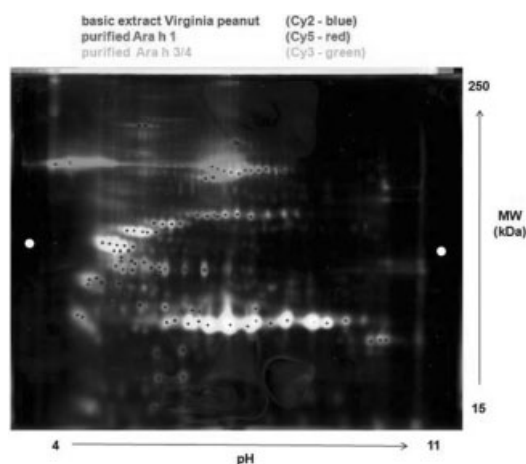
**PD19 – GENETIC AND ENVIRONMENTAL ALLERGIES AND IMMUNOTHERAPY IN ALLERGY AND ASTHMA****PD19/1 ALLERGEN CONTENT OF EXTRACTS FROM PEANUT VARIANTS ANALYZED BY 2D-DIGE ANALYSES**H. Schmidt<sup>1</sup>, C. Gelhaus<sup>2</sup>, M. Nebendahl<sup>1</sup>, A. Petersen<sup>3</sup>, M. Leippe<sup>2</sup>, W.-M. Becker<sup>3</sup>, O. Janssen<sup>1</sup><sup>1</sup>University Hospital Schleswig-Holstein Campus Kiel, Institute for Immunology, Kiel, Germany, <sup>2</sup>Christian-Albrechts University, Zoological Institute, Department of Zoophysiology, Kiel, Germany, <sup>3</sup>Research Center Borstel, Molecular and Clinical Allergy, Borstel, Germany

**Objectives:** Over the past decade, an increasing prevalence of peanut allergies was observed worldwide. Peanuts are meanwhile categorized among the most dangerous food allergens. This is particularly relevant since peanut-derived ingredients are widely used in industrial food production. To minimize the problem of hidden food allergens causing severe anaphylactic reactions, food companies search for strategies to reduce the allergenicity of peanut-derived food additives either by genetically altering the allergen content or by identifying peanut varieties with low levels of major allergens.

**Methods:** In the present study, we investigated extracts from Indonesian peanuts that apparently contain lower levels of the major allergens Ara h 1 and Ara h 2. Basic extracts of standard Virginia type and Indonesian peanuts were compared by two-dimensional difference gel electrophoresis (2D-DIGE) and subsequent mass spectrometric analysis of individual spots.

**Results:** We identified more than hundred individual components in peanut extracts by mass spectrometry and thus provide the first high-resolution allergen map that also includes so far unknown fragments of major peanut allergens. In addition, we demonstrate that Indonesian peanut seeds indeed contain much less Ara h 1 and Ara h 2. Apparently, the reduction in these storage proteins is compensated by higher contents of Ara h 3/4. The reduced level of Ara h 1 associated with a significantly lower abundance of the most potent peanut allergen Ara h 2 in various Indonesian peanuts was confirmed by Western blotting with monoclonal antibodies and sera of allergic patients.





[Peanut DIGE]

**Conclusion:**

1. Peanut variants from different locations differ significantly in the composition of allergenic seed storage proteins. This is important for plant breeders in order to minimize major allergens in next-generation peanut varieties.
2. The characterization and identification of allergen isoproteins require a high-resolution technology. Clearly, 2D-DIGE is suited to not only identify alterations in peanut variants or differentially processed peanuts but also in purified allergen preparations.

PD19/2

**TREATMENT OF ALLERGIC DISEASE WITH HELMINTH IMMUNOMODULATORS**M. Lendner<sup>1</sup>, C. Schnöller<sup>1</sup>, E. Hamelmann<sup>2</sup>, R. Lucius<sup>1</sup>, S. Hartmann<sup>1</sup><sup>1</sup>Humboldt-Universität zu Berlin, Department of Molecular Parasitology, Berlin, Germany, <sup>2</sup>Ruhr-University Bochum, University Children's Hospital, Bochum, Germany

Parasitic worms use intricate mechanisms to manipulate their host's immune system and suppress inflammatory responses directed against them. Previously, we characterized Cystatin and Tropomyosin of filarial nematodes and could show that these proteins exhibit immunomodulatory properties. Both molecules target macrophages and stimulate anti-inflammatory immune responses associated with high levels of IL-10 *in vitro*.

Here, we report on the influence of recombinantly expressed filarial cystatin and filarial tropomyosin on a murine model of ovalbumin-induced airway hyperreactivity, a disease governed by Th2-responses.

Application of cystatin during or after sensitization and prior to challenge with the model allergen suppressed allergic airway responsiveness, cellular recruitment to the lung (primarily eosinophils), mucus production and reduced allergen-specific IgE and IL-4 production. These effects were reversible by depletion of macrophages and by neutralization of IL-10 via anti-IL-10R antibodies. However, depletion of regulatory T cells had only limited effects. In comparison, filarial tropomyosin – although having intrinsic allergic activities – also showed immunomodulatory effects on the unrelated allergic disease. Among others drastically reduced levels of allergen-specific IgE were observed after treatment with this helminth protein.

Our data provide evidence that single immunomodulatory proteins of parasitic nematodes represent unique tools to combat inflammation and demonstrate novel strategies for the development of therapeutics to treat allergic diseases.

PD19/3

**DISTRIBUTION OF ALLERGENS AND NON-ALLERGENIC HOMOLOGUES WITHIN PROTEIN FAMILIES: THE ROLE OF SEQUENCE SIMILARITY TO HUMAN PROTEINS**C. Radauer<sup>1</sup>, H. Breiteneder<sup>1</sup><sup>1</sup>Medical University of Vienna, Department of Pathophysiology, Vienna, Austria

**Objectives:** Most allergens can be classified into a small number of families of evolutionary related proteins. However, most members of these allergen-containing families are non-allergenic. Using allergenic and non-allergenic members of important families of animal allergens, we sought to systematically examine the role of evolutionary distance from human proteins as a determinant of allergenicity.

**Methods:** Sequences from the tropomyosin family, the lipocalin superfamily (lipocalins and cytosolic fatty acid binding proteins – cFABPs) and the EF-hand superfamily (parvalbumins and troponins C) were included in the analysis. We collected allergen sequences using the AllFam database of allergen families ([www.meduniwien.ac.at/allergens/allfam/](http://www.meduniwien.ac.at/allergens/allfam/)) as the data source. Sequences of non-allergenic homologues were obtained by performing BLAST searches against vertebrate, mollusc, and arthropod sequences of the NCBI non-redundant (nr) protein database. All sequences were compared to human sequences from the nr database by BLAST. Percent identity to the closest human homologue was defined as number of identical aligned residues divided by total query length.

**Results:** Allergenic tropomyosins were confined to molluscs and arthropods showing 48-59% identity to human tropomyosins, while non-allergenic vertebrate homologues shared at least 73% of their sequences with human tropomyosins. Likewise, the lack of allergenicity among vertebrate proteins from the cFABP and troponin C families was reflected by their high conservation among vertebrates with sequence identities to human homologues of 31-100% and 41-100% for cFABPs and troponins C, respectively. Invertebrate allergens from both families showed sequence identities to human homologues below 46%. Lipocalins were confined to vertebrates with 22-99% identity to human lipocalins, while allergenic mammalian family members showed at most 60% identity to human proteins. Similarly, parvalbumins occurred exclusively among vertebrates with allergenic family members found among fishes and amphibians. With the exception of an alpha-parvalbumin from edible frog, all allergenic parvalbumins shared less than 57% of their sequences with human proteins.

**Conclusion:** With a single exception, all examined allergens displayed at most 60% sequence identity to human homologues. The connection between sequence conservation and distribution of allergens was demonstrated by comparing members of conserved (troponin C and cFABP) and variable (parvalbumin and lipocalin) families that belong to the same structural superfamily.

PD19/4

**SLC11A1 GENE POLYMORPHISMS ASSOCIATION WITH SUSCEPTIBILITY IN LEPROMATOUS LEPROSY MEXICAN PATIENTS**M. Montoya Buelna<sup>1</sup>, M. Fafutis Morris<sup>2</sup>, E. R. Ochoa Ramirez<sup>1</sup>, J. Becerra Conteras<sup>1</sup>, T.P.K. Reddy<sup>1</sup>, L. Sandoval Ramirez<sup>1</sup><sup>1</sup>Western Biomedical Research Center (CIBO), Mexican Institute of Social Insurance (IMSS), Guadalajara University, Genetics, Guadalajara, Mexico, <sup>2</sup>Guadalajara University, Immunology, Guadalajara, Mexico

Leprosy is a granulomatous disease that affects the skin and peripheral nerves caused by *Mycobacterium leprae*. The range of clinical forms varying from tuberculoid (LT) to lepromatous leprosy (LL) comes from the variations in the cellular immune response to the mycobacterium. Genetic factors of the host play an important role in the manifestation of disease susceptibility. Recent studies provide evidence that the human homologue of mice natural-resistance-associated macrophage protein (*Nramp1*) gene is implicated in the phagolysosomal function of macrophages as well as antigen presentation. The mechanism by which NRAMP1 molecule may modulate the Th1 and Th2 immune response is unclear. Information on the role of polymorphisms of *SLC11A1* (*NRAMP1*) gene in susceptibility to leprosy has been controversial. Family studies in Vietnamese patients, found weak evidence for linkage of *SLC11A1* to leprosy, however no evidence was found for linkage in Polynesian and Pakistani families; moreover, in African patients recently it was reported association of *SLC11A1* gene polymorphisms with leprosy type but no susceptibility to leprosy *per se*.

**Background:** The aim of this study was to analyze the association of *SLC11A1* polymorphisms with LL in population at the West of Mexico.

**Methods:** INT4 and D543N polymorphisms of *SLC11A1* gene were analyzed by polymerase chain reaction. In LL (n=81) patients, LT were excluded from analysis because there were few of them (n=4). Comparable numbers of healthy subjects (HS, n=60) were studied simultaneously.

**Results:** there was no difference from the genotype frequencies INT4 (p=0.095) and D543N (p=0.708) polymorphisms to the LL and control group (for INT4 genotype, P > 0.05; for D543N genotype, P > 0.05). The allelic frequencies of D543N polymorphism were statistically different (OR=8.3077, P< 0.05, 95% confidence interval), but not for INT4.

**Conclusions:** Differences between the allelic variant frequencies in D543N indicate a possible role in the susceptibility to leprosy. Further studies will be undertaken to increase sample sizes, and the screening of entire coding region of *SLC11A1* gene. There could be linkage disequilibrium with neighboring loci in leprosy patients.

# PD19/5 THE CONCEPT OF COMPONENT RESOLVED DIAGNOSIS APPLIED IN MELON ALLERGY – A RELEVANT ELICITOR OF FOOD ALLERGY IN SOUTHERN EUROPE

M. Bruckmueller<sup>1</sup>, C. Oberhuber<sup>1</sup>, S. Gaier<sup>1</sup>, S. Vazquez-Cortez<sup>2</sup>, M. Bublin<sup>1</sup>, M. Fernandez-Rivas<sup>2</sup>, K. Hoffmann-Sommergruber<sup>1</sup>

<sup>1</sup>Medical University Vienna, Dept. of Pathophysiology, Vienna, Austria, <sup>2</sup>Hospital Clinico San Carlos, Allergy Department, Madrid, Spain

**Background:** Up to date, food allergy diagnosis is performed using protein extracts instead of single purified well-defined food allergens. Within the EC funded IP EuroPrevall an allergen library was established. The allergens derived from allergenic food sources already known as well as new allergenic food sources. Among those, melon has been described as a relevant elicitor of allergic symptoms in Southern Europe.

**Objective:** The aim of the study was -) to define the panel of allergens in melon, -) to use single allergens for component resolved diagnosis for a group of well defined patients with melon allergy and -) to identify cross reactive structures.

**Methods:** Melon extract was prepared from Piel de Sapo melon (*Inodorus variety*) according to standard methods. Melon extract was characterized by SDS-PAGE, immunoblot and ELISA. Proteins from melon extract were fractionated by Con A affinity chromatography. Fifteen sera from patients allergic to melon were selected on the basis of clinical symptoms and positive CAP results and their IgE binding patterns investigated by immunoblot and inhibition assays.

**Results:** SDS-PAGE analysis of melon fruit extracts revealed distinct protein patterns with bands in the range from 9 to 72 kDa and no differences between non-reducing and reducing conditions. IgE immunoblotting with individual patient's sera showed several IgE binding proteins of approximately 11-72 kDa. Cucumisin, 67 kDa subtilisin-like protease was recognized by specific IgE in 53% of the sera from the allergic patients. IgE reactivity to profilin (13 kDa) and to a pathogenesis-related protein family 1 (16 kDa) was found in 80% and 13% respectively.

Cross reactivity between the PR 1 protein and a 17 kDa protein from grass pollen was detected.

**Conclusion:** So far, 3 food allergens were identified in melon. Cross reactivity of the pathogenesis related protein from melon to a 17 kDa protein from grass pollen was identified. Whether this has implications on the onset or persistence of melon fruit allergy needs to be further investigated.

**Acknowledgements:** This study was supported by the EC-project EuroPrevall, FOOD-CT-2005-514000.

# PD19/6 CLASS II HLA ALLELES AND SINGLE NUCLEOTIDE CYTOKINE GENE POLYMORPHISMS IN ASTHMA BRONCHIALE IN SLOVAK POPULATION

M. Buc<sup>1</sup>, M. Krivošíková<sup>1</sup>, J. Javor<sup>1</sup>, M. Dzurilla<sup>1</sup>, M. Vrlík<sup>2</sup>

<sup>1</sup>Comenius University School of Medicine, Department of Immunology, Bratislava, Slovakia, <sup>2</sup>Martin Centre of Immunology, Martin, Slovakia

**Objectives:** Atopic diseases, including *asthma bronchiale* (AB), develop in genetically predisposed individuals. Out of many genes involved in its predisposition those of the HLA complex and cytokines genes are of paramount importance. Studies on associations between AB and genetic polymorphisms had been absent in Slovakia what made us to investigate its association to HLA-DQB1, -DRB1 alleles and to single nucleotide polymorphism (SNP) of 13 cytokine genes (IL-1 $\alpha$ , IL-1 $\beta$ , IL-1R, IL-1RA, IL-4R $\alpha$ , IL-12, IFN- $\gamma$ , TGF- $\beta$ 1, TNF, IL-2, IL-4, IL-6 and IL-10), respectively.

**Methods:** 51 patients (28 men and 23 women) sensitive to pollen allergens and suffering from AB were investigated. The age of patients varied from 8-54 years. The diagnosis was based on anamnesis, clinical investigation (GINA criteria), skin tests, and investigation of IgE specific antibodies. The control group comprised 140 unrelated healthy individuals. The HLA typing and was carried out by a low resolution PCR-SSP using GenoVision primer sets (Olerup SSP™ AB Sweden); similarly, the cytokine SNP genotyping was performed by a PCR-SSP using commercially available kits (Invitrogen Corp., USA). P-values were calculated using the Fisher's exact test.

**Results:** The statistically significant positive association of DRB1\*01 (16.70% vs. 7.30%; p = 0.0107) and negative association of DRB1\*11 (10.80% vs. 20.40%; p = 0.0324) were observed in the investigated group of patients. From among all cytokine SNPs investigated, an association with the IL-10 polymorphism was observed only. The statistically significantly increased occurrence rates of the IL-10 -1082 A allele (68.63% vs. 56.79%; p = 0.0446) and the IL-10 -1082 A/-819 C/-592 C haplotype (42.16% vs. 30.00%; p = 0.0277), respectively, were found in the patient's group compared to controls. No other statistically significant differences were found. The results on HLA-DRB1\*01 and the IL-10 polymorphism associations with AB are agreement with similar reports in the literature.

**Conclusion:** Preliminary results of our study show that pollen sensitive patients suffering from atopic AB compared to healthy population have an increased frequency of DRB1\*01 allele. Further, our results indicate that polymorphisms of the immunosuppressive IL-10 gene can be ones of many genetic factors influencing a predisposition to AB too.

# PD19/7 PREVALENCE OF FOOD ALLERGY AMONG UNIVERSITY STUDENTS IN AHVAZ CITY, IRAN

M.A. Assarehzadegan<sup>1</sup>, A. Shakurnia<sup>1</sup>

<sup>1</sup>Ahvaz Jundishapur University of Medical Sciences, Immunology Department, Ahvaz, Iran, Islamic Republic of

**Objectives:** The aim of the study, the first of its kind in Southwest Iran, was to estimate the prevalence of Food allergy in Ahvaz city, the capital of Khuzestan province.

**Methods:** A cross-sectional, descriptive, questionnaire-based study was conducted in Ahvaz universities. The questionnaires (3500) were distributed in two main universities in Ahvaz. The return rate was 99.5% (3482).

**Results:** Out of 3482 participants 1536 (44.1%) and 1946(55.9%) were men and women, respectively. The main foods reported as causing adverse reactions were Egg plant (20.4%), melon (16.1%), Cow milk (15.2%), Kiwi (9.9%), Tomato (6.6%), and Grape (6.3%).

**Conclusion:** We suggest standard test to diagnosis fruit allergy and molecular and immunocharacterization of common allergenic foods to production of recombinant allergen for diagnostics and therapeutic targets.

# PD19/8 PREVALENCE OF FOOD ALLERGY IN SOUTHWEST IRAN, KHUZESTAN PROVINCE: A QUESTIONNAIRE STUDY

M.A. Assarehzadegan<sup>1</sup>, A. Shakurnia<sup>1</sup>

<sup>1</sup>Ahvaz Jundishapur University of Medical Sciences, Immunology Department, Ahvaz, Iran, Islamic Republic of

**Objectives:** The aim of this study has been to report the prevalence of food allergy in adults in Khuzestan province, to identify common food allergens.

**Methods:** A cross-sectional, descriptive, questionnaire-based study was conducted in Southwest Iran, Khuzestan province. We used standardized questionnaire. In total, 2235 unselected adults were concluded in our study. However, 2231 participants completed the questionnaire, and then the return rate was 99.8%.

**Results:** Out of 2231 subjects 904(40.5%) and 1327(59.5%) were men and women, respectively. The main foods reported as causing adverse reactions were egg plant (20%), melon (17.7%), cow milk (14.5%), processed meat (14.1%), kiwi (10.1%), tomato (7.5%), grape (6.4%), egg (6.4%) and cantaloupe (6.2%).

**Conclusion:** Egg plant, melon and Cow milk were the most reported allergens. We suggest standard test to diagnosis fruit allergy and molecular and immunocharacterization of common allergenic foods to production of recombinant allergen for diagnostics and therapeutic targets.

# PD19/9 A MODIFIED ADENINE CHEMICALLY COUPLED TO THE ALLERGEN MOLECULE OF DERMATOPHAGOIDES PTERONYSSINUS DOWN-REGULATES ALLERGEN-SPECIFIC TH2 RESPONSES

L. Fili<sup>1</sup>, E. Cardilicchia<sup>1</sup>, P. Fanti<sup>2</sup>, L. Maggi<sup>1</sup>, C. Manuelli<sup>1</sup>, A. Matucci<sup>1</sup>, A. Vultaggio<sup>1</sup>, F. Annunziato<sup>1</sup>, E. Occhiato<sup>2</sup>, A. Guarna<sup>2</sup>, E. Maggi<sup>3</sup>, P. Parronchi<sup>3</sup>

<sup>1</sup>Center for Research, Transfer and High Education DENOthe, University of Florence, Internal Medicine, Immunoallergology Unit, Firenze, Italy, <sup>2</sup>University of Florence, Dept. of Organic Chemistry 'Ugo Schiff', Firenze, Italy, <sup>3</sup>Center for Research, Transfer and High Education DENOthe, Internal Medicine, Immunoallergology Unit, Firenze, Italy

**Background:** Allergen-specific immunotherapy is the only treatment able to cure allergic diseases and recently TLR ligands (CpG-ODNs, MPL-A) have been used as novel adjuvants controlling the overexpression of Th2 cytokines and chemokines providing clinical benefit. As we recently found that an 8-OH substituted adenine acting as TLR7-ligand downregulates human allergen-specific Th2 responses in vitro and airway inflammation in OVA-sensitized mice, we have studied a new compound able to be coupled with allergenic molecule(s) as a possible new adjuvant for vaccination protocols where responses other than Th2 are hopeful.

**Methods:** The 8-OH-substituted adenine SA-26E was chemically coupled with Dermatophagoides pteronyssinus extract (DP-Conj), recombinant Der p2 (rDer-Conj) or purified natural Der p2 (nDer-Conj). NF-kB induction by the three conjugates was evaluated in CD14+ cells. Cytokine and chemokine production by Conj-stimulated BDCA4+ and CD14+ cells was assessed by ELISAs. Th-regulation by the different conjugates were investigated in allergen-specific human T cell lines. TLR-triggering was evaluated in transfected HEK293 cell line.

**Results:** The chemical derivative of 9-benzyl-2-butoxy-8-hydroxyadenine (SA-2) with a critical substitution in position 2 (SA-26E, ester) coupled either with Dermatophagoides pteronyssinus extract or purified Der p2 (natural or recombinant origin) induced innate cells to produce type I interferons (IFN) and IL-12 at similar levels than dispersible TLR ligands (R-848 or LPS). Lower levels of inflammatory IL-6 and TNF- $\alpha$  than R-848 were seen. As a consequence, the three conjugates downregulated Th2-prone allergen T cell lines as assessed by cytokine production in the supernatants, intracellular cytokine expression and Th2-related transcription factor expression (GATA-3 and T-bet). Unconjugated SA-26E was ineffective in the different culture systems.

**Conclusion:** 8-OH-adenine(s) represent new compounds inducing NF-kB translocation and downregulating Th2-responses in vitro. A system of chemical conjugation to relevant proteins as allergens has been set up with maintenance of regulatory effects of these complexes thus allowing the development of new vaccine strategies.

# PD19/10 THE NEUTROPHIL ACTIVATING PROTEIN OF HELICOBACTER PYLORI DOWN-MODULATES TH2 INFLAMMATION IN OVALBUMIN-INDUCED ALLERGIC ASTHMA

M. de Bernard<sup>1</sup>, G. Codoletti<sup>1</sup>, P. Mazzi<sup>2</sup>, A. Amedei<sup>3</sup>, G. Del Prete<sup>3</sup>, M. M. D'Elia<sup>3</sup>

<sup>1</sup>Venetian Institute of Molecular Medicine, Padova, Italy, <sup>2</sup>University of Verona, Department of Pathology, Section of General Pathology, Verona, Italy, <sup>3</sup>University of Florence, Department of Internal Medicine, Florence, Italy

**Objective:** The Helicobacter pylori Neutrophil Activating Protein (HP-NAP) is able in vitro to elicit IL-12 and IL-23 production via interaction with TLR2, and to promote Th1 polarization of allergen-specific T-cell responses. In order to evaluate in vivo whether HP-NAP might be a possible new tool for therapeutic strategies aimed to redirect Th2 into Th1 responses, herein we address the hypothesis that the administration of HP-NAP can suppress the Th2-mediated bronchial inflammation using a mouse model of allergic asthma induced by inhaled ovalbumin (OVA).

**Methods:** HP-NAP was administered intra-peritoneally or via mucosal route in mice sensitized to the allergen OVA. The eosinophils lung infiltration, serum IgE levels and Th2 cytokines production were evaluated after the sacrifice of animals.

**Results:** Systemic HP-NAP delivery markedly reduced the lung eosinophilia in response to repeated challenge with aerosolized OVA. Likewise, the production of IL-4, IL-5, and GM-CSF was significantly lower in the bronchoalveolar lavage of animals treated with systemic HP-NAP plus OVA than that of animals treated with OVA alone. Systemic HP-NAP also resulted in significant reduction of total serum IgE. Mucosal administration of HP-NAP was equally successful as the systemic delivery in reducing eosinophilia and Th2 cytokine levels in bronchoalveolar lavage. However, no suppression of lung eosinophilia and bronchial Th2 cytokines was observed in TLR2 knock-out mice following HP-NAP treatment.

**Conclusion:** The results suggest that both systemic and mucosal administration of HP-NAP strongly inhibit the development of airway eosinophilia and bronchial inflammation. Likewise, HP-NAP treatment strongly affected the lung cytokine release, reducing the production of IL-4, IL-5, and GM-CSF. These findings provide the indication that both systemic and mucosal administration of HP-NAP is effective in preventing allergic asthma. We propose HP-NAP as possible candidate for novel strategies of prevention and treatment of allergic diseases.

# PD19/11 CTB AS CANDIDATE REGULATORY ADJUVANT FOR ALLERGEN IMMUNOTHERAPY

G. Bakdash<sup>1</sup>, H. H. Smits<sup>1</sup>, D. Sibiryak<sup>1</sup>, T. M. M. van Capel<sup>1</sup>, H. P. M. van der Kleij<sup>2</sup>, D. J. E. Opstelten<sup>2</sup>, E. C. de Jong<sup>1</sup>, M. L. Kapsenberg<sup>1</sup>

<sup>1</sup>Academic Medical Centre, University of Amsterdam, Amsterdam, Netherlands, <sup>2</sup>HAL Allergy, Leiden, Netherlands

**Objectives:** In search for immunomodulatory adjuvants that improve the effectiveness of allergen immunotherapy, we focused on the non-toxic mucosal adjuvant cholera toxin B (CTB) produced by *Vibrio cholera*. CTB binds the asialo-GM-1 receptor on various celltypes including dendritic cells. Mucosal application of antigen coupled to CTB prevents immunity, or suppresses on-going responses in pre-immunized mice through an increase in TGF- $\beta$  production as well as through the induction of Foxp3<sup>+</sup>CD25<sup>+</sup> or Foxp3<sup>+</sup>CD25<sup>+</sup> regulatory T cells. Here we studied to what extent CTB confers tolerance in an *in vitro* model of dendritic cell-driven human naive CD4<sup>+</sup> T cell responses.

**Methods:** Human monocyte-derived dendritic cells (MoDCs) were stimulated with a combination of LPS, TNF- $\alpha$  and IL-1 $\beta$  with or without CTB for two days and subsequently co-culture with allogeneic naive CD4<sup>+</sup> T cells. After 10-14 days, these T cells were phenotypically analyzed and tested for regulatory activity, as determined by their ability to suppress the proliferation of bystander T cells.

**Results:** CTB did not affect the maturation of MoDCs as determined by CD83, CD86 and class II MHC expression, but led to an increased expression of PD-L2 on the MoDC and to an increased IL-10 production in response to CD40 ligation. Furthermore, CTB-priming of MoDCs reduced their ability to induce proliferation of naive T cells and conferred production of IL-10 and regulatory activity on these T cells, which was blocked by inhibition of IL-10, PD-L2 and TGF- $\beta$ .

**Conclusions:** CTB confers tolerance by programming human DCs for the induction of IL-10-producing regulatory T cells. Since the induction of IL-10 in T cells is considered to be a hallmark of successful allergen desensitization, CTB may represent a suitable candidate adjuvant in allergen-specific immunotherapy.

# PD19/12 SYNTHETIC LIPOPEPTIDE DERIVED FROM COWSHED BACTERIA SUPPRESSES ALLERGIC SENSITIZATION IN A MURINE ASTHMA MODEL

M. Peters<sup>1</sup>, S. Voss<sup>2</sup>, K.-H. Wiesmüller<sup>2</sup>, A. Bufer<sup>1</sup>, R. Spohn<sup>2</sup>

<sup>1</sup>Ruhr-University Bochum, Department of Experimental Pneumology, Bochum, Germany, <sup>2</sup>EMC Microcollections GmbH, Tübingen, Germany

**Objectives:** Epidemiologic studies have demonstrated an allergy-protective effect of farm life early in childhood. The so-called 'hygiene hypothesis' suggests, that infections and unhygienic contact in early childhood might protect against the development of allergic illnesses [1].

It has been shown, that inhalation of extracts prepared from cowshed dust including common farm microbes prevents allergen induced airway inflammation and hyperresponsiveness [2].

Microbes contain immunomodulating molecules like lipopolysaccharide, CpG nucleotides, double stranded RNA or lipoproteins. These molecules are recognized by pattern recognition receptors, mainly Toll-like receptors (TLRs).

The aim of this study was to evaluate ligands for TLR1/TLR2 heterodimers for their potency to induce allergy-protective mechanisms.

**Methods:** Lipoproteins derived from cowshed bacteria were selected according to the DOLOP database of lipoproteins. Lipopeptide analogues, containing the N-terminal unusual liposaccharide S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-N-palmitoyl-(R)-cysteine, were synthesized. The biological activity of these lipopeptides was determined by induction of IL-8 secretion from THP-1 cells. A systematic approach was performed to investigate different structural elements with respect to their influence on physico-chemical properties and on their ability to induce cytokine release. The most promising lipopeptide was investigated for its effect on allergic airway inflammation in a mouse asthma model.

**Results:** Within the group of the newly synthesized lipopeptides we found significant differences in the biological activity. *In vitro*, one compound exhibited a stimulatory activity almost three orders of magnitude higher compared to other synthetic bacterial lipopeptides. A lipopeptide with optimized physicochemical properties and biological activity was studied in a mouse model of allergic asthma. Inhalation of this lipopeptide during sensitization resulted in decreased airway reactivity, eosinophilic inflammation and sensitization *in vivo*. Interestingly, mice show no signs of endotoxaemia due to treatment with lipopeptide.

**Conclusion:** Our data support that TLR1/TLR2 ligands prevent from allergic sensitization, airway inflammation, and airway hyperresponsiveness in a murine asthma model.

[1] W. Eder and E. von Mutius (2004) Curr Opin Allergy Clin Immunol 4, 113

[2] M. Peters et al. (2006) Thorax 61, 134

# PD19/13 EFFECT OF CO-EXPRESSION OF TGF- $\beta$ 1 AFTER GENE GUN-MEDIATED DNA IMMUNIZATION ON TRANSGENE-SPECIFIC IMMUNE RESPONSES

V. Raker<sup>1</sup>, C. Barwig<sup>1</sup>, E. Montermann<sup>1</sup>, P. Scholtes<sup>2</sup>, S. Finotto<sup>2</sup>, S. Grabbe<sup>1</sup>, A. B. Reske-Kunz<sup>1</sup>, S. Sudowe<sup>1</sup>

<sup>1</sup>Johannes Gutenberg-University Mainz, Dermatology – Clinical Research Unit Allergy, Mainz, Germany, <sup>2</sup>Johannes Gutenberg-University Mainz, I. Medical Clinic – Asthma Core Facility SFB548, Mainz, Germany

**Background:** Biolistic gene transfer using plasmids encoding  $\beta$ -galactosidase ( $\beta$ Gal) under control of the fascin promoter (pFascin- $\beta$ Gal) induced type 1 immune responses, which counteracted systemic (IgE) as well as local (pulmonary eosinophilia)  $\beta$ Gal-specific Th2 immune responses, but in return triggered neutrophilia in the lung and airway hyperreactivity (AHR) after intranasal provocation. The immunosuppressive cytokine transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) has been implicated in tolerance induction, predominantly due to the activation of regulatory T cells.

**Objective:** To analyze the effect of TGF- $\beta$ 1 co-expression on transgene-specific immune responses after biolistic DNA immunization.

**Methods:** TGF- $\beta$ 1-encoding plasmids (pCMV-TGF $\beta$ 1) and pFascin- $\beta$ Gal were co-precipitated on gold particles and applied to abdominal skin of BALB/c mice via the gene gun.  $\beta$ Gal-specific IgG subclasses in sera of immunized mice were determined. After subsequent intranasal provocation with  $\beta$ Gal-protein bronchoalveolar lavage (BAL) was performed and local immune responses in the lung were analyzed. Airway reactivity was determined after methacholine challenge by non-invasive whole body plethysmography. Gene gun-immunized mice were subjected to repeated intraperitoneal injection of  $\beta$ Gal adsorbed to aluminiumhydroxide ( $\beta$ Gal/alum) to provoke antigen-specific IgE production. Antibody titers in sera were determined after the fifth application of  $\beta$ Gal/alum.

**Results:** The humoral response following co-application of pFascin- $\beta$ Gal and pCMV-TGF $\beta$ 1 was not significantly different from that of control mice immunized with pFascin- $\beta$ Gal alone or in combination with an irrelevant plasmid. However, the induction of AHR, as being manifest in the control mice, was almost completely abolished by co-application of pCMV-TGF $\beta$ 1. Similarly, neutrophilic infiltration in the BAL was reduced, altogether indicating that Th1/Tc1-mediated airway inflammation was decreased in those animals. After immunization with  $\beta$ Gal/alum IgE and IgG1 production were efficiently inhibited by prophylactic genetic vaccination with pFascin- $\beta$ Gal and pCMV-TGF $\beta$ 1. According to the notion that the induction of type 1 responses in mice vaccinated with pFascin- $\beta$ Gal was suppressed by simultaneous expression of TGF- $\beta$ 1, the enhancement of IgG2a production following sensitization with  $\beta$ Gal/alum was significantly alleviated in mice co-vaccinated with pCMV-TGF $\beta$ 1.

**Conclusion:** DNA-based co-expression of allergens and immunomodulatory cytokines might represent an alternative strategy to current protein-based desensitization protocols to mediate allergen-specific immunoregulation. A possible role of regulatory T cells in this scenario remains to be investigated.



**PD19/14 IN VIVO BLOCKADE OF OX40L INHIBITS ASTHMATIC LUNG INFLAMMATION**I. Wollenberg<sup>1,2</sup>, A. Agua-Doce<sup>1,2</sup>, J. Duarte<sup>1,2</sup>, L. Graça<sup>1,2</sup><sup>1</sup>Instituto de Medicina Molecular, Lisboa, Portugal, <sup>2</sup>Instituto Gulbenkian de Ciência, Oeiras, Portugal

**Objective:** OX40-OX40L interaction is an important step in the activation of effector T cells by antigen presenting cells. In our study we addressed the long-term effects of OX-40L blockade in the prevention of ovalbumin (OVA)-induced allergic airways disease in mice.

**Methods:** Differential cell counts of cells recovered from the airways as well as quantification of lung immunoglobulins and cytokines were performed. Hyperplasia of lung goblet cells was revealed using PAS staining of lung sections and respiratory mechanics of the mice were analyzed by determining airway responsiveness to methacholine, a bronchoconstrictor.

**Results:** Our data show that treatment with anti-OX40L antibody during the sensitization phase of the allergic response can reduce the manifestations of allergic airways disease, namely airways eosinophilia, and production of IL-4, IL-5 and IL-13 in the lungs, as well as serum IgE levels. Lung histology as well as measurement of the respiratory mechanics in response to inhaled methacholine could confirm that the airways of mice treated with anti-OX40L are morphologically and physiologically similar to healthy unmanipulated controls. However, a similar beneficial effect of anti-OX40L treatment was not observed long term, suggesting immune tolerance is not achieved. In fact, anti-OX40L treatment, did not prevent allergic airways disease following a sensitization 30 days following initial exposure of OX40L in the presence of OVA.

**Conclusions:** Our data show that blockade of OX40-OX40L interaction effectively prevents allergic airways disease in the short-term, but without achieving long-term antigen-specific tolerance.

**PD19/15 TREATMENT WITH LARVAL E/S DERIVED FROM THE NEMATODE *TRICHURIS SUIS* PREVENTS ONSET OF AIRWAY HYPERREACTIVITY IN A MOUSE DISEASE MODEL**M.R. Hepworth<sup>1</sup>, E. Hamelmann<sup>2</sup>, R. Lucius<sup>1</sup>, S. Hartmann<sup>1</sup><sup>1</sup>Humboldt Universität zu Berlin, Molekular Parasitologie, Berlin, Germany, <sup>2</sup>Clinic for Paediatric Medicine, Bochum, Germany

The prevalence of allergic and autoimmune diseases in the developing world has been proposed to be directly correlated to the loss of intestinal nematode infections which may act to tune or regulate the development of an immune response. *Trichuris suis* is a naturally occurring nematode of pigs which has been previously successfully exploited to induce remission in patients suffering from Crohn's disease and Ulcerative Colitis. Here it is shown that Excretory/Secretory products (E/S) of *T. suis* larvae are sufficient to downregulate the onset of OVA-induced airway hyperreactivity in a mouse model. Treatment of mice with multiple doses of *T. suis* E/S during sensitisation to OVA resulted in significant decreases in challenge induced airway hyperreactivity, cellular infiltration into the Bronchoalveolar lavage fluid and a reduction in lung pathology and inflammation. This was further associated with a dramatic reduction in OVA-specific Th2 associated cytokine responses and IgE production – but, unlike many helminth immunomodulators, no increase in FoxP3+ T regulatory cell mediated immunoregulation was observed in the draining lymph node or lung tissue. This data provides new evidence that immunomodulators derived from the promising, clinically relevant, *T. suis* can directly suppress the onset of allergic disease. Furthermore, characterisation of nematode immunomodulators such as these will allow development of refined treatments with no need for parasitic exposure.

**PD19/16 BISPECIFIC DESIGNED ANKYRIN REPEAT PROTEINS – A NOVEL STRATEGY TO INHIBIT IGE-MEDIATED ALLERGY**A. Eggel<sup>1</sup>, B.M. Stadler<sup>1</sup>, M. Vogel<sup>1</sup><sup>1</sup>University Institute of Immunology, DOLS, Bern, Switzerland

**Objectives:** IgE has been shown to interact via two binding-sites with its high-affinity receptor (Fc epsilon RI). Allergens cross-linking receptor-bound IgE on mast cells or basophils induce Fc epsilon RI aggregation. Subsequent cell degranulation and the release of pro-inflammatory mediators result in an allergic response. In order to prevent this allergy cascade several monoclonal anti-Fc epsilon RI alpha antibodies inhibiting the binding of IgE have been described. However, such antibodies are anaphylactogenic due to their bivalency. In this study we selected designed ankyrin repeat proteins (DARPs) representing a novel non-immunoglobulin binding scaffold against Fc epsilon RI alpha. We assessed whether such DARPs may block the IgE-binding sites on the receptor without being anaphylactogenic.

**Methods:** DARPs are based on a consensus sequence derived from natural ankyrin proteins occurring in organisms of all phyla. Ankyrin repeat proteins evolved as natural fusion proteins to anchor other proteins to specific sites. The modular architecture of DARPs and the defined randomized interaction residues in the consensus sequence allowed to generate DARP libraries containing high diversities. Using ribosome display, we isolated anti-Fc epsilon RI alpha binders from these libraries. Different anti-Fc epsilon RI alpha DARPs were fused via a standard protein linker to generate bivalent and bispecific constructs. Specificity of the binders was tested by ELISA. Surface Plasmon resonance was used to measure the affinities and to assess the ability to inhibit IgE-binding to the receptor. The capability of anti-Fc epsilon RI alpha DARPs to inhibit cell degranulation was investigated *in vitro* using rat basophilic leukemia cells stably transformed with human Fc epsilon RI alpha.

**Results:** Selected DARPs recognized different epitopes on Fc epsilon RI alpha with high-affinity and did not show cross-reactivity to other tested proteins. All tested DARPs interfered with IgE-binding to the receptor. One bispecific DARP prevented IgE-induced cell degranulation with high efficacy. Moreover, the bispecific DARP was not anaphylactogenic *per se*.

**Conclusions:** Monovalent DARPs with different epitope or target specificities may be used to generate bispecific molecules with improved affinity and functionality. Such DARPs represent a suitable alternative to antibodies in cases where the use of immunoglobulin scaffolds has limitations.

**PD19/17 A VACCINE CONSISTING OF HYPOALLERGENIC HYBRID PROTEINS FOR THE TREATMENT OF GRASS POLLEN ALLERGY**B. Linhart<sup>1</sup>, M. Focke-Tejkl<sup>1</sup>, K. Fleischmann<sup>1</sup>, H. Mayrhofer<sup>1</sup>, K. Blatt<sup>2</sup>, P. Valent<sup>2</sup>, R. Valenta<sup>1</sup><sup>1</sup>Medical University of Vienna, Dept. of Pathophysiology, Div. of Immunopathology, Vienna, Austria, <sup>2</sup>Medical University of Vienna, Dept. of Internal Medicine I, Div. of Hematology and Hemostaseology, Vienna, Austria

**Objectives:** More than 10% of the world population suffer from allergy to grass pollen. Here we describe the development of a vaccine based on recombinant hypoallergenic hybrid molecules which were constructed out of elements from the four major timothy grass pollen allergens Phl p 1, Phl p 2, Phl p 5, and Phl p 6 for the treatment of grass pollen allergy.

**Methods:** The DNA molecules encoding building blocks and combinations thereof were designed according to epitope mapping, computer-aided surface predictions and structural data and subsequently prepared by gene synthesis in a codon-optimized manner for expression in *Escherichia coli*. The recombinant molecular building blocks and hybrids thereof were then expressed in *E. coli* and purified by affinity chromatography. The recombinant molecules were further characterized using biochemical and immunological methods (i.e., SDS-PAGE, mass spectrometry, circular dichroism, IgE antibody reactivity testing by ELISA and immunoblot analysis, basophil activation assays, lymphocyte transformation tests and immunization experiments) in order to identify those molecules which exhibited the least allergenic activity and upon immunization of animals induced IgG antibodies which blocked allergic patients' IgE-reactivity to grass pollen allergen and allergen-induced basophil degranulation. Several hybrid molecules consisting of combinations of the recombinant building blocks were evaluated in the same manner to identify the recombinant hypoallergenic hybrid molecules suitable for allergy vaccination.

**Results:** We constructed recombinant hypoallergenic hybrid molecules consisting of elements of the major grass pollen allergens. The hybrid molecules showed almost no allergenic activity when compared to grass pollen extract and to a mix of the four wildtype allergens. Upon immunization of mice and rabbits the hybrids induced IgG antibodies which inhibited IgE-binding to natural grass pollen allergens.

**Conclusion:** The hypoallergenic hybrids are candidate molecules for vaccination against grass pollen allergy. This study was supported by a research grant from Biomay and by the Christian Doppler Research Association, Vienna, Austria.\*contributed equally.

**PD19/18 IDENTIFICATION AND CHARACTERIZATION THE ALLERGENS OF *SENECIO JACOBAEA* POLLEN**C. Gámez<sup>1</sup>, O. Luengo<sup>2</sup>, E. López<sup>1</sup>, B. Sastre<sup>1</sup>, E. Aguado<sup>1</sup>, C. Lahoz<sup>1</sup>, V. del Pozo<sup>1</sup><sup>1</sup>Fundación Jiménez Díaz-Capio/CIBERES (ISCIII), Immunology, Madrid, Spain, <sup>2</sup>Hospital Vall d'Hebron, Internal Medicine, Allergy Section, Barcelona, Spain

**Objective:** The aim of this study was to investigate the allergens of *Senecio jacobaea* pollen and to determine its clinical relevance. Moreover, we sought to identify and isolate the genes encoding the *Senecio jacobaea* allergens.

**Methods:** Fifty patients with positive skin prick test (SPT) to *Senecio* were recruited. Allergens were characterized by SDS-PAGE, 2D analysis and immunoblotting. Furthermore, identification of the allergens was carried out by mass spectrometry. After cDNA synthesis and RT-PCR amplification, cDNA cloning and DNA sequencing was performed.

*In vitro* inhibition test were executed to evaluate cross-reactivity with other pollen types.

**Results:** Three predominant allergens, both in intensity of reaction and frequency of recognition by allergic sera, were 59 (60%), 42 (50%) and 31 kDa (50%) respectively. The 2D analysis allowed the identification of several allergens. One spot around 42 kDa was identified as a protein homologous to pectate lyase and other three spots are homologous to malate dehydrogenase by mass spectrometry. We confirmed that these allergenic proteins were a malate dehydrogenase and a pectate lyase by immunoblotting (using commercial enzyme) and by microarray, respectively. Also, we have obtained malate dehydrogenase fragment cDNA of 750 pb. Sequence analysis showed the presence of a 513 pb frame that encodes a fragment of *S. jacobaea* malate dehydrogenase protein that is homologous of *Perilla frutescens* malate dehydrogenase.

Furthermore *S. jacobaea* proteins showed cross-reactivity with other proteins of the *Asteraceae* family and also with *P. judaica*.

**Conclusion:** *S. jacobaea* constitute a newly discovered allergenic source. A pectate lyase and a malate dehydrogenase were identified as two major allergens of *S. jacobaea*. The cloning of allergen malate dehydrogenase has been partially performed and future results could be important for diagnosis and treatment of *S. jacobaea* pollen allergy.

#### PD19/19 REPROGRAMMING THE IMMUNE SYSTEM WITH MONOCLONAL ANTIBODIES IN A MOUSE MODEL OF PEANUT INDUCED ANAPHYLAXIS

J. Duarte<sup>1,2</sup>, M. Caridade<sup>1,2</sup>, V. Oliveira<sup>1,2</sup>, A. Água-Doce<sup>1,2</sup>, L. Graca<sup>1,2</sup>

<sup>1</sup>Molecular Medicine Institute, Cellular Immunology, Lisbon, Portugal, <sup>2</sup>Instituto Gulbenkian de Ciência, Oeiras, Portugal

**Objective:** It has been reported that non-depleting monoclonal antibodies, such as non-depleting anti-CD4, can induce transplantation tolerance. We have recently shown that the same approach can be used to induce tolerance to a model allergen. We have now investigated whether peanut induced anaphylaxis can be prevented through anti-CD4-induced immune tolerance.

**Methods:** C3H/HeJ mice sensitized with crude peanut extract in aluminium hydroxide (alum) develop an anaphylactic response following peanut exposure. The anaphylactic response leads to edema and non-responsiveness to external stimulation, as well as to a reduction in body temperature (between 6 and 8 degrees). The disease is also correlated with increased serum IgE levels.

**Results:** Mice treated with non-depleting anti-CD4 at the time of peanut extract administration are protected from the clinical manifestations of anaphylaxis, and decrease in body temperature. Moreover, the serum concentration of IgE in anti-CD4 treated mice are similar to naive animals not exposed to the allergen, and significantly lower when compared to control mice not treated with the monoclonal antibody.

**Conclusions:** Our data suggest that non-depleting anti-CD4 monoclonal antibodies can be an important tool to prevent severe allergic responses, as in the case of anaphylaxis. Our preliminary data suggests that the tolerance mechanism is directed towards a reduction of the number of antigen-specific T cells, without interfering with T cells specific for unrelated antigens.

#### PD19/20 THERAPEUTIC EFFECTS OF DENDRITIC CELLS-MODULATED BY IL-10 AND IL-12-EXPRESSING ADENOVIRUSES ON AIRWAY INFLAMMATION IN ASTHMATIC MICE

H.-C. Su<sup>1</sup>, H.E. Liu<sup>2</sup>, Y.-L. Lee<sup>3</sup>

<sup>1</sup>Graduate Institute of Medical Sciences, College of Medicine, Taipei Medical University, Taipei, Taiwan, Republic of China, <sup>2</sup>Graduate Institute of Clinical Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan, Republic of China, <sup>3</sup>College of Medicine, Taipei Medical University, Department of Microbiology and Immunology, Taipei, Taiwan, Republic of China

Asthma is characterized by allergen-induced airway inflammation orchestrated by Th2 cells. Dendritic cells (DCs) have been found to efficiently prime naïve T-helper cells. Thus, modification of DC function may be used as a tool to prevent allergic asthma by changing CD4<sup>+</sup> T-cell differentiation or suppressing Th2 development. In this study, we examined whether DCs producing both IL-10 and IL-12 can apply a DC-based vaccine to modulate the immune response of allergic asthma. Bone marrow-derived DCs were genetically modified with IL-10- and IL-12-expressing adenoviruses and such cytokine gene-modulated DCs pulsed with ovalbumin (OVA) were intra-tracheally (i.t.) injected into mice after sensitization with OVA. Then these treated mice were three challenges with OVA and assayed. Herein we show that cytokine gene-modified DCs efficiently inhibited the development of airway hyperresponsiveness, reduced pulmonary infiltration of eosinophils. However, expression of Th2 cytokines and eotaxin in bronchoalveolar lavage fluid was not significantly suppressed in OVA-immunized mice with the combined cytokine gene-modified DCs treatment. Taken together, these results suggest that IL-10 and IL-12 gene-modulated DCs might be a potential therapeutic approach for asthma.

#### PD19/21 CD4 BLOCKADE PREVENTS INKT CONTRIBUTION TO ALLERGIC AIRWAYS DISEASE

C. Almeida<sup>1,2</sup>, M. Monteiro<sup>1,2</sup>, L. Graca<sup>1,2</sup>, A. Agua-Doce<sup>1,2</sup>

<sup>1</sup>Instituto de Medicina Molecular, Immunologia Celular, Lisboa, Portugal, <sup>2</sup>Instituto Gulbenkian de Ciência, Oeiras, Portugal

**Objectives:** Our previous results indicate that a non-depleting anti-CD4 monoclonal antibody (Mab) can prevent allergic sensitization in a well established murine model of allergic airways disease (AAD), leading to allergen-specific tolerance. Given that invariant Natural Killer T Cells (iNKT) were shown to contribute for the development of AAD and can express CD4, we investigated whether tolerance induced by CD4 blockade might also have an impact within the iNKT-cell compartment.

**Methods:** Female BALB/c mice were sensitized with ovalbumin (OVA)-alum on days 1 and 14 and challenged intranasally with OVA on days 20, 21 and 22. Experimental animals were treated with anti-CD4 at the time of sensitization. Bronchoalveolar lavage (BAL), lungs, mediastinal lymph nodes (MedLN) and spleen were collected 24 hours after the last OVA challenge and analysed by flow cytometry. iNKT cells were identified using a CD1d/PBS57 tetramer. Statistical significance was determined using a Mann-Whitney test.

**Results:** Mice treated with the anti-CD4 Mab showed a reduction in iNKT-cell infiltrates in lungs and BAL, but not in MedLN or spleen. Although MedLN from anti-CD4 treated mice contained similar numbers of iNKT cells as AAD controls, we observed changes in the phenotype of iNKT cells: there was a marked reduction of CD62L<sup>+</sup> and CD25<sup>+</sup> iNKT cells from treated animals. Moreover, the antibody was able to downmodulate the CD4 expression on iNKT cells from lungs, BAL and MedLN and spleen. However it did not lead to direct lysis of CD4<sup>+</sup> iNKT cells (or CD4<sup>+</sup> T cells), as these populations in the spleen remained unchanged.

**Conclusion:** Our results show that CD4 blockade has effects both on CD4<sup>+</sup> T cells and iNKT cells. The Mab treatment impairs the recruitment of iNKT-cell to the airways, thus contributing to the prevention of AAD, without affecting the splenic iNKT-cell population.

#### PD19/22 WEED POLLEN ALLERGY: CROSS- OR CO-SENSITIZATION TO MUGWORT AND RAGWEED POLLEN?

B. Jahn-Schmid<sup>1</sup>, N. Wopfner<sup>2</sup>, G. Gadermaier<sup>2</sup>, M. Egger<sup>2</sup>, R. Asero<sup>3</sup>, C. Ebner<sup>4</sup>, F. Ferreira<sup>2</sup>, B. Bohlé<sup>5</sup>

<sup>1</sup>Medical University of Vienna, Institute of Pathophysiology, Wien, Austria, <sup>2</sup>University of Salzburg, Christian Doppler Laboratory for Allergy, Diagnosis and Therapy, Salzburg, Austria, <sup>3</sup>Clinica San Carlo, Ambulatorio di Allergologia, Paderno Dugnano, Italy, <sup>4</sup>Allergieambulatorium Reumannplatz, Vienna, Austria, <sup>5</sup>Medical University of Vienna, Christian Doppler Laboratory for Immunomodulation, Wien, Austria

Ragweed and mugwort are important allergenic weeds belonging to the *Asteraceae* plant family. Allergy to mugwort and ragweed pollens is often associated in patients from areas where both weeds are spread; e.g. in Austria. A possible reason for this clinical observation may be an immunological cross-reactivity between homologous pollen allergens present in the related plants.

To analyze whether patients allergic to both weeds are co- or cross-sensitized, the humoral and cellular immune response to the major weed pollen allergens was investigated. IgE-reactivity of mugwort and/or ragweed pollen-allergic individuals was assessed in immunoblots and ELISA. T cell lines and clones specific for the major allergens Art v 1 (mugwort) and Amb a 1 (ragweed) were established and mapped for T cell epitopes using 12-mer peptides covering the respective protein sequences. In addition, these T cell cultures were stimulated with the respective homologous molecules (proteins and/or peptides) of each species.

Art v 1 was recognized by IgE from 95% of mugwort allergic patients, while the homologous ragweed protein was recognized by less than 50% of the sera. 97% of ragweed patients recognized Amb a 1, but only 15% the homologue in mugwort. The T cell response to Art v 1 was characterized by one immunodominant epitope, Art v 1<sub>22-36</sub>. T cells specific for this epitope did not cross-react with the corresponding peptide from the ragweed homologue. In contrast, Amb a 1 harbours more than 30 different T cell epitopes of which only 5 were cross-reactive with the Amb a 1-homologue in mugwort.

In summary, cross-reactivity between major ragweed and mugwort pollen allergens is very limited at the humoral and also at the cellular level, indicating that patients with allergic reactions to pollen of both weeds are co-, but not cross-sensitized. Consequently, specific immunotherapy in co-sensitized patients should be performed with both allergens.

This work was supported by the Austrian Science fund FWF (NFN-S8802, S8808, P20011-B13 and Biomay)

#### PD19/23 LACTOFERRIN, INTERFERON ALPHA, TRANSFORMING GROWTH FACTOR BETA AND INTERLEUKIN 17 MEASUREMENTS IN THE BREAST MILKS AND SERA OF ALLERGIC AND NON-ALLERGIC HUMAN MOTHERS. THE ADVANTAGEOUS EFFECT OF THE PROLONGED CONTINUOUS ENTERAL TREATMENT OF MICE WITH LACTOFERRIN AND INTERFERON ALPHA ON AN ALLERGIC REACTION

S. Sipka<sup>1</sup>, E. Gyimesi<sup>1</sup>, I. Kovács<sup>1</sup>, E. Polonkay<sup>2</sup>, A. Csillag<sup>3</sup>, J. Kovács<sup>1</sup>, G. Balla<sup>2</sup>, A. Bácsi<sup>3</sup>

<sup>1</sup>University of Debrecen, 3rd Department of Internal Medicine, Debrecen, Hungary, <sup>2</sup>University of Debrecen, Department of Pediatrics, Debrecen, Hungary, <sup>3</sup>University of Debrecen, Department of Immunology, Debrecen, Hungary

**Objectives:** We aimed to test the amounts of lactoferrin (LF), IFN $\alpha$ , TGF $\beta$  and IL-17 in the breast milks of allergic and non-allergic human mothers, and to observe the effects of the prolonged continuous enteral treatment of mice with LF and IFN $\alpha$  on an allergic reaction.

**Patients:** The LF, IFN $\alpha$ , TGF $\beta$  and IL-17 contents of 20 women with allergy, 20 women without allergy were measured in the breast milks by ELISA 5 days after the deliveries without any complication.

**Mice:** 7-7animals/ group of 4 weeks old Balb/c female mice were treated for 6 weeks by the addition of 0.1% LF and 200 IU/ml of IFN $\alpha$  or their combination to the drinking water.

**Allergy model in mice:** The intraperitoneal sensitization by two injections of 150 $\mu$ g ragweed allergen (RWE) occurred. The intranasal challenge took place on day 11 with 100  $\mu$ g of RWE. Three days later the cell counts (macrophages and neutrophils/ml) of bronchial lavage (BAL) were measured.

**Results:** The amounts of LF (3.43 vs. 2.0 ng/mg protein  $P=0.019$ ) and IL-17 (5.57 vs 1.32 pg/ml  $P<0.05$ ) in the milk of allergic mothers were significantly higher than those in the healthy subjects, but only 2 of 21 children became allergic by the age of 2 years. IFN  $\alpha$  was slightly but not significantly higher, the level of TGF $\beta$  was slightly but not significantly lower in the allergic women than those in the controls. The 6 weeks long continuous enteral treatment of mice with LF and IFN $\alpha$  alone, or with their combination, resulted in significant decreases in the cell counts of BAL compared to the controls in the experimental allergy model of mice. The largest decrease occurred when LF and IFN $\alpha$  were combined concurrently (45625 vs 86719 cells/ml,  $p=0.005$ ). In these animals the serum level of IL-17 was also significantly decreased (1.08 vs 3.78 pg/ml,  $p=0.006$ ).

**Conclusion:** These observations demonstrate remarkable connections of LF, IFN $\alpha$ , TGF $\beta$  and IL-17 contents of breast milk with the atopic background in human mothers. In addition, the continuous enteral application of LF and IFN $\alpha$  in the drinking water can result in a significant reduction in the allergic response and the serum level of IL-17 in mice.

#### PD19/24 STUDIES ON THE HYPOALLERGENIC NATURE OF A RECOMBINANT TRIMER OF THE MAJOR BIRCH POLLEN ALLERGEN BET V 1

R. Campana<sup>1</sup>, S. Vrtala<sup>1</sup>, B. Maderegger<sup>2</sup>, Y. Dall'Antonia<sup>3</sup>, K. Blatt<sup>4</sup>, M. Focke<sup>1,5</sup>, I. Swoboda<sup>5</sup>, S. Scheiblhofer<sup>6</sup>, A. Gieras<sup>1</sup>, A. Neubauer<sup>2</sup>, W. Keller<sup>3</sup>, P. Valent<sup>4</sup>, J. Thalhammer<sup>6</sup>, S. Spitzauer<sup>7</sup>, R. Valenta<sup>1,5</sup>

<sup>1</sup>Division of Immunopathology, Department of Pathophysiology, Center of Physiology and Pathophysiology, Vienna General Hospital (AKH), Medical University of Vienna, Vienna, Austria, <sup>2</sup>Biomay AG, Vienna Competence Center, Vienna, Austria, <sup>3</sup>Division of Structural Biology, Institute of Chemistry, Karl Franzens University of Graz, Graz, Austria, <sup>4</sup>Division of Hematology and Hemostaseology, Department of Internal Medicine I, Vienna General Hospital (AKH), Medical University of Vienna, Vienna, Austria, <sup>5</sup>Christian Doppler Laboratory for Allergy Research, Vienna General Hospital (AKH), Medical University of Vienna, Vienna, Austria, <sup>6</sup>Christian Doppler Laboratory for Allergy Diagnosis & Therapy, Department of Molecular Biology, University of Salzburg, Salzburg, Austria, <sup>7</sup>Institute of Medical and Chemical Laboratory Diagnostics, Vienna General Hospital (AKH), Medical University of Vienna, Vienna, Austria

It has been demonstrated for many important allergens that it is possible to reduce their allergenic activity by chemical treatment or recombinant DNA technology based on the destruction of IgE epitopes. In the case of the major birch pollen allergen Bet v 1, the reduction of allergenic activity has been typically achieved by fragmentation, mutation or denaturing treatment resulting in a destruction of conformational IgE epitopes. Here we have studied an unusual hypoallergenic derivative of Bet v 1, i.e., a recombinant trimeric Bet v 1 molecule obtained by expression of three Bet v 1-encoding cDNAs linked by a few amino acids. In solid phase immunoassays, the Bet v 1 trimer exhibited even stronger IgE reactivity than the Bet v 1 monomer and reacted equally well several Bet v 1-specific antibody probes specific for different epitopes. When used in fluid phase IgE competition experiments the trimer and monomer inhibited IgE reactivity to each other equally well. However, the Bet v 1 trimer exhibited a more than 10fold reduced allergenic activity compared to the Bet v 1 monomer when tested for the cross-linking of basophil bound IgE using CD203c activation assays. The physicochemical and structural characterization of the Bet v 1 trimer revealed that it exhibited an altered fold when compared to the monomer by circular dichroism, which was reminiscent of that of a non-allergenic Bet v 1 homolog. Furthermore, gel filtration experiments revealed that Bet v 1 trimer occurred in defined aggregates whereas Bet v 1 appeared strictly as monomeric species. Our results indicate that in contrast to all other previously known hypoallergenic derivatives where reduction of the allergic activity has been obtained by destruction of IgE epitopes, the Bet v 1 trimer represents a hypoallergenic allergen derivative due to an altered presentation and/or orientation of IgE epitopes.

Supported by the Austrian Science Fund, FWF.

#### PD19/25 T CELL RECOGNITION OF DER P 2 IN INDIVIDUALS WITH ATOPIC DERMATITIS

L.R. Crack<sup>1</sup>, H.W. Chan<sup>1</sup>, T. McPherson<sup>1</sup>, G.S. Ogg<sup>1,2</sup>

<sup>1</sup>Weatherall Institute of Molecular Medicine, University of Oxford, MRC Human Immunology Unit, Oxford, United Kingdom, <sup>2</sup>NIHR Oxford Biomedical Research Centre, Oxford, United Kingdom

**Background:** Atopic dermatitis (AD) is a chronic inflammatory skin disorder. Current evidence suggests that both skin barrier dysfunction and T cell abnormalities, particularly those of a T<sub>H</sub>2 phenotype, contribute to disease pathogenesis. Despite house dust mite-derived Der p 2 being a common source of IgE reactivity in atopic individuals, there is little data that has addressed potential specific T cell responses. Here we investigate whether Der p 2-specific T cells differ in atopic dermatitis patients and healthy controls.

**Methods:** Ex vivo IL-4 and IFN- $\gamma$  ELISpot analysis of peripheral blood mononuclear cells (PBMC) from adult cohorts of AD patients and healthy controls. PBMC from both cohorts were also stimulated *in vitro* with pools of overlapping Der p 2 peptides, followed by cultured IFN- $\gamma$  and IL-4 ELISpots. Intracellular cytokine staining assays were performed to determine the responding T cell subtype. HLA blocking antibodies and peptide truncations were used in conjunction with cultured ELISpots in order to identify possible MHC class II-restricted T cell epitopes.

**Results:** Der p 2-specific T cells were detected in both atopic dermatitis patients and healthy controls. However, IL-4 responses were stronger and observed more frequently in AD patients compared with healthy controls. Responses were sufficiently robust to map novel Der p 2 CD4<sup>+</sup> T cell epitopes. Within one pool of Der p 2 peptides, one 20mer peptide (D11) elicited the most robust responses and that anti-HLA-DP and HLA-DR antibodies could block this response. Responses to truncated peptides of this 20mer identified a shorter sequence within this 20mer as a potential CD4<sup>+</sup> T cell epitope.

**Conclusions:** T<sub>H</sub>2 responses to house dust mite allergen Der p 2 were more frequent in atopic dermatitis patients compared to healthy controls, providing further evidence of the key role of T cells in the pathogenesis of atopic dermatitis. Mapping of new epitopes within Der p 2 may be of value for future therapeutic approaches based on peptide immunotherapy.

#### PD19/26 CAPPING OF ANTI-ALLERGIC RNA VACCINES IS NECESSARY FOR THE GENERATION OF ANTIBODIES AND THE PREVENTION FROM IGE INDUCTION IN VIVO

E. Roesler<sup>1</sup>, E. Weinberger<sup>2</sup>, S. Scheiblhofer<sup>2</sup>, A. Fruehwirth<sup>2</sup>, J. Thalhammer<sup>1</sup>, R. Weiss<sup>1</sup>

<sup>1</sup>University of Salzburg, Christian Doppler Laboratory of Allergy Diagnosis and Therapy, Salzburg, Austria, <sup>2</sup>University of Salzburg, Molecular Biology, Salzburg, Austria

**Objectives:** Most cellular and eucaryotic mRNAs have a cap structure at their 5' end that is critical for translation. Cap structures also contribute to polyadenylation, transcription initiation, mRNA transport, splicing, and protect the mRNAs from degradation by 5' exonucleases.

In the current study we evaluated the influence of the 5'-terminal cap structure on the immunogenicity of conventional as well as self-replicating RNA vaccines and also investigated the vaccines' potential to protect from an allergic disease.

**Methods:** Capped mRNAs were created in synthetic *in vitro* transcription reactions using Vaccina Virus Capping Enzyme (VCE) that can achieve a capping efficacy of up to 100%. Translation efficacy was investigated by transfection of BHK-21 cells followed by a Western Blot analysis.

For *in vivo* studies, BALB/c mice were immunized with capped/uncapped conventional or self-replicating RNA vaccines encoding Phl p 5 followed by subcutaneous sensitization with recombinant Phl p 5 in alum and intranasal allergen provocation. IgG1 and IgG2a serum antibody levels were measured by ELISA, IgE levels by a basophil degranulation assay. Splenocytes were prepared and analyzed for proliferation and cytokine secretion. Moreover, lung inflammation was evaluated by measurement of bronchoalveolar lavage (BAL) cell numbers and BAL cytokines.

**Results:** Western Blot analysis showed that capped RNA transcripts triggered high production of protein compared to uncapped RNA which only displayed marginal protein levels.

*In vivo*, only capped mRNA-based vaccines proved their anti-allergic efficacy in terms of IgG subclass distribution, functional IgE suppression, reduction of IL-4 and IL-5, induction of IFN- $\gamma$  producing cells, and reduction of eosinophils in the lung in contrast to uncapped vaccines. Surprisingly, sensitization with recombinant Phl p 5 in alum led to a high and even significant (uncapped conventional RNA) IgG2a induction in both uncapped groups indicating for re-activation of vaccine-induced Th1 memory cells. Nevertheless, no prevention from IgE induction could be observed within these groups.

**Conclusion:** The results point to a crucial importance of mRNA capping for RNA translation *in vitro* and the immunogenicity of RNA vaccines *in vivo*. Consequently, capping is mandatory for efficient prevention from allergic responses.

#### PD19/27 PHENOTYPIC AND FUNCTIONAL CHARACTERIZATION OF ALLERGEN SPECIFIC CD4<sup>+</sup>CD25<sup>+</sup> REGULATORY T IN ALLERGIC PATIENTS TO ARTEMISIA VULGARIS

M.V. Martínez-Sánchez<sup>1</sup>, S. Soriano<sup>1</sup>, G. Salgado-Cecilia<sup>1</sup>, M. Muro-Amador<sup>1</sup>, J.A. Campillo-Marquina<sup>1</sup>, A.M. García-Alonso<sup>1</sup>, M.R. Álvarez-López<sup>1</sup>, J.A. Pagán-Alemán<sup>2</sup>, A. Minguela<sup>1</sup>

<sup>1</sup>Hospital U. Virgen de la Arrixaca y Centro de Investigaciones Biomédicas en Red de Enfermedades Hepáticas y Digestivas (CIBERehd, ISCIII), Servicio de Inmunología, Murcia, Spain, <sup>2</sup>Hospital U. Virgen de la Arrixaca, Servicio de Alergología, Murcia, Spain

Naturally occurring CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells (Treg) are important in the regulation of immune response in autoimmunity, allergy and infections. Ag-specific rather than polyclonal Tregs, have superior suppressive capacity. In the Mediterranean area *Artemisia vulgaris* pollinate all over the year so that it can be an insidious disease. In the present work we have compared the *ex vivo* phenotype and function of Tregs specific to *A. vulgaris*.

**Results:** Expression of CD27, CD29, CD49d, CD62L, CRTh2, CXCR3, CCR4 and CCR5 functions on Tregs, effector-CD4, CD8 and CD19 lymphocytes was analyzed in blood samples from healthy controls (n=11) and allergic patients to *A. vulgaris* (n=13), previous to and 9-12 months after the immunotherapy, by using a LSRII (BD) 10 colours flow cytometer. *In vitro* specific suppressive capacity of CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>+</sup> Tregs, highly purified on a MoFlow Sorter (Coulter), was analyzed previous to and after immunotherapy on CD25<sup>-</sup> (CD4<sup>+</sup>CD25<sup>-</sup>CD127<sup>+</sup>) and CD25<sup>+</sup> (CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>+</sup>) effector cells stimulated with the extract of *A. vulgaris* used for immunotherapy and autologous antigen presenting cells (APC) to a Treg:Effector:APC cell ratio of 1:1:1.



**Results:** Allergic patients before and after immunotherapy had similar numbers of Tregs on peripheral blood. Tregs phenotype CD62L<sup>high</sup>CD49d<sup>+</sup>CD27<sup>+</sup> was more frequent in patients than in controls ( $p < 0.05$ ), but immunotherapy bring it back to levels observed in health controls. No clear differences in the expression of chemokine receptors were observed. CD25<sup>+</sup> and CD25<sup>+</sup> effectors show similar proliferative response to extracts of *A. vulgaris* in controls and in patients before and after immunotherapy. Before immunotherapy, Tregs from patients showed lower specific suppressive capacity (62% of inhibition) than controls Tregs (73% of inhibition). Surprisingly, specific suppressive capacity of CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>+</sup> Tregs after immunotherapy was strongly diminished (25% of inhibition) for CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>+</sup> effectors, and completely abolished for CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>+</sup> effectors.

**Conclusions:** Patients allergic to *A. vulgaris* did not show clear alterations in the number or phenotype of their Tregs in peripheral blood. However specific suppressive function of Tregs was diminished in patients before immunotherapy, and this difference was strongly remarked after treatment. So that Tregs did not seem to play a critical role in the immunomodulation induced by the specific immunotherapy in patients allergic to *A. vulgaris*.

#### PD19/28 RECOMBINANT ALLERGENS FOR FOLLOWING ANTIBODY-RESPONSES DURING IMMUNOTHERAPY WITH A GRASS POLLEN VACCINE AND AFTER FIVE YEARS OF DISCONTINUATION

E. Gadermaier<sup>1</sup>, J. Staikuniene<sup>2</sup>, S. Scheiblhofer<sup>3</sup>, J. Thalhammer<sup>3</sup>, M. Kundl<sup>4</sup>, K. Westritschnig<sup>1</sup>, I. Swoboda<sup>1</sup>, S. Flicker<sup>1</sup>, R. Valenta<sup>1</sup>

<sup>1</sup>Medical University of Vienna, Division of Immunopathology, Department of Pathophysiology, Center for Physiology and Pathophysiology, Vienna, Austria, <sup>2</sup>Kaunas University of Medicine, Department of Pulmonology and Immunology, Kaunas, Lithuania, <sup>3</sup>University of Salzburg, Department of Molecular Biology, Division of Allergy and Immunology, Salzburg, Austria, <sup>4</sup>Medical University of Vienna, Institute of Environmental Health, Center for Public Health, Vienna, Austria

**Objectives:** Follow-up of antibody responses in serum samples from grass pollen allergic patients having received one course of injection immunotherapy (SIT) with an aluminium hydroxide-adsorbed grass pollen extract ( $n=12$ ) or only anti-inflammatory treatment ( $n=7$ ). Serum samples were taken before treatment, after five months of treatment and after five years.

**Methods:** Specific IgE, IgG1-IgG4, IgM, IgA and light chain responses were monitored using purified recombinant timothy grass (i.e., Phl p 1, Phl p 2, Phl p 5, Phl p 6, Phl p 7, Phl p 12, Phl p 13)- and birch pollen (Bet v 1) allergens.

**Results:** After five months of SIT but not after inflammatory treatment we found increases of IgG1>IgG4>IgG2 antibody responses in particular for the major grass pollen allergens Phl p 1, Phl p 5, Phl p 6 and Phl p 2 but no relevant increases against the other tested allergens (Phl p 7, Phl p 12, Phl p 13, Bet v 1). No relevant induction of allergen-specific IgA, IgM or IgG3 responses were found in the SIT group. The increases of allergen-specific IgG responses were accompanied by allergen-specific light chain binding involving both kappa and lambda chains in the SIT group. An inhibition of allergen-dependent basophil degranulation was only obtained with sera from the SIT-group containing therapy-induced allergen-specific IgG antibodies but not with sera from the group having received only anti-inflammatory treatment. A decrease of Phl p 1- and Phl p 5-specific IgE levels was found in the SIT group after the grass pollen season but not in the group of patients receiving only anti-inflammatory treatment. After five years allergen-specific (Phl p 1, Phl p 5) IgE and IgG antibody levels in the SIT group had returned to baseline levels.

**Conclusion:** Our results demonstrate that only SIT but not anti-inflammatory treatment can prevent boosts of allergen-specific IgE production in allergic patients. Thus only SIT but not anti-inflammatory treatment has disease-modifying effects.

Supported by grant 813003 of the Austrian Research Promotion Agency and by a research grant from BIOMAY, Vienna, Austria and the Christian Doppler Research Association, Austria.

#### PD19/29 BRAZILIN INHIBITED MITOGEN-INDUCED T<sub>H</sub>2 RESPONSES IN VITRO AND SUPPRESSED THE ALLERGIC INFLAMMATION AND HYPER-RESPONSIVENESS IN MURINE MODEL OF ASTHMA

C.-C. Lee<sup>1,2</sup>, C.-N. Wang<sup>2</sup>, P.-A. Chen<sup>1</sup>, B.-L. Chiang<sup>3</sup>, J.-J. Kang<sup>4</sup>

<sup>1</sup>China Medical University, School of Medicine, Taichung, Taiwan, Republic of China, <sup>2</sup>China Medical University, Graduate Institute of Basic Medical Science, Taichung, Taiwan, Republic of China, <sup>3</sup>National Taiwan University, Graduate Institute of Clinical Medicine, Taipei, Taiwan, Republic of China, <sup>4</sup>National Taiwan University, Institute of Toxicology, Taipei, Taiwan, Republic of China

The aim of the study is to determine whether brazilin exhibits the anti-inflammatory effects could inhibit T helper cell type II (T<sub>H</sub>2) responses and suppress allergic reaction in murine model of asthma. We investigated the effects of brazilin on mitogen-induced T<sub>H</sub>2 cytokines release, role of transcriptional factors and MAPK kinases, and the therapeutic effects on murine model of asthma. We found that brazilin dose-dependent inhibited PMA combined cAMP induced IL-4 and IL-5 expression in RNA and protein level in EL-4 T cells. The suppression was mediated through the inhibition of upstream T<sub>H</sub>2 related transcriptional factors GATA-3 and c-Maf mRNA and protein expression but not involvement in T<sub>H</sub>1 related transcriptional factor T-bet activation. After intratracheal instillation of brazilin in OVA immunized murine, we found that brazilin treated mice showed that decrease of IL-4 and eotaxin release in bronchial alveolar lavage fluid and attenuated the OVA-induced lung eosinophilia. These results suggest that brazilin exhibited the anti-T<sub>H</sub>2 reaction in vitro and in vivo study showed the therapeutic potential to allergic diseases.

#### PD19/30 THE BOVINE IMMUNE MILK FROM HYPERIMMUNIZED COWS INHIBITS AIRWAY HYPERRESPONSIVENESS AND AIRWAY ALLERGIC INFLAMMATION OF OVA-SENSITIZED BALB/C MICE

C.-H. Wu<sup>1</sup>, B.-F. Lin<sup>1</sup>

<sup>1</sup>National Taiwan University, Institute of Microbiology and Biochemistry, Taipei, Taiwan, Republic of China

It has been reported that the biological function of colostral antibodies from immunized cows, give newborns an immunological protection against environmental pathogens. The cytokines produced by T lymphocytes are the certain regulatory factor on immune responses. To investigate the effects of bovine immune milk on cytokine productions LPS-stimulated RAW 264.7 were treated with different concentration (0, 4, 8, 10 mg/mL) of Stolle Milk Protein Concentrate (SMPC) for 48 hours. SMPC had dose-dependent inhibitory effect on TNF $\alpha$  production. Then, primary splenocytes from BALB/c mice were treated with SMPC (0, 0.8, 4, 8 mg/mL) under ConA stimulation for 24 hours and the cytokines content in supernatant were analyzed by ELISA. SMPC significantly decreased the Th2 cytokines, IL-4 and IL-5 production and increased the Th1 cytokines, IL-2 and IFN $\gamma$  production, and thus the IFN $\gamma$ /IL-4 ratio was significantly higher ( $P < 0.01$ ). Further, the OVA-sensitized BALB/c mice were fed SMPC (4 or 8 mg/day) for 2 weeks to investigate its effects on allergic responses. The results showed that mice fed with SMPC had significantly lower splenocytic IFN $\gamma$  and IL-4 production and lower serum levels of total IgE, anti-OVA IgE and anti-OVA IgG<sub>1</sub>. On the other hand, SMPC significantly inhibited airway hyperresponsiveness (AHR) and decreased IL-13 and IFN $\gamma$  concentrations and less eosinophil in bronchoalveolar lavage fluid (BALF) after inhalation of nebulized OVA. This study showed that SMPC decreased serum anti-OVA IgE and IgG<sub>1</sub> levels, AHR, and airway allergic inflammation in mouse model of asthma, suggesting an immunomodulatory effect on allergic disease.

#### PD19/31 THE ROLE OF ALTERED PEPTIDE LIGANDS IN INDUCTION OF TOLERANCE IN OVA-INDUCED MURINE MODEL OF ASTHMA

H.-Y. Huang<sup>1</sup>, B.-L. Chiang<sup>2</sup>

<sup>1</sup>Graduate Institute of Clinical Medicine, National Taiwan University, Taipei, Taiwan, Republic of China, <sup>2</sup>National Taiwan University Hospital, Department of Pediatrics, Taipei, Taiwan, Republic of China

**Background:** Asthma is caused by aberrant Th2 responses to harmless inhaled allergens underlain a disturbance in the balance between Th1- and Th2-mediated immune responses. Th1 cells and regulatory T cells are thought to modulate the allergen-induced Th2 responses. Peptide alterations of T cell epitopes with single or few amino acid variations can have drastic effects on the outcome of this recognition. Altered peptide ligands (APLs) can act as modulators of immune responses through induction of different cytokines production profile or differentiation of different lineage of T cells.

**Objectives:** In this study, we would like to design APLs, which modified from OVA<sub>323-339</sub> peptide, major epitope of DO11.10 TCR transgenic mice, and investigate whether APLs affect the function of allergen-specific T cells, such as differentiating toward Treg or Th1 and then we will investigate whether APLs could modulate the airway inflammation in OVA-induced murine of asthma mice, as an allergen-specific immunotherapy approach.

**Methods:** We synthesized six APLs with a single amino acid substitution of the OVA<sub>323-339</sub> (WT) and examined the phenotypes of OVA-specific T cells activated by these peptide analogues.

**Results:** Five of six peptide analogues (E333A, H331Q, H331F, H331R, and H331E) did not induce proliferation, TCR internalization, and cytokines production of DO11.10 T cells, being null peptides. Whereas, N335A induced activation of T cells in high concentration, likely being a weak agonist. Furthermore, N335A-primed T cells produced higher level of INF- $\gamma$  and lower level of IL-4 compared with WT-primed T cells upon WT restimulation. However, T cells stimulated with six peptide analogues did not show any properties of Treg cells.

**Conclusion:** We found that the OVA peptide analogous, N335A, could induce OVA-specific T cells to differentiate into Th1 cells and then we will deliver N335A into OVA-sensitized mice to investigate whether N335A could regulate the allergic responses and inhibit the airway inflammation in murine model of asthma.

#### PD19/32 IMMUNOLOGICAL RESPONSE TO ORAL SPECIFIC IMMUNOTHERAPY

A. Kazimova<sup>1</sup>

<sup>1</sup>Azerbaijan Medical University, Baku Clinical Hospital N5, Allergy, Baku, Azerbaijan

**Background:** Appraisal of effectiveness of oral and parenteral SIT in patients with perennial allergic rhinitis (PAR) and allergic bronchial asthma (ABA).

**Methods:** One-centered open randomized public study was held on base of the Hospital N5 in Baku from September 1, 2007 to November 31, 2008. Research consisted from 43 patients (24 PAR; 19 ABA) at the age of 12-49 (middle age 28.9), 18 women and 25 men. Control group-10 healthy people aged 13-46 (middle age 28.7). Patients were randomized into 2 groups: 1st -peroral SIT – 22 patients (PAR-12; ABA-10), 2nd-parenteral SIT-21 patients (PAR-12; ABA-9). There were used

medications of the “Biomed” (Moscow, Russia) for the parenteral SIT realization. There was used the mixture of 4 allergens (*Dermatophagoides farinae*, *Dermatophagoides pteronyssinus*, house dust and pillow feather) in equal parts. Patients took allergen in a dose of  $10^{-12}$  to  $10^{-1}$  PNU within 5 months. There was selected the medication of “Immunolog” (Vinnitsa, Ukraine) for the peroral SIT realization. The medication is a mixed-dragee, which also consist of 4 allergens. Patients had the allergen in a dose of 0.2 to 1000 PNU also during 5 months. There was tested the analysis of immunological indicators in blood as IL-4; IL-10; TNF- $\alpha$ ; IFN- $\gamma$ ; IgE; IgA; IgM; IgG in all patients before and after the immunotherapy. Immunological analyses were carried out by method ELISA, on analyzer Stat-fax “2100” (USA). **Results:** The comparison of the received results of the immune status features has revealed a prevalence of the immune answer of type Th1 in a group of patients with peroral SIT, though was not strongly pronounced. It was evidenced by more pronounced increase of formulating IFN- $\gamma$ , IgG and the reduction of IL-4, IL-10, TNF- $\alpha$  and IgE, IgA after the immunotherapy. Levels of indicators IL-4, IL-10 and IFN- $\gamma$ , IgG kept within norms before and after the immunotherapy though they had changes in positive direction. While indicators IgE, IgM, TNF- $\alpha$  turned out more significant in the positive relation. **Conclusion:** According to the results of the study, the immunological blood indices in patients with PAR and ABA having SIT peroral method did not differ from those ones by the parenteral method, and they even exceed it slightly.

#### PD19/33 MURAMYL PEPTIDE GMDP NORMALIZES TH1/TH2 BALANCE IN PATIENTS WITH ATOPIC BRONCHIAL ASTHMA

S.V. Guryanova<sup>1</sup>, I.G. Kozlov<sup>2</sup>, T.M. Andronova<sup>1</sup>

<sup>1</sup>Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Peptide Chemistry, Moscow, Russian Federation, <sup>2</sup>Russian State Medical University, Pharmacology, Moscow, Russian Federation

**Aims:** The key process leading to atopic bronchial asthma (ABA) is hyperactivation of T-helper 2 (Th2) accompanied with decreasing T-helper 1 (Th1) activity. Therefore a search for drugs normalizing Th1/Th2 balance is of considerable interest. In this study the effect of glucosaminylmuramyl dipeptide (GMDP) on the production of main Th1 and Th2 cytokines was observed.

**Methods:** Th1 activity was registered on IFN- $\gamma$  production by intact and stimulated with PHA (10  $\mu$ g/ml) and anti-CD3 (5  $\mu$ g/ml) *in vitro* mononuclear cells (MNC) from peripheral blood of healthy donors and ABA patients. Th2 activity was evaluated on IL-4 secretion in the same conditions.

**Results:** It was shown that GMDP (0.05–5  $\mu$ g/ml) *in vitro* modulates proliferation of PHA- and anti-CD3-stimulated MNC from healthy donors and ABA patients and dose dependently increases 3–8 fold production of IFN- $\gamma$ . It was also observed that muramyl peptide significantly decreases secretion of IL-4 by MNC from ABA patients both intact and mitogen-stimulated. This effect of muramyl peptide does not appear to be due to lack of co-stimulatory signals: MNC healthy donors does not react to GMDP in the absence of mitogens.

**Conclusions:** The above data suggests, that the positive clinical effect of GMDP on the patients with ABA, detected in previous investigations, results from normalization of the Th1/Th2 balance.

#### PD19/34 ANALYSIS OF THE IMMUNE MODULATORY FUNCTION OF PHYCOBILIPROTEIN OF RED ALGAE WITH THE FUNCTIONAL ASSAY WITH DENDRITIC CELLS

C.-J. Chang<sup>1</sup>, B.-L. Chiang<sup>2</sup>

<sup>1</sup>Graduate Institute of Clinical Medicine, National Taiwan University, Taipei, Taiwan, Republic of China

**Background:** BaPC, a pure protein purified from red algae *Bangia atropurpurea*, is a phycobiliprotein (PC). So far, several biological functions of PC derived from *Spirulina* have been reported to exhibit a variety of pharmacological properties, such as antioxidant, anti-inflammatory, neuroprotective and hepatoprotective effects. In the present study, we investigated the effects of BaPC in the modulation of immune responses.

**Methods:** Therefore, we isolated mouse bone marrow derived dendritic cells (DCs) and cultured with BaPC to study its effect on dendritic cells and other immune effector cells.

**Results:** Treatment of DC with BaPC resulted in slightly increase of the expressional level of cellular surface markers such as CD40, CD80, CD86, CD205 and MHCII molecules and the level of cytokine IL-12 also increased but not IL-10. In addition, BaPC-induced cytokine production of IL-12 was reduced in neutralization assay with antibodies against Toll-like receptor 4 (TLR-4). Further, the result of inhibitory drug treatment showed that IL-12 and IL-10 production were decreased after Hellenalin treatment. Furthermore, the capacity for endocytosis is reduced in DC treated with BaPC. We also found that BaPC treated DC induced significantly higher levels of T-cell proliferation in analysis of mixed lymphocyte reaction (MLR). Interestingly, the production of Th1 cytokine IFN $\gamma$  is increased in MLR with the time, but the production of Th2 cytokine IL-4 is decreased.

**Conclusion:** Thus, our data indicated that BaPC potentially promote the activation and maturation of immature DCs and preferred to drive T helper1 immune responses for activated T cells. From the results here, BaPC might be used for the future application of immune modulation.

#### PD19/35 THE CONCLUSIVE THERAPEUTIC FOLLOW UP OF THE CHRONIC URTICARIA WITH TRIPLE REGIMEN(H1,H2 RECEPTORS ANTAGONISTS & HYDROXYZINE) & THERAPEUTIC IMMUNOTHERAPY

M. Ishaq<sup>1</sup>, S. Ishaq<sup>1</sup>, I.K. Munir<sup>1</sup>

<sup>1</sup>Al-Junaid Hospital, Allergy/Pulmonary, Nowsehra, Pakistan

**Rationale:** Chronic urticaria an intractable allergic problem demands a calculated clinical approach helping in the aversion of recurrent morbidity. The significance of its economical & physical impact is without reservation.

**Methods:** 10 Patients in the study had a prolonged history of urticaria (a chronic lingering cascade with episodic outburst in association with airways allergies) had been included. Clinically presenting as interact able itching/urticaria, recurrent coryza, post nasal drips while taking onion, chillies & citrus fruits, intake of aspirin sulpha drugs, showers with warm waters. A likewise features had been also found in other family members with a variable degree of severity. Skin prick test with diagnostic allergy extracts had shown outstanding skin reaction in some with a variable degree of itching & flare up around the skin test site. Therapeutic approach with triple regimen (e.g.) Cimetidine, Loratadine (double dose till the severity of symptomatology) & Hydroxyzine followed by the specific therapeutic allergy extracts resulted in a remarkable improvement of the clinical manifestations as under.

**Results:** Dramatic regression of the clinical manifestations (e.g.) urticaria (itching/burning) in 70% in first 24 hours with complete improvement in next 36 hours while rather slow improvement in upper airways allergies 50–60% in first 10 hours with significant relief in the next 24 hours observed. The degree of regression of all allergies were inversely proportional to the age of the patients.

**Conclusions:** Topical application of anti-pruritics & corticosteroids preparation has significantly relieved not only the symptomatology of urticaria but also the incidence of post scratch scar formation.

#### PD19/36 THE EFFECTS OF ORAL DELIVERY OF RECOMBINANT HOUSE DUST MITE ALLERGEN ON AIRWAY INFLAMMATION IN MURINE MODEL OF ASTHMA

C.-H. Jian<sup>1</sup>, B.-L. Chiang<sup>2</sup>

<sup>1</sup>Graduate Institute of Oral Biology, College of Medicine, National Taiwan University, Taipei City, Taiwan, Republic of China, <sup>2</sup>Graduate Institute of Clinical Medicine, College of Medicine, National Taiwan University, Taipei City, Taiwan, Republic of China

**Objectives:** Asthma is one of the most common allergic diseases in children; in addition, about 80% of asthmatic patients in Taiwan are sensitized by house dust mite – *Dermatophagoides pteronyssinus* (*Der p*). The characteristics of asthma including airway hyperresponsiveness (AHR), eosinophils infiltration, antigen-specific T helper 2 cells activation, increased mucus secretion and even airway remodeling. Oral tolerance, oral administration of antigens induce antigens-specific immune tolerance, might be a therapeutic strategy for allergic asthma. Therefore, we aimed to apply oral administration of recombinant *Der p* 1 and recombinant *Der p* 2 to decrease the airway inflammation induced by *Der p*.

**Methods:** We used female BALB/c mice and gave the mice with crude extraction of *Der p* as a murine model of allergic asthma. In this study, we sensitized mice with peritoneal injection, then challenged with intratracheal injection of crude extraction of *Der p*.

**Results:** The results showed that peritoneal injection with high-dose could induce the clinical features of asthma significantly, such as elevated *Der p*-specific IgE in serum, production of Th2-cytokine (IL-5, IL-13), and *Der p*-specific lymphoproliferation. Furthermore, the high-dose group of mice had severe airway inflammation including increased airway hyperresponsiveness, interleukin 5 production both in lung, and lymphocytes infiltration. Further, we orally delivered different doses of crude mite or recombinant proteins to the mice and followed the changes in immunological parameters.

**Conclusion:** In conclusion, these data indicated that sensitization with high-dose of crude extraction of *Der p* can establish a murine model of asthma. We will further investigate the effects of oral administration of recombinant allergens on airway inflammation.

#### PD19/37 IL-10-MEDIATED IMMUNE RESPONSE INDUCED BY DNA-HSP65 THERAPY DOWN REGULATES ALLERGIC RESPONSE

D.M. Fonseca<sup>1</sup>, L.W. Campos<sup>1</sup>, P.F. Wowk<sup>1</sup>, M.O. Paula<sup>1</sup>, A.F. Gembre<sup>1</sup>, W.M. Turato<sup>1</sup>, M. Russo<sup>2</sup>, M. Dias-Baruffi<sup>3</sup>, C.L. Silva<sup>1</sup>, V.D. Bonato<sup>1</sup>

<sup>1</sup>Medical School of Ribeirão Preto/São Paulo University, Ribeirão Preto, Brazil, <sup>2</sup>Institute of Biomedical Sciences, São Paulo, Brazil, <sup>3</sup>Pharmaceutical Sciences School of Ribeirão Preto, Ribeirão Preto, Brazil

**Objective:** It has been reported that immunization with DNA plasmid codifying distinct allergens as well as mycobacterial heat shock proteins down-modulates experimental allergy. In this work, we evaluated the mechanisms involved on modulation of allergic response by DNA plasmid codifying a 65kDa heat shock protein from *Mycobacterium leprae* (DNA-HSP65).

**Methods:** BALB/c mice, previously sensitized with ovalbumin (OVA), were OVA-challenged by intranasal route. After 72h, mice were treated with 3 doses of DNA-HSP65 or DNA-vector by intramuscular route (15-day intervals). Fifteen days after treatment, they received a second OVA-challenge. After this challenge, we evalu-

ated the airway hyperresponsiveness, cell recruitment and cytokine concentrations on bronchoalveolar lavage fluid (BALF), serum antibody production and cytokine secretion by OVA-stimulated spleen cells. A cell transfer experiment was also performed.

**Results:** DNA-HSP65-treated mice exhibited a significant decrease in airway hyperresponsiveness compared to untreated-allergic and DNA-vector-treated mice. It was observed a significant decrease of eosinophilia, IL-4, IL-5, IL-13, TSLP and eotaxin, and an increase in IL-10 on BALF from DNA-HSP65-treated mice. IL-10 secretion was also increased in spleen cell cultures stimulated with OVA. The DNA-HSP65 therapy also reduced allergen-specific IgG1 and IgE antibodies. An Hsp65-specific immune response was induced by DNA-HSP65 therapy, this response was characterized by IFN- $\gamma$  and IL-10 production by spleen cells, and specific IgG1 and IgG2a antibodies. The role of IL-10 mediating the modulation of allergic response was confirmed when the therapeutic effect of DNA-HSP65-treatment was impaired in IL-10 knockout mice. Moreover the protective role of Hsp65-specific cells was demonstrated by a cell transfer experiment. The intravenous transference of spleen cells from Green Fluorescent Protein (GFP) transgenic mice immunized with DNA-HSP65 to allergic wild type mice down-modulated the eosinophilia and Th2 cytokine production on BALF. GFP+ lymphocytes were detected on BALF, lungs and draining lymph nodes from allergic mice which received cells from DNA-HSP65 immunized GFP transgenic mice, but not in mice that received GFP+ cells from non-immunized transgenic mice.

**Conclusion:** These results show that DNA-HSP65 down-modulated Th2 allergic response. This effect is IL-10-dependent and seems to involve the migration of IL-10-producing cells to the allergic response site.

**Financial support:** FAPESP, CNPq, FAEPA

#### PD19/38 IFN- $\gamma$ -MEDIATED IMMUNE RESPONSE INDUCED BY MYCOBACTERIAL ANTIGENS THERAPY DOWN REGULATES ALLERGIC RESPONSE

D. M. Fonseca<sup>1</sup>, L. W. Campos<sup>1</sup>, P. F. Wowk<sup>1</sup>, M. O. Paula<sup>1</sup>, A. F. Gembre<sup>1</sup>, W. M. Turato<sup>1</sup>, M. Russo<sup>2</sup>, M. Dias-Barufi<sup>3</sup>, C. Horn<sup>4</sup>, C. L. Silva<sup>1</sup>, G. Marchal<sup>5</sup>, V. D. Bonato<sup>1</sup>

<sup>1</sup>Medical School of Ribeirão Preto/São Paulo University, Ribeirão Preto, Brazil, <sup>2</sup>Institute of Biomedical Sciences/São Paulo University, São Paulo, Brazil, <sup>3</sup>Pharmaceutical Sciences School of Ribeirão Preto/São Paulo University, Ribeirão Preto, Brazil, <sup>4</sup>Oswaldo Cruz Foundation, Rio de Janeiro, Brazil, <sup>5</sup>Institute Pasteur, Paris, France

**Objective:** It has been described that immunization with distinct mycobacterial species could control experimental allergy. In this work, we evaluated the mechanisms involved on modulation of allergic response by culture filtrated proteins (CFP) from *Mycobacterium tuberculosis* in the presence of CpG-Oligodeoxynucleotides (CpG/CpG).

**Methods:** BALB/c mice, previously sensitized with ovalbumin (OVA), were challenged with OVA by intranasal route. After 72h, mice were treated weekly with 3 doses of CFP/CpG, by subcutaneous route; CpG-Oligodeoxynucleotides were used as control. Fifteen days after treatment, they received a second OVA-challenge. After this challenge, we evaluated the airway hyperresponsiveness, cell recruitment and cytokine concentrations on bronchoalveolar lavage fluid (BALF), serum antibody production and cytokine secretion by OVA-stimulated spleen cells. A cell transfer experiment was also performed.

**Results:** CFP/CpG-treated mice exhibited a significant decrease in airway hyperresponsiveness compared to untreated-allergic and CpG-treated mice. It was observed a significant decrease of eosinophilia, IL-4, IL-5, IL-13, TSLP and eotaxin, and an increase of IFN- $\gamma$  on BALF from CFP/CpG-treated mice. IFN- $\gamma$  secretion was also increased in OVA-stimulated spleen cell cultures. The CFP/CpG therapy also reduced allergen-specific IgG1 and IgE antibodies and increased IgG2a. No lung tissue damage was observed after CFP/CpG treatment, which induced a CFP-specific immune response characterized by IFN- $\gamma$  and IL-10 production by spleen cells, and specific IgG1 and IgG2a antibodies. The role of IFN- $\gamma$  mediating the modulation of allergic response was confirmed when the therapeutic effect of CFP/CpG treatment was impaired in IFN- $\gamma$  knockout mice. Moreover the protective role of CFP-specific cells was demonstrated by a cell transfer experiment. The intravenous transference of spleen cells from Green Fluorescent Protein (GFP) transgenic mice immunized with CFP/CpG to allergic wild type mice down-modulated the eosinophilia and Th2 cytokine production on BALF. GFP+ lymphocytes were detected on BALF, lungs and draining lymph nodes from allergic mice which received cells from CFP/CpG-immunized GFP transgenic mice, but not in mice that received GFP+ cells from non-immunized transgenic mice.

**Conclusion:** These results show that CFP/CpG down-modulated Th2 allergic response. This effect is IFN- $\gamma$ -dependent and seems to involve the migration of IL-10-producing cells to the allergic response site.

**Financial support:** FAPESP, CNPq, FAEPA

#### PD19/39 EVALUATION OF DIFFERENT PARTICULATE DELIVERY SYSTEMS AND ADMINISTRATION ROUTES FOR IMPROVED ALLERGEN-SPECIFIC IMMUNOTHERAPY

D. Mohanan<sup>1</sup>, Y. Perrie<sup>2</sup>, W. Jiskoot<sup>3</sup>, T. Kündig<sup>1</sup>, B. Gander<sup>4</sup>, P. Johansen<sup>1</sup>

<sup>1</sup>University Hospital of Zurich, Department of Dermatology, Zurich, Switzerland, <sup>2</sup>Aston University, Birmingham, United Kingdom, <sup>3</sup>Leiden/Amsterdam Center for Drug Research, Leiden, Netherlands, <sup>4</sup>Institute of Pharmaceutical Science, ETHZ, Zurich, Switzerland

Although allergen-specific immunotherapy (SIT) has been around for almost a hundred years and remains in use worldwide for the treatment of allergic rhinitis and asthma, it's only recently that we have a better understanding of the mechanisms of its action. The immunological mechanisms by which specific immunotherapy is effective is thought to be by induction of allergen-neutralising IgG antibodies and by modulation of T-cell responses; SIT shifts the immune response against the allergen from a predominant Th2-like response towards a more Th1-like response. However, the main disadvantage of current allergen SIT resides in the high number of subcutaneous injections required over several years, which is associated with high costs and occasional local adverse effects. Thus, there is an increasing interest in studying alternative administration routes as well as delivery systems.

Microspheres, nanoparticles and liposomes are novel dosage forms that through its particulate nature may target the immune system and at the same time provide a depot of the entrapped allergen. In the present study, we tested the immunotherapeutic potential of these different delivery systems upon intramuscular, intradermal, subcutaneous and intralymphatic administration. Balb/c mice were immunised on Day 0 and boosted on Day 42 with different ovalbumin-containing formulations and bled at different timepoint for analysis of OVA-specific IgG1, IgG2a and IgM responses. Lymphocyte proliferation and cytokine secretion were assessed by culturing splenocytes of immunised mice.

From our data, it has been shown consistently that the intralymphatic route is the best route to induce an early IgG2a regardless of the delivery system used. However the same can't be said about the IgG1 responses. It was also observed that mice that had received the nanoparticles via the intralymphatic route, made significantly higher IgG2a responses when compared to their IgG1 response early in the experiment. It was also noted that nanoparticles regardless of route used, had induced a better IgG2a response overall when compared to the other 2 delivery systems. Hence our data suggests that the nanoparticles given via the intralymphatic route might be a "potential diamond in the rough" for the treatment of allergies.

#### PD19/40 SCHISTOMIASIS AND ASTHMA

M. Ritter<sup>1</sup>, Clarissa da Costa<sup>1</sup>, Laura Layland<sup>1</sup>

<sup>1</sup>Institute of Medical Microbiology, Immunology and Hygiene, Munich, Germany

Over the last century and in strong contrast to third world countries, Western populations have shown a consistent rise in diseases such as allergy (asthma) and intestinal disorders (Crohn's disease). The underlying etiological characteristics of these diseases remains unclear although it is suspected that there are misdirected Th2 responses or inadequate Th1 and Th17 immune reactions. Interestingly, epidemiological studies and *in vivo* animal models have demonstrated that due to host immunomodulation, helminth infections may be a reason for the lower prevalence of asthma and other allergic illnesses. Important immune cell populations responsible for modulating the Th1/Th2 balance include the Foxp3<sup>+</sup> regulatory T cells (Treg) and distinct sub-populations of dendritic cells. We investigated the effects of *Schistosoma mansoni* and components thereof, in inducing such regulatory populations and preventing asthma.

We used an established mouse model of allergic airway inflammation with Ovalbumin as the model antigen, and could show that a chronic schistosome infection can dramatically reduce leucocyte influx into the lung, reduce pathology, reduce systemic and local OVA-specific T and B cells responses. All this correlates with an accumulation of Foxp3-positive T cells in the draining lymph nodes. Interestingly, the protective effects of schistosomes were dependent on antihelminthic treatment implying that strong regulatory responses are driven by constant antigen trigger. Furthermore, reduction of allergic airway inflammation by schistosomes required immunization at least the onset of egg-laying demonstrating that only the immunization with OVA into an immunocompromised (or Th2-Treg-biased) host results in suppression of allergic diseases.

#### PD19/41 FORMALDEHYDE INHALATION ACTIVATES SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION-5 IN THYMOCYTES

Y. Yoshida<sup>1</sup>, D. Ning<sup>1</sup>, J. Noguchi<sup>1</sup>, N. Kunugita<sup>2</sup>, T. Sugiura<sup>1</sup>

<sup>1</sup>University of Occupational and Environmental Health, Kitakyushu, Japan, <sup>2</sup>National Institute of Public Health Ministry of Health, Labour and Welfare, Wako, Japan

It has been reported that the number of people suffering from occupational asthma and skin rashes triggered by various chemicals in the indoor air have increased markedly. Formaldehyde (FA) is known to be an indoor air pollutant and its influence on health is of a great concern. However, there are very few studies that report its effect on transcription factors in immune cells. In this study, we investigated the effects of FA on immune responses, specifically on the regulation of transcription factors. The proliferation of splenocytes and thymocytes were not affected by FA exposure. The level of interleukin (IL)-2 in thymocytes of FA-exposed mice was induced, while IL-2 and IL-12 production was not induced in splenocytes. FA exposure activated Signal Transducer and Activator of Transcription 5 (STAT5) in thymocytes. These suggest that STAT5 is a candidate for a biomarker for inhalation of FA in the indoors.