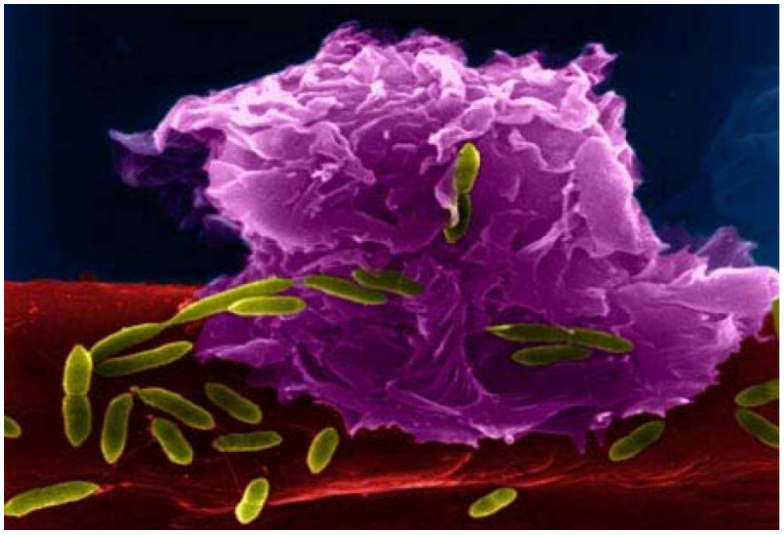
Comprehensive Center for Infection, Immunity and Transplantation

Infektiologie-Immunologie-Transplantation

# 4th CIIT SCIENCE DAY

June 27th, 2013

# Abstract book



tilak Universitätskliniken LKH Innsbruck

Medizinische Universität Innsbruck

## Welcome Remarks

In 2009, the ´Comprehensive Center for Infection, Immunity, and Transplantation (CIIT) was founded at the Innsbruck Medical University to promote the collaboration between science and teaching as well as to support diagnostics, therapy and prevention of infectious diseases. The CIIT is co-ordinated by a speakers´ team from various disciplines and strengthens the interdisciplinary exchange by organizing lectures of local and external speakers and clinically oriented case studies (Grand Rounds).

A highlight in the CIIT event calendar is the CIIT Science Day. On June 27th, 2013, the 4th CIIT Science Day will again bring together allocated researchers with a special focus on infection, immunity and transplantation. On this day an overview of current scientific topics and projects in respective research areas is provided and on-going projects and studies are presented by scientists during guided poster tours. The organizers are proud that this year 59 abstracts have been submitted, which guarantees a broad scientific exchange and lively discussions.

Additionally, we are very glad that this year Prof. Michael Sixt from the Institute of Science and Technology Austria (IST Austria) will give the keynote lecture. Prof. Sixt is an outstanding expert focussing on molecular and mechanical principles underlying leukocyte dynamics.

Finally, we want to thank all people involved in the organization of this 4th CIIT Science Day, especially Susanne Rofner, Sonja Harm, the members of the CIIT speakers´ team and the poster chairs. Furthermore, we want to acknowledge the Austrian Society of Allergology and Immunology (ÖGAI) and the companies, which supported us financially. They are listed in the back of the abstract book.

We wish all participants and guests an interesting and stimulating meeting and a lively scientific exchange.

*Doris Wilflingseder* and *Thomas Sonnweber*

on behalf of the CIIT speakers´ team

## Program

LOCATION: großer Hörsaal and Seminarräume MZA (Medizinzentrum Anichstraße)

Großer Hörsaal:

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| 14.00-14.15 | Introducing words |
| | Opening of the 4th CIIT DAY: Rektor Univ. Prof. Dr. H. Lochs |
| 14.15-15.15 | Keynote Lecture: Univ.-Prof. Dr. Michael Sixt |
| | "Molecular Regulation of Shape, Polarity and Motility of Leukocytes" |
| 15.25-18.30 | Moderated Postersessions (Seminarräume 1+2) |

| | | **Postermoderator** | **location** |
|---|---|---|---|
| 15.25-16:20 Postersession I+II | Poster 1-10 | Reinhard Würzner | SR1 |
| | Poster 11-20 | Cornelia Speth | SR2 |
| 16.30-17.25 Postersession III+IV | Poster 21-30 | Jakob Troppmair | SR1 |
| | Poster 31-40 | Nikolaus Romani | SR2 |
| 17.35-18.30 Postersession V+VI | Poster 41-50 | Ernst Werner | SR1 |
| | Poster 51-59 | Katja Kotsch | SR2 |

*Coffee and drinks will be available throughout the Postersessions.*

18.30-20.00 Posterdiscussions with light buffet and drinks

# Index

| | | |
|---|---|---|
| 25 | T cell responses rely on Langerin+ dermal dendritic cells in a spontaneous mouse melanoma model | D.G.Mairhofer |
| 26 | The Erythropoietin-Analogue ARA290 Ameliorates the Course of Experimental Colitis | M. Nairz |
| 27 | The Anti-apoptotic Protein Mcl-1 is Essential for the CD8 T Cell Response upon Chronic Viral LCMV Infection. | E. Ottina |
| 28 | Lipidomics Profiling and Risk of Cardiovascular Disease in the Prospective Population-based Bruneck Study | R. Pechlaner |
| 29 | In vivo and in vitro evaluation of the effect of PRE-084 on inflammation-sensitized hyperoxia-induced developing brain injury | A. Posod |
| 30 | Bone marrow T cells from the femur are similar to iliac crest derived cells in old age and represent a useful tool for studying the aged immune system | T. Pritz |
| 31 | An Integrative Analysis of Renal miRNA- and mRNA-Expression Signatures in Progressive Chronic Kidney Disease | M. Rudnicki |
| 32 | Tetrahydrobiopterin Compounds Modulate Intracellular Signaling and Reactive Oxygen Species Levels in an In Vitro Model of Ischemia-Reperfusion Injury | P. Santer |
| 33 | INVESTIGATING THE ROLE OF Bcl2a1/A1/BFL-1 IN LEUKOCYTE DEVELOPMENT | M. Sochalska |
| 34 | Interferon Type I Signalling Mediated Crosstalk Between Intestinal Epithelium and Gut Microbiota in Mice | M. Tschurtschenthaler |
| 35 | Impact of the prosurvival Bcl-2 family member A1 on T cell immunity | S. Tuzlak |
| 36 | The Ontogeny and Differentiation Pathways of Tumor-Associated Macrophages in a Model of Spontaneous Mammary Carcinogenesis | P. Tymoszuk |
| 37 | Protein Kinase C θ Regulates Th1/Th17 Plasticity | K. Wachowicz |
| 38 | The role of Type I Interferons in alcoholic liver disease | V. Wieser |

## Transplantation/Therapy/Diagnostics

| | | |
|---|---|---|
| 39 | Evaluation of Cryopreserved and Thawed Human Ovarian Tissue after Xenotransplantation in SCID Mice | S. Ayuandari |
| 40 | Reduction of Complement Factor H Binding to CLL Cells Improves the Induction of Rituximab-Mediated Complement-Dependent Cytotoxicity | Z. Bánki |
| 41 | VSV-GP Is A Potent Oncolytic Virus Platform For The Treatment Of Melanoma | I. Barnstorf |
| 42 | Liver Grafts From Anti-HBc Positive Donors Are Safe For Anti-HBc Negative Recipients In Liver Transplantation Using Combined Antiviral Prophylaxis | A. Brandl |
| 43 | LCMV-GP Pseudotyped VSV for Oncolytic Virotherapy of Ovarian Cancer | C. Dold |
| 44 | The Functional Influence of Activating NK Cell Receptors in Solid Allograft Rejection | C. Fabritius |
| 45 | The Therapeutic Potential Of The Pharmacological Inhibition Of PBEF/NAMPT/Visfatin in Inflammatory Bowel Disease | R. R. Gerner |
| 46 | Innate Lymphocyte Activation by Targeted Manipulation of the Mevalonate Pathway: Implications for Cancer Immunotherapy | G. Gruenbacher |
| 47 | Treatment With Oral Active Vitamin D Is Associated With Decreased Risk Of Peritonitis And Improved Survival In Patients On Peritoneal Dialysis | J. Kerschbaum |
| 48 | Clinical Application of CSF PCR Testing for Diagnosis of CNS-disorders - A Retrospective 11-Years Experience | M. Kleines |
| 49 | Rituximab Reduces Relapses and the Dose of Immunosuppressants in Steroid-Dependent and Frequently Relapsing Minimal Change Disease and Focal Segmental Glomerulosclerosis in Adults: A Systematic Review | A. Kronbichler |
| 50 | Metallothioneins as Markers for Biological Organ Age in Preimplantation Kidney Biopsies | J. Leierer |
| 51 | The Effect of Preservation Solutions HTK, HTK-N and TiProtec on Various Tissues after Rat Hind-Limb Allotransplantation | F. Messner |

| | | |
|---|---|---|
| 52 | Tetrahydrobiopterin Preconditioning Saves Murine Pancreatic Isografts from Brain Death Exacerbated Ischemia Reperfusion Injury | R. Oberhuber |
| 53 | Donor brain death results in differentially modulated immune activation in solid organs | P. Ritschl |
| 54 | HIV-1 Gene Therapy using Zinc -Finger Nucleases (ZFN) and Transcription Activator Like Effector Nucleases (TALENs) | K. Schmidt |
| 55 | The Viral Vector Vaccine VSV-GP Boosts Immune Response Upon Repeated Applications | R. Tober |
| 56 | LCMV-GP Pseudotyped Oncolytic Vesicular Stomatitis Virus for the Treatment of Prostate Cancer | C.R. Urbiola |
| 57 | Recall responses to tetanus and diphtheria vaccination in elderly persons depend on adequate B cell memory and plasma cells generated earlier in life | B. Weinberger |
| 58 | Austrian Drug Prescription Report 2006-2011: Antibiotics | G. Wietzorrek |
| 59 | Effect of Storage Temperature and Antibiotic Impregnation on the Quantity of Bone Morphogenetic Protein-7 in Human Bone Grafts | A. Wurm |

# Infection

## Heme regulation in Aspergillus fumigatus

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**Background:** Sufficient iron supply is indispensable for survival of almost all organisms. However, an excess of iron is potentially toxic. In the opportunistic human-pathogenic fungus Aspergillus fumigatus the ability to adapt to iron limitation represents a crucial virulence factor. Iron regulation is tightly interconnected with heme metabolism, as iron-containing heme is an essential cofactor of a variety of cellular processes, e.g. respiration, sterol biosynthesis, oxidative stress detoxification and also reductive iron assimilation. Most knowledge on fungal heme regulation derives from studies in Saccharomyces cerevisiae. A. fumigatus, as well as most other fungal species, lack homologs of key heme regulators found in S. cerevisiae. The goal of this study is to elucidate heme-dependent regulation in A. fumigatus (wt).

**Methods:** As a first step, we generated a mutant strain (Δ hemA) lacking the gene encoding aminolevulinic acid synthase (HemA), which catalyzes the committed step in heme biosynthesis. This mutation offers the possibility to control the cellular heme content by supplementation with aminolevulinic acid (ALA).

**Results:** Growth of Δ hemA was blocked at ALA concentrations below 20 µM, but fully restored by addition of 200 µM on solid as well as in liquid media. Supplementation with protoporphyrin IX (PpIX), the iron free heme precursor, and hemin (chloroprotoporphyrin IX iron(III)) supported growth of Δ hemA. Under iron starvation, ALA supplementation led to a tremendous accumulation of PpIX in both Δ hemA and wt. Furthermore, in Δ hemA, ALA restriction transcriptionally increased the heme-biosynthetic coproporphyrinogen(III)oxidase and the putative heme receptor CFEM3. Additionally, ALA limitation decreased the resistance of Δ hemA to oxidative stress and the triazole antifungal drug posaconazole.

**Conclusion**: Our results prove that A. fumigatus is able to utilize exogenous porphyrins, although it is in contrast to several other fungal species is not able to utilize hemin as iron source. Furthermore, the observed accumulation of PpIX indicates that HemA represents the major rate limiting step in heme biosynthesis when iron is scarce. Moreover, our results underline the crucial role of heme in detoxification and sterol biosynthesis in A. fumigatus.

This work was supported by the Austrian Science Foundation grant FWF P21643-B11 to HH.

# Human Plasmatic Coagulation is Significantly Impaired by EHEC-derived Shiga Toxin 2

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**Background:** Infection with Shiga toxin producing Escherichia coli (EHEC) is the most important cause for typical hemolytic uremic syndrome (HUS). HUS is defined by renal injury due to damage of renal endothelial cells caused especially by Shiga toxin 2 (Stx2). Consecutively the coagulation system is activated. It has also been postulated, that Stx itself acts as an activator of blood platelets. However, there are also hints that the plasmatic coagulation system is affected in HUS. In this study we investigated the effect of Stx2 on platelets, on the plasmatic coagulation and on antithrombin (AT), a strong inhibitor of the plasmatic coagulation cascade.

**Methods:** Stx2 was incubated with platelet-rich-plasma (PRP) and aggregometry was performed to evaluate the aggregation inducing capacity of Stx2. In flow cytometry, activation of platelets was shown measuring the expression of two platelet-activation markers, CD62P (P-selectin) and CD63. For evaluation of a possible impairment of the plasmatic coagulation due to Stx2 thromboelastometry (ROTEM) analyses with platelet-poor-plasma (PPP) were performed and Coagulation Time (CT) and Clot Formation Time (CFT) were measured. ELISA was performed to evaluate a binding of antithrombin (AT) to Stx2.

**Results:** Neither in aggregation, nor in the expression of the investigated activation markers CD62P and CD63, a Stx2-induced effect on platelets could be observed. In ROTEM a clear reduction of both, the Coagulation Time (CT) and the Clot formation Time (CFT) in PPP was observed. However, ELISA revealed a strong binding of AT to Stx2.

**Conclusion:** Stx2 is not able to activate human platelets in-vitro. However, a strong binding of AT to Stx2 was observed. A reduction of both CT and CFT in ROTEM under influence of Stx2 points towards a significant functional correlate of the Stx2-AT binding. We hypothesize, that in HUS-associated thrombotic disorders, Stx2 has a direct and possibly significant impact on the plasmatic branch of the coagulation system.

# In Aspergillus fumigatus, BolA-deficiency causes growth defects dependent on temperature, oxidative stress as well as supply of iron and oxygen.

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**Background:** Environmental adaptation is of paramount importance to all microbes, especially those inhabiting host niches during infection. Stress conditions encountered by pathogens within the host microenvironment are multifactorial and include changes in temperature, macro- and micro nutrient availability, oxidative stress, pH, and oxygen tension (hypoxia). Maintenance of optimal iron levels inside the cell is critical for all eukaryotes and most prokaryotes, as iron is both essential and, in excess, potentially toxic. Therefore, cells must be able to sense iron levels and maintain iron homeostasis with sufficient yet non-toxic levels of this key nutrient. Iron-sensing is best characterized in S. cerevisiae, where the transcription factor Aft1 senses mitochondrial Fe-S cluster biosynthesis activity involving the monothiol glutaredoxins (Grx3/4) and the BolA protein Fra2. Most other fungal species, including Aspergillus fumigatus, lack Aft1 homologs and control iron homeostasis using different transcription factors, homologs of A. fumigatus SreA and HapX. Nevertheless, most fungal species possess Fra2 homologs but information on their functions is lacking. In this study, we analysed the function of the A. fumigatus Fra2 homolog, termed BolA.
**Materials:** The function of BolA in A. fumigatus was studied by deletion and reconstitution of the encoding bolA gene with a GFP-tagged version followed by phenotyping of the strains under different growth conditions and subcellular localization.
**Results:** BolA-deficiency decreases siderophore production and growth during iron starvation. Remarkably, the growth defect is most pronounced at 25°C and partly cured in hypoxic conditions and raising the incubation temperature. GFP-tagged BolA localizes to the cytoplasm.
**Conclusions:** Growth defects caused by BolA-deficiency are affected by temperature, oxidative stress as well as supply of iron and oxygen.

## Variants of HPV 16 among Women with Abnormal PAP Finding in Tirol

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**Background**: Infection with HPV type 16 is the most common high risk type infection responsible for malignant transformation of cervical lesions. But why do some infections with type 16 develop to precancerous lesions or cancer and some do not? Why do we observe higher cervical cancer incidence in eastern districts of Tirol (Unterland) than the rest of the districts. One possible explanation might be mutational variations of HPV genotypes causing cervical cancer. In this study we aimed to detect HPV 16 variants from cervical swab samples collected from December 2012 until Feb 2013. We studied the variations in particular between the different geographical districts in Tirol where variations in cervical cancer incidences have been reported.

**Methods**: A total of 53 cervical swab DNA extracts, which are HPV-16 positive were sequenced at the E6 open reading frame a region known to be associated with malignant transformation. The resulting sequences were compared with the prototype for various mutations. We also collected clinical data for each patient using the hospital information system. Statistical analyses were performed with MEGA andSPSS.

**Results**: Out of 53 HPV 16 positive samples available, we succeeded to amplify 45 samples. Comparison with the reference strand revealed that all samples belonged to the European lineage (n=45). We have found 4 variations in the sequence analysis and they had all been published previously. The sequences were sub-grouped into two of the European branches, namely the EUR-350 T and EUR-350 G. We observed a statistically significant predominance of the EUR-350-T variant (OR=3.72; p=0.046) in Tiroler Unterland where significantly more cervical cancer is diagnosed as compared to the rest of the regions.

**Conclusion**: In this small study we conducted among women in Tirol we were able to show significant variations in the distribution of variants of HPV 16. Further larger studies may be needed to explain the observed geographical as well as pathological variations.

# SNaPAfu: A novel Single Nucleotide Polymorphism Multiplex assay for Aspergillus fumigatus direct detection, identification, and genotyping in clinical specimens

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**Background:** Early diagnosis of invasive aspergillosis is essential for patient management. Likewise genotyping of fungal isolates responsible for the disease is of great advantage for outbreak control in clinical setting. The “gold standard method” for A. fumigatus genotyping is MultiLocus Sequence Typing (MLST). This assay detects genetic diversity and elucidates population structures based on seven housekeeping genes. Nevertheless the method is known to have a high discriminatory power; the approach is expensive and laborious. Hence, the purpose of this study was to improve existing MLST panel, combine it in a multiplex assay and merge it together with a identification marker into once assay that is able to directly detect and genotype

**Methods:** We designed a molecular assay that combines detection, identification, and genotyping (DIG) of Aspergillus fumigatus in a single reaction. To this aim, 20 highly polymorphic markers present in the MLST genes were chosen and combined in a multiplex PCR-based assay. Amplicons we read by mini-sequencing. A genotyping reference set of 113 clinical and environmental A. fumigatus strains previously genotyped by microsatellite-based strategy isolated were tested and used for the set-up of the SNaPAfu assay. Reference strains for A. fumigatus, section Fumigati and from other sections were also included to verify the specificity of the assay. Pure culture extracts were tested, followed by Aspergillus-DNA spiked clinical specimens obtained from patients with possible, probable, or proven aspergillosis according to EORTC/MSG criteria.

**Results:** A new set of designed primers allowed MLST gene amplification in a single-tube reaction format. The newly proposed SNaPAfu assay had a specificity of 100%, a sensitivity of 92% and detection limit of 1 ITS copy/mL (~0.5 fg genomic Aspergillus-DNA/mL). The marker A49_F was detected in 89% of clinical samples. The SNaPAfu assay is accurately performed on clinical specimens using only 0.5 µL of DNA extract, whit DNA concentrations ranging from 0.5 ng to 2.5 ng.

**Conclusions:** The first highly sensitive and specific, time- and cost-economic multiplex assay was implemented that allows DIG of A. fumigatus strains in a single amplification followed by mini-sequencing reaction directly in clinical specimens. The new test is suitable to clinical routine and will help improving patient management.

# HapXcess and C-terminal Truncation Impairs Aspergillus fumigatus´ Iron Homeostasis

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**Background:** The maintenance of iron homeostasis is indispensable as iron is essential for various cellular processes but can be toxic at iron excess. In A. fumigatus the bZIP-like transcription factor HapX is important for adaption to iron starvation and consequently virulence due to its role in repression of iron consuming pathways (i.e. heme biosynthesis, TCA cycle, respiration) and activation of iron uptake (i.e. siderophore biosynthesis and uptake, reductive iron assimilation).

**Methods:** The gene encoding HapX was conditionally overexpressed using the xylose-inducible xylP promoter and transcriptional regulation of HapX target genes was monitored after one hour of induction. To analyze the importance of specific domains of the HapX protein the encoding gene was C-terminally truncated. The localization of the HapX protein under different iron supply was monitored through N-terminal tagging of the gene with Venus, a GFP derivative.

**Results:** hapX overexpression leads to repression of genes involved in iron consumption (i.e. heme biosynthetic hemA and leucine-biosynthetic leuA) and activation of iron acquisition-related genes (i.e. siderophore-biosynthetic sidG and siderophore transporter-encoding mirB) within one hour of induction. In agreement, elevated hapX expression decreased the cellular accumulation of protoporphyrin IX, the iron-free precursor of heme, and increased production of the extracellular siderophore TAFC. HapX-truncation studies revealed that the C-terminal 93 amino acid residues are essential for its activating as well as repressing functions. HapX N-terminally tagged with Venus localized to the nucleus during iron starvation but was undetectable after a one hour-shift to iron sufficiency.

**Conclusions:** Our data demonstrate tight iron-regulation of hapX expression at the protein level as previously shown at the transcript level. Consistently, HapX-deficiency is detrimental only during iron limitation. Two hapX copies and in particular xylP promoter-mediated overexpression of hapX caused growth defects independent of the iron availability, which underscores the importance of a precisely regulated HapX level.

This work was supported by the Austrian Science Foundation grant FWF P21643-B11 to HH.

# Amphotericin-resistant Aspergillus terreus isolates exhibit a higher adaption to cellular stress

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**Background:** The majority of Aspergillus (A.) terreus isolates is intrinsically resistant to amphotericin B (AmB), the most common used antifungal drug. In this study we are aiming to understand the resistance mechanisms of A. terreus.

**Methods:** For this, microarray analyses were performed to evaluate gene expression levels in an A. terreus isolates resistant to AmphotericinB (AmB) upon sublethal treatment with the compound. The differences in gene expression levels from the resistant A. terreus (ATR) isolate were compared to data obtained from an AmB-susceptible A. terreus (ATS). Up-regulation of genes was verified by relative quantification using real-time RT-PCR and tubulin as reference gene as well as by Western Blot analyses to determine the levels of differentially regulated proteins. The functionality of up-regulated genes was measured by ROS production, blocking experiments using antioxidants and inhibitory reagents.

**Results:** We found that upon sublethal AmB treatment 284 out of 10.000 genes were differentially expressed in ATR compared to an untreated control, while in ATS only 79 genes showed a differential expression pattern. The majority of up-regulated genes in ATR were involved in cell stress adaptation, sugar metabolism and signal transduction. Among the highest up-regulated genes associated with cell stress adaption or cell wall integrity in AmB-treated ATR but not ATS were found to be aox, hsp70, hsp90, erg1 and erg3. The increase in those genes was confirmed by relative quantification. To set the results into a functional context respecting the resistance mechanisms involved, we performed inhibition assays, ROS measurements and additionally treated the fungal isolates with antioxidative agents. Lastly, the signalling mechanisms in ATS and ATR were investigated.

**Conclusion:** In summary we found that AmB resistance is based on the oxidative character of this compound and the resistant A. terreus isolate is able to cope with this challenge due to significantly increased adaption of the cellular stress machinery.

# NMDA-receptor mediated excitotoxicity is involved in the pathogenesis of experimental cerebral malaria

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A major cause of morbidity and mortality of Plasmodium falciparum malaria is cerebral malaria (CM). The current study investigates the role of NMDA-receptor mediated excitotoxic cell death in the brain of mice with CM.

C57BL/6J mice were infected with Plasmodium berghei ANKA parasitized red blood cells. Cerebral Microdialysis was performed and glutamate levels were measured. Animals with CM were randomized for treatment with artesunate, MK801 (a non-competitive NMDA-receptor antagonist), artesunate/MK801 or vehicle. Survival and clinical outcome was scored. Brains were further processed for histochemistry.

Glutamate levels were significantly elevated in mice with CM compared to control animals. Glutamate peaks were noted before and after clinical signs of CM developed. In the treatment experiment no animal survived in the vehicle group. In contrast, 33.3% of the animals in the artesunate group and 74,1% in the artesunate/MK801 treatment group survived. Kaplan-Meier survival curves yielded a significantly longer survival of the animals in the artesunate/MK801 group compared to the vehicle or MK801 group. In addition MK801 treated animals showed significantly prolonged survival compared to vehicle treated animals. Histological analyses yielded a lower number of microhemorrhages and Fluoro-Jade B positive cells in the artesunate/MK801 treated animals compared to artesunate treated mice.

In conclusion, glutamate levels in the brain are increased early in the course of CM. Treatment with MK801, rescues mice from CM. Therefore, NMDA-receptor mediated excitotoxicity may play a role in the pathogenesis of CM and could represent a target for adjunctive treatment strategies.

# N-chlorotaurine, a long-lived endogenous oxidant, inactivates Panton Valentine Leukocidin (PVL) of Staphylococcus aureus

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**Background** N-chlorotaurine (NCT) is an endogenous long lived antiseptic. Since it has already been demonstrated that NCT inactivates Shiga toxin from Enterohemorrhagic Escherichia coli (EHEC), it is most likely that also other bacterial toxins such as PVL from S. aureus can be affected by N-chloro amino acids. Recently, the NCT analogs N-monochloro-2,2-dimethyltaurine (NVC-612) and N-dichloro-2,2-dimethyltaurine (NVC-422) have been developed, which offer higher stability. N,N-dichlorotaurine (NDCT) which is generated by disproportionation of NCT was tested too. The aim of our study was to investigate if PVL can be affected directly by our test compounds.

**Methods** SDS-polyacrylamidgelelectrophoresis (SDS-PAGE) was performed with purified PVL (1,5mg/ml). The gels (16%) were stained with Coomassie blue. A549 cells (human alveolar basal epithelial cells) were treated with mock treated and NCT treated supernatant of three PVL positive MRSA strains. Analysis was done via direct counting of cytopathic cells, LDH assay and FACS. Human granulocytes (PMNs) were incubated with mock treated and NCT treated purified PVL. Analysis was done via FACS and LDH assay.

**Results** Tests with A549 cells revealed that N-chloro amino acids (5.5 mM) are able to inactivate virulence factors in the supernatant of PVL positive MRSA strains. FACS analysis of PMNs clearly demonstrated the inactivation of purified PVL by NCT and its analogs. NVC-422 offered the strongest activity (inactivation of PVL by 0,55mM NVC-422). SDS-PAGE disclosed the structural alterations, which led to the loss of function of PVL.

**Conclusions** All tested chloramines (NCT, NDCT, NVC-612 and NVC-422) showed sufficient activity to inactivate PVL. It has already been shown that PVL positive MRSA strains are strongly associated with severe cases of skin and soft tissue infections that need to be treated surgically and with antimicrobial agents. Therefore, inactivation of PVL would be a great benefit in the treatment of such infections.

# Galleria mellonella as alternative model to study the in vivo efficacy of amphotericin B treatment of Aspergillus terreus infections and its influence of on larval immune response

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**Background:** Infections with *Aspergillus (A.) terreus* are of major concern, due to its high likelihood of dissemination and its intrinsic resistance to amphotericin B (amB). The reason for this resistance is not known yet. Recently, three clinical isolates, with distinct morphological variations, have been found to be amB susceptible *in vitro*.

**Methods:** Efficacy of amB treatment and its influence on the larval immune system were investigated in the invertebrate model *G. mellonella*. Proteomic analysis of larval haemolymph, haemocyte counts and post-treatment infection studies were performed according to Kelly & Kavanagh 2011. Additionally, putative difference in virulence potential of the respective isolates was analyzed by correlating survival rates with physiological attributes and *in vitro* killing ability of larval haemocytes.

**Results:** Treatment with amB only showed success in the groups infected with amB-susceptible strains, which reflected our *in vitro* data. Furthermore, amB administration resulted in an increased number of circulating haemocytes. Proteomic studies showed different protein expression of proteins which have immune function. Pre-treatment of larvae with different antifungals also increased their resistance to *Staphylococcus (S.) aureus* infection, indicating a general ability of antifungals to prime the insect´s immune system. Larval survival rates differed in the early time points of infection for the various isolates tested. The amB-resistant isolate T90, showed lowest mortality rates in the early time points of infection. This is in correlation with slower germination rate of T90, and the highest fungal damage caused by haemocytes *in vitro*.

**Conclusion:** This work demonstrated that *G. mellonella* is a useful model to study (1) the *in vivo* efficacy of amB treatment and (2) virulence potential of different *A. terreus* isolates. Our results showed that *in vivo* antifungal treatment correlate with *in vitro* susceptibility data. Furthermore, one has to be aware of non specific influence of antifungal drugs on the larval immune response.

# The Role of Heme Oxygenase 1 in regulating Iron Homeostasis and innate immune response to Salmonella Infection

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**Background:** Macrophages play an essential role in sensing and elimination of microbes. Heme oxygenase-1 (HO-1, hmox1) the enzyme cleaving heme to ferric iron, billiverdin and carbon monoxide, is involved in the regulation of stress response, iron homeostasis and host pathogen interactions. However, underlying regulatory effects of hmox1 in the course of infection in macrophages and associated changes of iron homeostasis and innate immune function are incompletely understood.

**Methods/Results:** Using the murine macrophage cell line RAW264.7 as model and lenti- virus based tetracyclin inducible shRNA knock downs of the hmox, we observed alterations in iron regulatory gene expression, with an up-regulation of the iron exporter ferroportin-1 (FPN1) mRNA and protein expression. To study the relevance of these observations for host response to infection we infected macrophages with Salmonella enterica serovar Thyphimurium. Interstingly, the knock down of hmox reduced the survival of Salmonella in macrophages whereas it had no effect on pathogen uptake. Preliminary results suggest that the improved pathogen control could be traced back to reduced iron availability for intra-macrophage bacteria and partly improved innate immune function as a consequence of increased ferroportin expression and intracellular iron restriction.

**Conclusion:** Taken together, our data highlight the central role of HO-1 in host response towards intracellular pathogens in it's capacity to modulate anti-bacterial immune effector pathways and cellular iron homeostasis.

## Innate Dendritic Cell Sensing of Opsonized HIV-1 to Activate Th17 Cells

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**Background:** Early on in HIV-1 infection, gut Th17 cells are massively depleted leading eventually to compromised intestinal barrier function and excessive immune activation. In contrast, the functional Th17 cell compartment of the gut is well-maintained in non-pathogenic SIV infection as well as HIV-1 long-term non-progressors.

**Methods:** Microarray analyses revealed that after HIV-C treatment a Th17-inducing pathway was induced in dendritic cells (DCs). Expression of Th17-activating cytokines IL-1β, IL-6 and IL-23 was confirmed by relative quantification of mRNA using real-time RT-PCR and intracellular FACS staining (ICS) of proteins. Supernatants of HIV-C treated DCs were tested by Cytometric Bead Array and ELISA and transferred to naive CD4 T cells.Differentiation to T helper cells was characterized by ICS.

**Results**: Here, we show that dendritic cells exposed to complement-opsonized HIV-1 primed Th17 cells more effectively than DCs exposed to non-opsonized HIV-1. DCs loaded with HIV-1 bearing high surface complement levels, following incubation in plasma from HIV-infected individuals, secreted significantly higher concentrations of Th17-polarizing cytokines compared to DCs exposed to nonopsonized HIV-1 or HIV-1 exhibiting low levels of surface complement following opsonization in patient plasma.

**Conclusion:** These in vitro and ex vivo data, therefore, indicate that complement-opsonized HIV-1 exerts beneficial effects during HIV-1 infection by simultaneously triggering Th17 expansion as well as stronger CTLs via DCs as illustrated earlier by our group. Thus, specifically modifying the complement signaling pathway could strenghten the cellular arm of the immune system and serve as a therapeutic target for HIV-1 infection.

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## Interaction of Different Mucormycetes Species With the Complement System

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**Background:** Mucormycoses are emerging angioinvasive infections caused by ubiquitous filamentous fungi belonging to the class of Mucormycetes. In the bloodstream, they come in contact with the complement system, which is a central component of the soluble innate immunity. The multiple antimicrobial effector functions that can be exerted by the complement system include fungal damage, opsonization and support of phagocytosis, as well as attraction and activation of immune cells. However, complement-induced exaggerated inflammation may also cause tissue damage and contribute to the pathology of fungal infection. We therefore aim to study in detail this interaction between mucormycetes and the complement system.
**Methods:** Different Mucormycetes species (Lichtheimia corymbifera, L. ramosa, Rhizopus microsporus, R. oryzae, Rhizomucor pusillus and Mucor spp) were compared for their capacity to activate the complement system and to be opsonized by the various complement factors. In addition, a putative intra-species variation was assessed by testing up to three different clinical isolates from each species. For comparison, Aspergillus fumigatus and A. terreus were included in all experiments. Spores were opsonized in human serum; opsonization on the surface was detected by flow cytometry. The interaction of neutrophil granulocytes with native and opsonized spores was examined microscopically.
**Results:** We found that sporangiospores of all tested species were able to induce complement activation with subsequent opsonization of the spores; deposition of the complement factors C1q, C3 and Terminal Complement Complex (TCC) on the spore surface could be detected. The degree of opsonization considerably varied between the different species, and variations also occurred within the same species. On the average, the Mucormycetes species Rhizopus microsporus, Rhizopus oryzae, Rhizomucor pusillus and Mucor spp all showed substantial opsonization intensity on their spore surface. The complement deposition on the two Lichtheimia species L. corymbifera and L. ramosa was weaker than on the other Mucormycetes species. Formation of the Terminal Complement Complex could be detected on the spores of all examined species.
Covering of the Mucormycetes spores with complement proteins strongly influences the interaction with neutrophil granulocytes. While virtually no interaction between neutrophils and non-opsonized Rhizopus microsporus and Rhizomucor pusillus spores was detected, opsonization of the spores resulted in strong adherence to or even phagocytosis by granulocytes.
**Conclusion:** These preliminary findings indicate that complement may play an important role in the pathogenesis of Mucormycoses and encourage further study efforts in this topic.

# Complement opsonization of fungi modifies dendritic cell up-take and cytokine secretion

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**Background:** In this study, interactions of dendritic cells (DCs) with differentially opsonized and non-opsonized Aspergillus fumigatus strains were investigated. Two different mutants were used in this context, one lacking the pigment melanin (pksP mutant) and one lacking the hydrophobin layer (rodA mutant). The opsonization pattern of the different strains as well as the binding and internalization by dendritic cells were investigated.

**Methods:** Fungi were opsonized using normal human serum as complement source. The opsonization pattern, binding of conidia to DCs and intenalization were characterized by FACS analyses. Inhibition of fungal growth in presence of DCs and interactions with complement receptors were detected using live science and confocal microscopy.

**Results:** As demonstrated in this study, melanin has the highest impact on the fungal virulence compared to the wildtype Aspergillus strain. Surprisingly, the rodlet layer showed a minor impact on the complement activation process. With respect to dendritic cell binding and internalization complement-opsonization of conidia enhanced these processes compared to their non-opsonized counterparts independent on the fungal strain used. As analysed by confocal microscopic analyses, this more efficient binding and up-take was probably mediated by complement receptors 3 and 4 (CR3 and CR4) abundantly expressed on DCs. Additionally, the incubation of these wildtype and mutant strains with DCs led to different immune responses, which were analyzed by cytokine ELISAs.

**Conclusions:** Data obtained in this study, revealed that melanin is one of the key effectors of masking complement deposition and binding of conidia by DCs. However opsonization of swollen conidia with normal human serum, containing C3 opsonins favors the uptake and internalization as well as the production of pro-inflammatory cytokines, resulting in a favorable $T_H1$ immune response. These in vitro studies have proposed that it could be possible to use immune cells, like dendritic cells or neutrophils, as vaccines against invasive aspergillosis with the help of complement opsonins and maybe also with help of antifungal drugs, like voriconazol or amphotericin B, to prevent the host of fungal infections.

# A lower CD4 Cell Count at HIV Diagnosis is Associated with Increased Mortality for Most Causes of Death

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**Objective:** We investigated changes in mortality rates and factors associated with all-cause mortality and specific causes of death among HIV-positive individuals within the Austrian HIV Cohort Study (AHIVCOS) in the combination antiretroviral therapy (cART) era.

**Methods:** We studied mortality among the Austrian HIV Cohort Study (AHIVCOS) (1997-2010). CD4 cell count was divided into four categories: CD4 cells ≥350, 200-349, 50-199 and <50 cells/mm$^3$. Observation time was divided into three periods: period 1: 1997-2000; period 2: 2001-2004; and period 3: 2005-2011. Mortality rates are presented as deaths per 100 person-years (PY). Potential risk factors associated with all-cause mortality and specific causes of death were identified by using multivariable Poisson regression models. Patients lost to follow-up were checked with death registry data.

**Results:** Of 5743 patients (36,919 person-years of observation), 876 died: 311 (35.5%) from AIDS-defining causes. Overall mortality rates decreased from 3.61/100 PY in period 1 to 2.42/100 PY in period 2 and 1.93/100 PY in period 3, respectively. However, deaths due to non-AIDS-defining tumours increased over time. A steady rise in the proportion of deaths from non-AIDS-defining conditions was found, but AIDS-defining diseases were still the most common cause of death. Compared with a CD4 count ≥350 cells/mm$^3$ an increased incidence rate ratio (IRR) for mortality was observed with a lower CD4 cell count at HIV diagnosis and/or initiation of cART for all causes of death except for hepatitis B/C. However, the risk of HBV/HCV-associated mortality was higher in patients starting cART at a lower CD4 cell count. These analyses were adjusted for sex, age, calendar year, duration of cART, transmission category and population size of residence area and nationality.

**Limitations:** No adjustment for socio-economic and lifestyle factors was made because of missing or incomplete data. The relationship between health seeking behaviour and lifestyle factors and its impact on late presentation is not known. As a result of loss to follow-up of patients who left the country mortality may be underestimated.

**Conclusions:** The spectrum of mortality has changed with cART, risk of death decreased and causes of death changed over time. The association of a lower CD4 cell count at HIV diagnosis with increased mortality for most causes of death suggests an urgency to prevent individuals presenting late for care.

## The Role of Platelet Opsonisation in The Course of Invasive Aspergillosis

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**Background:** The fatality of invasive aspergillosis (IA) is high in immunosuppressed patients despite of antifungal therapies. Complement and platelets are important elements of the innate immunity that are likely to participate in the defence against invading fungal pathogens and should therefore be investigated in more detail. It has already been shown that conidia, hyphae and culture supernatants of Aspergillus induce activation of platelets. We studied whether this process results in recognition of platelet surface by the complement system and what putative consequences might be.

**Methods:** Aspergillus (A.) fumigatus was grown in RPMI medium; secreted fungal factors in the culture supernatant were prepared by filtration. Human platelets were available as concentrates; serum from a pool of healthy donors was used as complement source. Thrombocyte and granulocyte activation, presence of complement regulators and complement deposition were investigated by FACS analysis using specific antibodies. Cell viability was assessed by measuring mitochondrial activity (MTS assay) and by live-dead staining with a commercial kit. Platelet surface and interaction between granulocytes and thrombocytes were studied by scanning electron and life-time microscopy.

**Results:** Platelet activation, induced by A. fumigatus supernatant (SN), stimulates the complement system with subsequent deposition of complement factors on the thrombocyte surface. This effect was measured by quantifying opsonisation with complement factor C3. Triggering of the complement cascade is at least partially due to the fact that fungal SN masks or degrades the membrane-bound regulators on the thrombocyte surface that normally protect host cells from complement attack.

Putative consequences of fungus-induced thrombocyte opsonization might be reduced viability due to formation of the membrane attack complex (MAC), activation of granulocytes or enhanced clearance of opsonised platelets by phagocytes.

Quantification of mitochondrial activity in MTS assay showed that opsonization results in a significant decrease of cell viability. Similarly, live-dead staining with a dye binding to intracellular amines in dead cells clearly revealed that complement binding induces damage of the platelets. Electron microscopy confirmed the presence of dead cells in the presence of fungal SN and complement.

Contact of opsonised platelets with neutrophils did not modulate the activity of these immune cells. In contrast, the uptake of thrombocytes by the neutrophils was significantly enhanced when the platelets were pretreated with fungal SN and serum.

**Conclusion:** Secreted fungal factors induce activation and opsonisation of thrombocytes with complement, thus reducing their viability and enhancing their clearance by phagocytes. This mechanism might limit the immune function of the platelets in the course of invasive aspergillosis and contribute to the high lethality of this infectious disease.

# Immunology/Inflammation

## Effects of $VEGF_{121}$-Fibrin on ischemic lesions in UCD-206 chickens, an animal model for systemic sclerosis

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**Background:** Systemic sclerosis (SSc) is an autoimmune connective tissue disease characterized by microvascular damage, perivascular mononuclear cell infiltration, and fibrosis of skin and viscera. Microvascular alterations with intimal proliferation, occlusion of blood vessels, and capillary loss lead to decreased blood flow, a state of chronic ischemia, and to clinical manifestations such as fingertip ulcers. Tissue hypoxia normally induces neoangiogenesis, but in SSc vascular repair and angiogenesis are strongly disturbed. Thus, the expression of vascular endothelial cell growth factor (VEGF) seems to be uncontrolled and chronic. However, a very high VEGF expression is associated with the lack of fingertip ulcers. Therefore, local administration of VEGF in a form that allows controlled release might be a promising therapeutic approach.
**Methods:** Early and late ischemic comb and neck skin lesions of UCD-206 chickens, an animal model showing all hallmarks of the human disease, were treated with VEGF121-fibrin, fibrin alone or left untreated. After 7 days the clinical outcome was assessed, skin thickness measured, and skin biopsies were taken for further analyses. Angiogenesis was studied on frozen tissue sections by indirect immunofluorescence tests (IIF) using antibodies specific for the endothelial cell marker van Willebrand factor (vWF), and alpha smooth muscle actin (αSMA), a marker for mural cells. Expression of VEGFR-1, VEGFR-2, and Tal-1 by endothelial cells was analyzed by double staining with the respective antibody and anti-vWF antibody. Tissuequest® was used for quantitative analyses.
**Results:** One week after treatment approximately 80% of the VEGF121-fibrin treated lesions showed clear clinical improvement, whereas 82% of placebo controls and 97% of untreated lesions had deteriorated. Angiogenesis was significantly increased after treatment with VEGF121-fibrin compared to controls. Expression of VEGFR-2 and its regulator transcription factor Tal-1 where both significantly upregulated. The change in the ratio of the anti-angiogenic VEGFR-1 to the pro-angiogenic VEGFR-2 showed approximately 90% reduction in VEGF121-fibrin treated skin compared to untreated skin, whereas after fibrin treatment this ratio was only reduced by 15%.
**Conclusion:** In the avian SSc model, treatment with VEGF121-fibrin proofed to be clinically effective in early and late ischemic lesions by inducing the formation of morphological normal blood vessels. VEGF121 seems to differentially regulate the expression of VEGFR-1 and VEGFR-2 shifting the balance towards the pro-angiogenic VEGFR-2, thus increasing the angiogenic response. Long term effects of VEGF121-fibrin on vessel stability and possible adverse effects have to be investigated in a follow up study.
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# $CD8^+CD28^-$ T cells cannot be rescued by autophagy following antigenic stimulation

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**Background:** Macro-autophagy has been shown to be induced by antigen receptor-mediated activation of murine T cells, thus accommodating the increased bioenergetic requirements of the cells. However, little is known about the induction of autophagy upon antigen receptor mediated-activation of human T cells. It was therefore the aim of this study to analyze autophagy in human $CD8^+$ T cells. Additionally, since highly differentiated T cells, characterized by the lack of the co-stimulatory molecule CD28, have been shown to accumulate with human aging and display several defects such as a defective DNA damage repair, we separated $CD8^+$ T cells into $CD28^+$ and $CD28^-$ T cells in order to analyze the capability of highly differentiated $CD8^+CD28^-$ T cells to induce autophagy.

**Methods:** PBMCs were isolated from healthy donors and $CD8^+CD28^+$ and $CD8^+CD28^-$ T cells were isolated using the MACS technology. Autophagy was induced either via antigenic stimulation with anti-CD3 antibodies, in the presence or absence of costimulatory molecules, via inhibition of mTOR with rapamycin, or using spermidine and resveratrol. Autophagy and mTOR were assessed by immunoblotting, autophagy additionally by electron microscopy. Autophagy related genes were analyzed by quantitative real-time PCR. Proliferation was investigated using the CFSE dilution assay and FACS technology.

**Results:** We demonstrate that anti-CD3 treated human $CD8^+CD28^+$ T cells induce autophagy, as assessed by electron microscopy and LC3B-II immunoblotting. Costimulatory signals further increased autophagy upon anti-CD3 treatment. Increased autophagy was accompanied by activation of the mTOR pathway in spite of the extensive characterization of mTOR as inhibitor of autophagy. Inhibition of mTOR by rapamycin could still enhance activation-driven LC3B-II expression, demonstrating that autophagy can be regulated via different signaling pathways in $CD8^+CD28^+$ T cells. In contrast to $CD8^+CD28^+$ T cells, $CD8^+CD28^-$ T cells failed to induce autophagy upon anti-CD3 stimulation independently of the absence or presence of co-stimulatory signals. $CD8^+CD28^-$ T cells did also not up-regulate mTOR upon antigen receptor stimulation, but readily following stimulation with IL-15. Autophagy could also not be induced in $CD8^+CD28^-$ T cells by rapamycin or other classical inducers such as spermidine or resveratrol. The failure of $CD8^+CD28^-$ T cells to induce autophagy was not due to the decreased expression of genes regulating macro-autophagy such as Atg5, Atg7, Atg8, Atg16L1.

**Conclusion:** In conclusion, our results demonstrate that due to their inability to induce autophagy $CD8^+CD28^-$ T cells cannot meet the metabolic needs of antigen receptor-mediated activation. They are therefore unlikely to survive when confronted by their specific antigen in the absence of survival factors such as IL-15.

# A p38/MK2 signaling pathway controlling redox stress and cell death in ischemia/reperfusion injury

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**Background:** Formation of ROS is causal for initiation and progression of ischemia/reperfusion (IR)-associated tissue injury (IRI). Avoiding ROS damage by using antioxidants proved unsatisfactory in the clinical setting. IR is also characterized by changes in the activity of intracellular signaling pathways, whose role in the development and progression of IRI is often poorly defined. We recently obtained first evidence that increased p38 activity during reperfusion contributes to elevated mitochondrial ROS levels and cardiomyocyte death.

**Methods:** Hypoxia/reoxygenation (HR) (HL-1 cardiomycoytes, MEFs with different genotypes), ischemia/reperfusion (kidney clamping in the Lewis rat). p38 function was blocked by the low molecular weight inhibitor BIRB796 or conditional siRNA knockdown. Signaling analysis used phosphorylation specific antibodies. Serum creatinine, urea, NGAL and HSP70 were used as kidney function and damage markers, respectively. ROS was visualized by confocal imaging of MitoTracker Red CM-H2XRos labeled cells or antibodies specific for redox-modified proteins.

**Results:** siRNA-mediated knockdown demonstrated the pro-oxidant role of p38α signaling. MAPKAP kinase 2 (MK2) functions downstream of p38. In the kidney clamping experiments p38 activity increased upon reperfusion and p38 inhibition by BIRB796 almost completely prevented severe functional impairment caused by IR. Histological and molecular analyses suggested that protection resulted from decreased redox stress and apoptotic cell death.

**Conclusion:** This study establishes p38 as a critical regulator of early damage associated with IR. Analyses of the mechanisms underlying the protective effect of p38 inhibition point to a reduction in the oxidative damage and - possibly linked to this – decreased apoptotic cell death. Beyond that, inhibition of p38 signaling may also prevent inflammatory responses frequently occurring in the clinical setting at later time points of IR.

## Regulation of PPAR alpha in models of skin barrier disruption

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PPARs not only are key regulators of systemic lipid and glucose metabolism, but they are also important in cutaneous homeostasis. PPARs have been shown to play prominent roles in fetal epidermal development, they control epidermal lipid synthesis, keratinocyte differentiation and they inhibit skin inflammation. In line with these observations, agonists to various PPAR isotypes were found to elicit beneficial effects in mouse models of skin disease and in patients with atopic dermatitis. Filaggrin is one of the most essential structural proteins involved in skin barrier formation. Loss-of-function mutations in its gene cause ichthyosis vulgaris characterized by dry, scaly and flaky skin, and they are associated with atopic dermatitis, characterized by severe skin inflammation. In this work, we aimed to assess the expression of PPAR alpha, beta/delta and gamma in skin after chronic and acute skin barrier disruption. We studied filaggrin-deficient flaky tail mouse skin and wild type mice exposed to repeated tape stripping. Our murine models revealed a reduction of PPAR alpha whereas PPAR beta/delta and PPAR gamma expression levels were not altered as compared to controls. Similar results were obtained in chronic versus acute skin barrier disruption. These data indicate that PPAR alpha is regulated in skin by epidermal barrier requirements with potential implications for the common inflammatory skin disease atopic dermatitis.

## Immunoregulatory Impact of Food Antioxidants

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**Background**: Immune system activation and inflammation are deeply involved in the pathogenesis of a variety of diseases including infections, autoimmunity and malignancy as well as allergy and asthma.

The incidence of allergy and asthma has significantly increased during the past decades. Still the background of this phenomenon is not well understood. The contribution of life style and habits are heavily discussed. Among them is a too clean environment, which may predispose individuals to an increased sensitivity to allergic responses. Also dietary habits have changed drastically in the Western world, and it appears that especially the increased use of antioxidant food supplements, preservatives and colorants could be of relevance [1,2].

**Methods:** The effect of compounds on phytohemagglutinin-induced tryptophan breakdown via indoleamine 2,3-dioxygenase (IDO) and neopterin production by GTP-cyclohydrolase I (GCH) was analyzed by using peripheral blood mononuclear cells (PBMC) isolated from healthy donors.

**Results:** In vitro experiments show that typical antioxidant compounds like vitamin C and E and the stilbene resveratrol as well as food preservatives such as sulfite, benzoate and sorbic acid and also colorants like curcumin exert significant suppressive effects on the T helper (Th)-type 1 immune activation cascades in freshly isolated human peripheral blood mononuclear cells.

**Conclusion:** Obviously, antioxidant compounds interfere with central immunoregulatory pathways such as tryptophan breakdown and neopterin production. Results show an anti-inflammatory property of antioxidants, which could shift the Th1-Th2-type immune balance towards Th2-type immunity that is of utmost importance in allergic responses. Additionally, food preservatives reduce the number of pathogens to which humans are exposed by their diet, so that in agreement with the hygiene hypothesis the likelihood of allergy might increase. Although effects of antioxidants may be beneficial for counteracting inflammatory diseases, their excessive intake might be related to the increase of allergy and asthma and of the obesity epidemic in the Western world.

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## COUP-TF member NR2F6 as a barrier against autoimmunity

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**Background:** Deregulated $CD4^+$ Th17 cells, a T cell subset producing the pro-inflammatory cytokine interleukin (IL)-17A, have been firmly implicated in T cell mediated autoimmune pathogenesis such as arthritis, multiple sclerosis, Crohn's disease, uveitis and psoriasis. Therefore, research has focused on the transcriptional regulation of the Il17a gene promoter. Nevertheless, the full molecular mechanisms underlying IL-17A expression control remained unknown.
**Methods:** We used both Nr2f6-deficient as well as our newly established Nr2f6 over-expressing transgenic mouse lines in order to investigate the binding behaviour of NR2F6, NFAT as well as RORgamma-t at the Il17a promoter during Th17 $CD4^+$ T differentiation via electromobility (EMSA) as well as chromatin immunoprecipitation assays (ChIP). In addition T cell intrinsic role of NR2F6 was proven via using the in vivo mouse multiple sclerosis model EAE.
**Results:** NR2F6 directly binds to multiple sites within the Il17a promoter locus, suppressing the DNA accessibility of the transcription factor NFAT. In addition NR2F6 directly competes with the Th17 lineage specific transcription factor RORgamma-t for the DNA accessibility to the hormone response elements within the Il17a promoter region. RORgamma-t binding within the Il17a locus was enhanced in Nr2f6-deficient $CD4^+$ Th17 cells but decreased in Nr2f6-overexpressing transgenic $CD4^+$ Th17 cells Expression profile analysis of knock out confirmed high Il17a, Il17f and Il21 cytokine expression in the Nr2f6-deficient $CD4^+$ cells. Adoptive EAE was performed with $MOG_{35–55}$-immunized $Nr2f6^{+/+}$ or $Nr2f6^{-/-}$ mice that were stimulated ex vivo with $MOG_{35–55}$ in the presence of IL-23 and then injected into naïve $Nr2f6^{+/+}$ recipient mice to confirm the biological relevance of Nr2f6 in lymphocytes as a gatekeeper for the transcriptional suppression of Th17 cells and autoimmunity.
**Conclusion:** Given the reported key role of the cytokine IL-17 in autoimmune diseases, our report closes an important gap in the understanding of how the expression of this cytokine is controlled via NR2F6. Furthermore, these findings support the idea that selective activation of NR2F6 could represent an innovative therapeutic regimen for attenuating IL-17A production as a treatment for certain Th17-mediated autoimmune disorders.

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# A Loss-of-Function Approach Identifies the miR-15 Family As a Central Regulator of Early B Cell Development

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**Background**: MicroRNAs (miRNAs) are ~22-nucleotide noncoding RNAs that mediate post-transcriptional silencing of a predicted 60 % of protein-coding genes in mammals. Although miRNAs are prominently expressed in cells of the immune system, only a few have been conclusively studied with respect to their function. This project aims to provide a conclusive roadmap for the role of all miRNAs throughout early B cell development.

**Methods**: A common experimental setup to investigate the function of a miRNA is a “gain-of-function” approach in which the miRNA of interest is overexpressed to analyze its impact on a given process. However, a major drawback of this technique is that miRNAs expressed significantly above endogenous levels may target genes that are not affected under physiological conditions. To circumvent this problem, we have generated a retroviral miRNA-knockdown library encompassing the majority of the miRNA families expressed throughout lymphocyte development. Using pre-B cells as a model system, we have applied this “loss-of-function” approach to characterize all early B cell miRNAs according to their functional role in processes such as proliferation, apoptosis and differentiation.

**Results**: We show that functional knockdown of the tumour-suppressive miR-15 family has only slight effects under cellular steady-state conditions. Upon withdrawal of the growth factor IL-7, however, loss of miR-15 function partially protects from apoptosis, enables prolonged proliferation and almost completely inhibits pre-B to immature B cell differentiation. In line with these observations, miR-15 knockdown confers a competitive advantage in suboptimal IL-7 concentrations, indicating that levels of miR-15 determine the sensitivity of cells towards growth factor receptor signaling.

Interestingly, IL-7 withdrawal seems to increase the activity of endogenous miR-15 family members in pre-B cells. Together with the knockdown data, this suggests that miR-15 functions as a central element in a positive feedback loop that reinforces cell-cycle arrest at low growth factor concentrations, which is considered a prerequisite for Rag1/2-mediated light chain gene recombination and differentiation.

**Conclusions**: Our data identify the miR-15 family as an essential factor of early B cell development by regulating proliferation, differentiation and cellular fitness. This suggests that aberrant expression of the miR-15 family might not only contribute to cancer, but may also play a role in immune diseases. Ongoing work is needed to elucidate how the miR-15 family is integrated into the regulatory transcriptional network in lymphocytes, especially in terms of its transcriptional regulation and relevant target genes.

# Role of Mitogen-activated Protein Kinases (MAPKs) in the Activation of p66SHC: A Novel Approach for the Prevention of ischemia/reperfusion Injury (IRI)

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**Background:** Excessive production of reactive oxygen species (ROS) during early reperfusion is an important trigger for the development of ischemia/reperfusion injury (IRI) in the course of solid organ transplantation. Apart from their direct damaging effects to biomolecules ROS have been implicated in mitochondrial damage, inflammasome activation and regulation of autophagy. Since currently available antioxidants yielded little clinical benefit, we have been pursuing strategies to limit or prevent the increase in ROS by targeting intracellular signaling pathways. Our work identified the stress kinase p38 MAPK as inducer of redox stress and cell death during ischemia/reperfusion (IR), while activation of the serine-threonine kinase RAF reduced redox stress. During reperfusion the cytosolic protein p66Shc must translocate to the mitochondria to produce $H_2O_2$ and cause cell death. This step is controlled by p66Shc phosphorylation and thus provides a target for intervention. Here we analyzed the role of mitogen-activated protein kinases (MAPKs) p38, c-Jun N-terminal kinase, JNK, and ERK in controlling p66SHC function.

**Methods:** We used two cell systems, HL-1 cardiomyocytes and mouse embryonic fibroblasts (MEFs) and the following stress conditions: ischemia/reperfusion (sIR) and pro-oxidant treatment. Intracellular signaling was monitored by phosphorylation-specific antibodies. Alterations in mitochondrial ROS were followed by fluorescent microscopy after loading with Mitotracker Red. Autophagy was assessed by the conversion of LC3-I into LC-3 II, a hallmark of autophagosome formation, detected by immunoblotting.

**Results:** Our data show activation of p66Shc and stress kinases as well as increased ROS production as consequences of sIR and pro-oxidant treatment. The inhibition of signaling through the MAPKs (p38, JNK and ERK) showed a pronounced decrease in p66Shc phosphorylation (S36) only in the case of JNK. Moreover, JNK inhibition could be linked to a decrease in ROS production. p66Shc-deficient MEFs were protected from prooxidant induced apoptosis and showed increased autophagy activation in comparison to the WT counterparts.

**Conclusion:** Interference with intracellular signaling may provide a therapeutic approach for decreasing damage to cells and organs during cellular stresses. Future experiments will probe into a possible link between JNK, ROS and the regulation of cell death and autophagy, respectively.

# T cell responses rely on Langerin+ dermal dendritic cells in a spontaneous mouse melanoma model

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**Background:** The mouse melanoma model tg(Grm1)EPv is based on the over-expression of the metabotropic glutamate receptor 1 (Grm1) leading to the development of spontaneous melanoma. This model is highly significant since over-expression of Grm1 has been described in over 60% of human melanoma samples. In this study we examined the role of skin dendritic cells in T cell responses against melanoma. Moreover, this mouse model will be interesting as a pre-clinical model for the development of immunotherapeutical approaches against melanoma.

**Methods:** In this study, we used tg(Grm1)EPv mice which develop spontaneous melanoma within 4-6 months of age in ear and tail skin. These tg(Grm1)EPv mice were crossed to transgenic Langerin-DTR mice which express the diptheria toxin receptor under the Langerin-promotor. This allowed us to deplete Langerin+ dermal DCs (L+ dDC) and Langerhans cells (LC) by injection of diphtheria toxin. Moreover, we utilized the transgenic mouse model pMEL-1 in which most CD8+ T cells are specific for gp100, a melanoma antigen that is expressed only in melanocytes. We employed adoptive T cell transfer followed by flow cytometric analyses of lymph nodes and skin to determine which antigen-presenting cells are responsible for initiating tumor-specific CD8+ T cell responses.

**Results:** Upon transfer of pMEL-1 CD8+ T cells into tumor-bearing tg(Grm1)EPv mice, cells start to proliferate massively. In tumor-free mice with only hyperpigmented skin, comparable CD8+ T cell proliferation required intradermal injection of an adjuvant combination consisting of poly (I:C) (TLR3 ligand) and an agonistic anti-CD40 antibody. Interestingly, proliferation of pMEL-1 T cells was also boosted in tumor-bearing animals upon adjuvant treatment. In addition, CD8+ T cells derived from pMEL-1 mice acquired an effector phenotype (CD44+ CD62L-) and were recruited to the site of skin injection. Yet, we did not observe a delay on tumor growth. Upon depletion of Langerin+DCs, however, we could observe a substantial impact on pMEL-1 T cells. We found less proliferation, fewer effector memory T cells and a decline in the number of skin infiltration CD8+ T cells. Strikingly, this inhibition occurred only when L+ dDCs, but not LCs, were absent.

**Conclusion:** In conclusion, L+dDC seem to be crucially involved in anti-tumor T cell responses and should thus be targeted in immunotherapeutical strategies against melanoma.

# The Erythropoietin-Analogue ARA290 Ameliorates the Course of Experimental Colitis

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**Background:** Erythropoietin (EPO) is a cytokine whose main function is to stimulate the production of red blood cells after binding of EPO to its homodimeric receptor (EPOR) on erythroid progenitors. Based on the fact that immune cells express and alternative, heterodimeric receptor composed of EPOR and CD131, we have recently reported that EPO ameliorates the course of experimental colitis due to its ability to reduce the binding activity of the transcription factor NF-κB in macrophages. The potential benefits of high-dose EPO therapy in humans are outweighed by the high risk of thromboembolic complications, though.

**Methods:** We used the EPO analogue ARA290, a nonapeptide known to selectively activate the heterodimeric EPOR, and tested its in vivo efficacy in the dextran-sulfate sodium (DSS) model of experimental colitis. Moreover, we are using cell line and primary macrophages to investigate the production of cytokines and signaling pathways involved in vitro.

**Results:** We could demonstrate that ARA290 ameliorates the clinical course of DSS-colitis as efficient as does EPO without affecting haemoglobin levels. DSS-exposed mice treated with solvent showed substantial weight loss and reduced survival. However, treatment with ARA290 or EPO resulted in significantly reduced weight loss and improved survival. Correspondingly, histopathologic analysis of colon samples revealed significantly reduced tissue damage and inflammation in DSS-exposed mice treated with ARA290 or EPO as compared to solvent-treated DSS-mice. When analyzing supernatants of colonic organ cultures for cytokine levels, we found that TNF, IL-1ß, IL-6, IL-12p70, IL-23, IFN-γ and IL-17A concentrations were significantly lower following treatment with ARA290 or EPO.

**Conclusion:** ARA290 is efficient in improving the clinical course of DSS-induced colitis. It inhibits the production of pro-inflammatory cytokines, which are key mediators in the pathogenesis of the disease, but does not stimulate erythropoiesis. Thus, ARA290 may be a promising agent for the therapy of humans affected by inflammatory bowel disease as it exerts potent anti-inflammatory effects without unintended thromboembolic side effects.

# The Anti-apoptotic Protein Mcl-1 is Essential for the CD8 T Cell Response upon Chronic Viral LCMV Infection.

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**Background:** Chronic infections with viruses such as hepatitis B virus, hepatitis C virus or HIV are in endemic steady state in the world population, constituting a major global public health problem. Studies of chronic viral infections in humans and mice show that persistent antigenic stimulation induces deregulation of T cell responses by a process called T cell exhaustion that culminates in deletion of virus-specific T cells. The ability to generate and retain sufficient numbers of functionally competent T cells relays on the balance between T cell proliferation and apoptosis. Apoptosis is regulated by the interplay of pro-survival and pro-apoptotic molecules of the Bcl-2 family. In particular a pro-apoptotic protein of the family, Bim, has been implicated in the deletion of virus-specific CD8 T. However is still unknown which of the pro-survival Bcl-2 family members is neutralized by Bim to mediate deletion of cytotoxic T cells.

**Methods:** The aim of this project is to determine the role of the anti-apoptotic protein Mcl-1 in promoting CD8+ T cell responses upon chronic viral infection. To address this question we infected Mcl-1 haploinsufficient mice with LCMV docile, which causes chronic infection in mice.

**Results:** Infected Mcl-$1^{+/-}$ mice show a reduction of viral-specific CD8+ T cells during chronic infections. In addition, also the ability to produce antiviral cytokine like gamma interferon (IFN-γ) is abolished in Mcl-$1^{+/-}$ null viral-specific CD8+ T cells.

**Conclusion:** Together our data indicate that Mcl-1 is essential for the CD8+ T cell response upon chronic viral infection. We propose that pharmacological targeting of Mcl-1, e.g. during anti-cancer therapy, may cause enhanced susceptibility to viral infection as one possible side effect.

# Lipidomics Profiling and Risk of Cardiovascular Disease in the Prospective Population-based Bruneck Study

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**Background:** The bulk of cardiovascular disease risk is not explained by traditional risk factors. Recent advances in mass spectroscopy allow the identification and quantification of hundreds of lipid species. Molecular lipid profiling by mass spectrometry may improve cardiovascular risk prediction.

**Methods:** Lipids were extracted from plasma samples of the prospective population-based Bruneck Study taken in 2000. Lipid species from eight different classes were profiled by shotgun lipidomics using a triple quadrupole mass spectrometer. Occurrence of cardiovascular events until 2010 was registered and its association with lipid levels was analyzed using Cox regression. The UK Twin Cohort served as a validation cohort.

**Results**: Samples from 685 participants were taken, measuring 135 lipid species. Levels of individual species of cholesterol esters (CE), lysophophatidylcholines (LPC), phosphatidylcholines (PC), phosphatidylethanolamines (PE), sphingomyelins (SM), and triacylglycerols (TAG) were associated with cardiovascular disease. TAGs and CEs with low carbon number and double bond content were among the lipid species with the highest predictive value. Consideration of TAG(54:2), CE(16:1) and PE(36:5) on top of traditional risk factors resulted in improved risk discrimination and classification (NRI 9.5%; ΔC-index 0.022) for cardiovascular disease. These findings were validated in 1412 participants of the UK Twin Registry.

**Conclusions**: Considering the lipidome on the level of individual species, as opposed to lipid classes, improves cardiovascular risk prediction. This challenges the current focus on lipid levels rather than lipid composition. Molecular lipid species constitute promising new biomarkers that outperform the conventional biochemical measurements of lipid classes currently used in the clinics.

# In vivo and in vitro evaluation of the effect of PRE-084 on inflammation-sensitized hyperoxia-induced developing brain injury

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**Background:** Supraphysiologic oxygen concentrations are toxic to the developing brain. Inflammatory processes increase the risk of brain injury. Sigma-1 receptor agonists are potent suppressors of inflammation-related events, powerful immunomodulatory and antioxidative agents. Neuroprotective effects of this class of pharmacological agents have already been shown in various models of neonatal and adult central nervous system pathology. The aim of this study was to assess the selective sigma-1 receptor agonist 2-(4-morpholinethyl) 1-phenylcyclohexanecarboxylate (PRE-084) in in vivo and in vitro models of inflammation-sensitized hyperoxia-induced developing brain injury.

**Methods:** For in vivo studies, rat pups were randomly presensitized with i) lipopolysaccharide or ii) vehicle on postnatal day 3. On postnatal day 6, pups received either i) PRE-084 or ii) vehicle and were subsequently exposed to hyperoxic conditions for 6, 12 or 24 hours. At the end of exposure, animals were sacrificed and brains were processed for caspase-3 analysis using immunohistochemistry and Western Blotting. For in vitro studies, oligodendroglial cells were subjected to hyperoxic conditions in the presence or absence of pro-inflammatory cytokines and PRE-084. Cell membrane integrity and cell viability were assessed by means of LDH and XTT assays.

**Results:** Inflammatory presensitization significantly increased hyperoxia-induced injury both in vivo and in vitro. PRE-084 administration did not attenuate damage.

**Conclusion:** Sigma-1 receptor agonists have been described as a promising therapeutic strategy in brain injury. We were not able to confirm this in the present model. The exact mechanisms of action of sigma-1 receptor agonists as well as the pathophysiologic pathways involved in hyperoxia-induced injury in the developing brain remain to be elucidated.

# Bone marrow T cells from the femur are similar to iliac crest derived cells in old age and represent a useful tool for studying the aged immune system

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**Background:** $CD4^+$ and $CD8^+$ T cells reside in the human bone marrow (BM) and show a heightened activation state. However, only small sample sizes are available from sources such as the iliac crest. Larger samples can be obtained from the femur in the course of hip replacement surgery. It was therefore the goal of the present study to compare the phenotype and function of BM T cells from different sources from elderly persons and to investigate how femur derived bone marrow T cells can serve as a tool to gain a better understanding of the role of adaptive immune cells in the BM in old age.

**Methods:** Bone marrow mononuclear cells (BMMC) were isolated from the iliac crest or femur of healthy donors by collagenase digestion and Ficoll gradient centrifugation. For intracellular cytokine staining BMMC and peripheral blood mononuclear cells (PBMC) of the same donor were stimulated with PMA/Ionomycin and analyzed by flow cytometry. Antigenspecific $CD8^+$ T cells were analyzed by pentamer technique. The composition of the T cell repertoire in different T cell subsets was investigated by CDR3 spectratyping.

**Results:** There were no phenotypic differences between BMMC from the two sources. Compared to PBMC, both BM sample types contained fewer naïve and more antigen experienced $CD4^+$ as well as $CD8^+$ T cells, which, in contrast to peripheral cells, expressed CD69. Cytokine production was also similar in T cells from both BM types. Larger sample sizes allowed the generation of T cell lines from femur derived bone marrow using non-specific as well as specific stimulation. The phenotype of T cell lines generated by stimulation with OKT-3 and IL-2 for two weeks was very similar to the one of *ex vivo* BM derived T cells. Such lines can be used for studies on the interaction of different types of BM cells as shown by co-culture experiments with BM derived stromal cells. Using $CMV_{NLV}$ specific T cell lines we additionally demonstrated that BM samples from the femur are suitable for the generation of antigen specific T cell lines, which can be used in studies on the clonal composition of antigen specific BM T cells.

**Conclusion:** Our results demonstrate that BMMC from the femur shaft are a useful tool for studies on the role of T cells in the BM in old age.

# An Integrative Analysis of Renal miRNA- and mRNA-Expression Signatures in Progressive Chronic Kidney Disease

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**Background:** MicroRNAs (miRNAs) significantly contribute to chronic kidney disease (CKD) progression via regulating mRNA expression and abundance but their association with clinical outcome remains poorly understood.

**Methods:** Large scale miRNA- and mRNA-expression profiling was performed on cryo-cut biopsy sections from 43 cases of proteinuric kidney diseases. Significantly differentially expressed miRNAs comparing progressive (doubling of serum-creatinine or end-stage renal disease during a mean follow up of 43 months) and stable (all other) cases were determined, and protein coding genes showing inversely correlated mRNA expression profiles were identified and further characterized.

**Results:** In progressive subjects downregulation of 12 miRNAs (miR-140-3p, -148a, -190, -192, -192*, -194, -204, -206, -216b, -30d, -30e-3p, -532-3p) was identified as major drivers for correspondingly upregulated mRNAs being involved in inflammatory response, cell-cell-interaction, metabolism, apoptosis, and intracellular signaling. Ten of these miRNAs were correlated with serum-creatinine at time of biopsy and at follow-up, and all 12 miRNAs correlated inversely with the degree of arteriolar hyalinosis and tubular atrophy/interstitial fibrosis. The corresponding significantly upregulated mRNA targets were further analyzed using bioinformatics network analysis identifying a set of 8 protein coding genes discriminating stable from progressive subjects (CCR7, CCL19, CXCL1, S1PR4, PLCB2, RASGRP1, RASGRP2, ITPR3).

**Conclusion:** We identified differentially expressed renal miRNA- and mRNA-profiles in progressive chronic kidney disease. These miRNAs and mRNAs were associated mainly with inflammatory pathways, and the degree of expression correlated with renal disease severity, suggesting an important role of these miRNA/mRNA-pairs in the pathogenesis of renal disease progression.

# Tetrahydrobiopterin Compounds Modulate Intracellular Signaling and Reactive Oxygen Species Levels in an In Vitro Model of Ischemia-Reperfusion Injury

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**Background:** The pteridines tetrahydrobiopterin ($BH_4$), an essential cofactor of several enzyme systems, including nitric oxide synthases (NOS), and its structural analogue 4-amino-tetrahydrobiopterin ($ABH_4$) prevented acute rejection during solid organ transplantation. Moreover, $BH_4$ also attenuated ischemia-reperfusion injury (IRI). The mechanisms underlying these protective effects, however, are poorly defined. Activation of intracellular signaling proteins, including the mitogen-activated protein kinases (MAPKs) ERK, p38 and JNK, and the excessive production of mitochondrial reactive oxygen species (ROS) critically regulate initiation and progression of IRI and are observed mainly during early reperfusion. While the role of ROS in the initiation and progression of IRI is well understood, the contribution of individual signaling pathways is less clear. Here we tested potential effects of $BH_4$ and $ABH_4$ on MAPK activity and mitochondrial ROS levels.

**Methods:** Pteridine effects were studied in HL-1 cardiomyocytes and human umbilical vein endothelial cells (HUVEC). Hypoxia-reoxygenation (HR) as an in vitro model for ischemia-reperfusion was used. Intracellular signaling was analyzed using phosphorylation-specific antibodies, mitochondrial ROS levels were determined by fluorescence microscopy and cell viability was assessed by annexin V-FITC/propidium iodide staining.

**Results:** During hypoxia and reoxygenation (H/R) all three MAPKs were activated during early reoxygenation in HL-1 cardiomyocytes and endothelial cells (HUVEC). p38 and JNK activation were further augmented by $BH_4$ and to a smaller degree by $ABH_4$, whereas ERK activation was not affected. Pretreatment with $BH_4$ and $ABH_4$ significantly reduced both basal mitochondrial ROS levels as well as the H/R-induced increase in ROS. Prolonged incubation with $ABH_4$, however, showed pro-apoptotic effects in cardiomyocytes.

**Conclusion:** These data suggest that a protective effect of $BH_4$ and $ABH_4$ pretreatment may be attributed mainly to their antioxidant capacity. Intracellular signaling on the other hand is influenced in distinct and complex ways.

# INVESTIGATING THE ROLE OF Bcl2a1/A1/BFL-1 IN LEUKOCYTE DEVELOPMENT

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**Background:** Programmed cell death plays an important role in maintaining homeostasis in the immune system. Bcl2 family proteins control mitochondrial-induced apoptosis by either promoting or preventing membrane integrity and cytochrome c release into the cytosol leading to caspases activation. Deregulation of B cell selection processes by impairment of Bcl-2 regulated cell death can cause immunodeficiency, autoimmunity or contribute to malignant transformation.

Murine A1 protein is an anti-apoptotic member of the Bcl2 family encoded in four genes in mice, A1-a, A1-b, A1-c, A1-d, with A1-c representing a processed pseudogene. A1 is mostly expressed during embryogenesis and in the hematopoietic system in adult mice. Activation of the BCR in B cells or the TCR in T cells is associated with increased levels of Bcl2a1, which suggests a cytoprotective function that is essential for the activation and survival of lymphocytes.

A1 is upregulated in mature B cells in periphery and during differentiation from transitional ($IgM^{high}$) to mature follicular ($IgM^{low}$) B cells in the spleen. It was shown that constitutive A1 knockdown of all A1 isoforms in mice leads to a decrease in the percentage of mature follicular B cells and impairment of proliferation upon mitogenic stimulation. Whether these phenomena are due to impaired differentiation or increased B cell death remains unclear at present.

**Methods:** To understand the role of A1 in hematopoietic system under physiological and pathological conditions we utilize Tet-regulated RNAi targeting all A1 isoforms in vivo. These double-transgenic animals harbor the miR30-embedded shRNA targeting A1 under control of the Tet-$CMV^{min}$ promoter (referred as TREA1 mice) and the reverse Tet-transactivator (rtTA) placed under control of the CAG promoter (referred to as DTr mice). Administration of doxycycline drives the expression of a mi-shRNA targeting A1 leading to a significant knockdown of A1a, A1b and A1d mRNA. The knockdown efficiency was evaluated at the mRNA in developing as well as mature leukocytes.

**Results :**FACS analysis of cells derived from primary and secondary lymphatic organs from DTr mice kept on doxycycline for 17 days revealed a variety of phenotypic abnormalities. We observed that inducible A1 KD show reduced percentages of total B cells in bone marrow, peripheral blood and lymph nodes mainly due to loss of mature (recirculating) B cells in bone marrow, lymph node and spleen. Most other cell types were not affected upon acute depletion of A1.

**Conclusion:** We conclude that A1 is essential for the survival of mature follicular B cells, but dispensable for their development.

## Interferon Type I Signalling Mediated Crosstalk Between Intestinal Epithelium and Gut Microbiota in Mice

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**Background:** Type I interferons (IFNs) signal through the heterodimeric IFN-alpha receptor complex IFNAR1 and IFNAR2 and are known for their antiviral, anti-proliferative, anti-tumour and immunomodulatory functions. These aspects take on even more significance considering the key attributes of intestinal epithelial cells (IECs): They are exposed to a unique environment in the body that harbours trillions of microbes and at the same time they are charged with the task of governing the immune system to discriminate between pathogens and commensals. Not surprisingly, IFNAR1 has been implied from GWAS as risk gene for Crohn's disease. However, little is known about IFN type I signalling in the intestinal epithelium.

**Methods:** To address the function of type I interferon mediated signalling in the gut epithelium, we generated mice with conditional deletion of Ifnar1 in IECs ($Ifnar1^{\Delta IEC}$). Phenotype was assessed at baseline condition for enteritis and proliferation. Moreover, $Ifnar1^{\Delta IEC}$ mice were used in model of dextran sulphate sodium (DSS)-induced colitis and colitis-associated-tumour (AOM/DSS). Gut microbiota was analysed by 16S rDNA ribotyping.

**Results:** Mice with deficient type I IFN signalling showed increased proliferation and expansion of lysozyme+ Paneth cells. Unexpectedly, IEC-specific deletion of Ifnar1 neither resulted in spontaneous inflammation nor affected the severity of DSS colitis. However, absence of IFNAR1 function in the intestinal epithelium resulted in increased tumour burden compared to IFNAR1-sufficient mice. This tumour-promoting effect was dependent on the microbial flora, as co-housing of $Ifnar1^{\Delta IEC}$ and $Ifnar1^{+/+}$ mice equalized the differences between genotypes. Accordingly, 16S ribotyping revealed profound differences in microbial composition between (epithelial) genotypes and their housing conditions.

**Conclusion:** IFNAR1 is indispensable for intestinal homeostasis through the regulation of cell proliferation and anti-bactericidal activity as well as accompanied suppression of tumourigenesis and alterations of gut microbiota ecosystem, respectively. However, IFN type I signalling does not seem to regulate gut inflammatory status.

## Impact of the prosurvival Bcl-2 family member A1 on T cell immunity

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**Background**: Apoptosis is a key mechanism to prevent the development of autoimmunity and cancer. It is induced either by death receptor ligation on the cell surface (extrinsic pathway) or by stress factors involving proteins of the Bcl-2 protein family (intrinsic pathway). A1/Bcl2A1 is an anti-apoptotic member of the Bcl-2 family that is mainly expressed in the hematopoietic system. Altered expression of A1 has been reported in context with autoimmune disorders as well as in different cancers in humans. In T cells A1 is thought to play a role during early T cell development and after TCR ligation upon activation. However, since no antibodies and knock-out models are available for the analysis of A1 function are available, the physiological function of A1 remains to be clarified.

Due to quadruplication in the mouse genome generation of A1 knockout mice is not feasible. Therefore, our laboratory has generated a mouse model that targets all functional A1 isoforms using an RNAi approach leading to a stable A1 knockdown in the hematopoietic system. Using this approach we are able to investigate the impact of diminished A1 expression on T cell maturation, differentiation and effector function.

**Methods**: Different T cell subsets were isolated from wild type mice and analyzed for A1 mRNA expression by qRT-PCR. The impact of A1 knockdown on T cell development was investigated in vivo by T cell subset distribution analysis, by using in vitro T cell development assays (OP9-DL1 differentiation system) as well as by using TCR-transgenic mice in which a mi-shRNA targeting A1 was expressed in the hematopoietic system. Furthermore, we analyzed the abundance of naïve, memory and Treg cells in the spleen and used Experimental Autoimmune Encephalomyelitis (EAE) as an in vivo model to study the role of A1 in T cell mediated autoimmunity.

**Results**: We confirmed strong A1 mRNA induction in T cells upon TCR-ligation and T cell activation. Additionally, A1-mRNA levels were found elevated in memory T cells when compared to naïve T cells. Although no impaired T cell development and T cell distribution pattern were observed in A1-knockdown mice under steady state conditions, we found a delayed onset of EAE, indicating an involvement of A1 in the development of this form of autoimmunity.

**Conclusion**: Diminished expression of A1 does not seem to grossly impair T cell development and T cell subset distribution under steady state conditions. This may be due to insufficient knockdown efficiency or counter-selection phenomena in our model system. However, we observed a delayed development of EAE in A1 knockdown mice compared to control mice or mice expressing a mi-shRNA targeting firefly luciferase. This strongly points towards an involvement of A1 in inflammatory responses.

# The Ontogeny and Differentiation Pathways of Tumor-Associated Macrophages in a Model of Spontaneous Mammary Carcinogenesis

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**Background:** Tumor-Associated Macrophages (TAMs) represent a major non-neoplastic cell population in a variety of animal and human tumors, and their abundance is linked with bad patient's prognosis and increased risk of recurrence and metastasis. They are considered as important contributors to the immunosuppressive environment of the tumor, support its growth, vascularization and systemic spread. Despite their potential impact on patient's outcome and therapy success, the mechanisms which regulate the accumulation of TAMs in tumors are not fully elucidated. Although in light of the accepted theory a critical contribution of inflammatory monocytes to the development of TAM populations is postulated, accumulating evidence suggests the existence of monocyte-independent, self-sustaining macrophage populations. In our research we sought to pin down the ontogeny of TAMs and identify growth factors which orchestrate their differentiation in a spontaneous model of mammary cancer.

**Methods:** FVBN/J MMTV Neu Stat1+/+ and Stat1-/- mice were used in our experiments. The mammary epithelium-directed over-expression of the rat homologue of HER2 drives the spontaneous development of mammary adenocarcinomas in these animals. In vivo experiments such as adoptive transfers, depletions, labeling of blood monocytes, and inhibition of Csf-1 receptor were performed to assess the dependence of TAMs on circulating monocytes, Stat1 and Csf-1R signaling.

**Results:** We show that MMTV Neu tumors are infiltrated by two populations of macrophages, characterized as CD11b$^{hi}$ F4/80$^{lo}$ and CD11b$^{lo}$ F4/80$^{hi}$, respectively. The second population accumulated in tumors in a Stat1-dependent manner. With help of long term BrdU labeling and depletion of circulating monocytes we demonstrate the longevity and relative autonomy of CD11b$^{lo}$ F4/80$^{hi}$ TAMs. The results of experiments with adoptive transfer of monocytes and in vitro-differentiated macrophages indicate that monocyte-derived CD11b$^{hi}$ F4/80$^{lo}$ macrophages may be the direct precursors for the F4/80$^{hi}$ population. Moreover, incorporation of BrdU in short-pulse experiments witness about an intensive proliferation of both TAM populations, which could be dampened by a Csf-1 receptor inhibitor and by the genotoxic drug doxorubicin. A prolonged therapy of tumor-bearing animals with those drugs strongly reduced the amount of CD11b$^{lo}$ F4/80$^{hi}$ TAMs, suggesting the vital role of the Csf-1-driven proliferation in TAM homeostasis. Furthermore, we identify Stat1 as an upstream regulator of Csf-1 expression, and this could be responsible for the lowered numbers of CD11b$^{lo}$ F4/80$^{hi}$ TAMs in Stat1-deficient MMTV Neu tumors.

**Conclusion:** Our results indicate that vigorous local in situ proliferation and low-rate monocyte recruitment contribute to the homeostasis of TAMs in MMTV Neu tumors. The process of differentiation of CD11b$^{hi}$ F4/80$^{lo}$ TAMs to CD11b$^{lo}$ F4/80$^{hi}$ and their in situ proliferation are driven by Csf-1 whose expression is transcriptionally controlled by Stat1.

## Protein Kinase C θ Regulates Th1/Th17 Plasticity

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**Background:** Mature CD4+ cells can polarize into functionally distinct T helper subsets (Th1/Th2/Th17/iTreg). Although this differentiation is often regulated by opposing molecular pathways, some plasticity between the subsets exists. This phenomenon is especially remarkable in case of Th1 versus Th17 lineage development, but underlying mechanisms are not fully understood. Protein Kinase C θ (PKCθ) is a well established player in proximal T cell receptor signaling, but less is known about its contribution to differentiation and function of particular Th subsets. In this study we investigated role of PKCθ in Th17 cells differentiating cells.

**Methods:** We used in vitro (polarization of isolated naïve CD4+ cells) and in vivo (induction of Experimental Autoimmune Encephalomyelitis (EAE) in mice) models to investigate phenotype of Th17 cells derived from PKCθ-/- and wild-type mice. Cells were analyzed by flow cytometry, RT-PCR and western blotting to examine their phenotype and genes expression patterns.

**Results:** According to our observations, in vitro differentiated PKCθ-/- CD4+ cells express normal levels of Th17 marker genes (IL-17, RORγt), but at the same time produce more factors typical for the Th1 subset (IFNγ, T-bet). Increased expression of IFNγ and T-bet in PKCθ-deficient cells was observed only under Th17-promoting, and not under Th1-promoting conditions. In concert with increased IFNγ production, also phosphorylation of STAT1 was enhanced and sustained in the cultures of PKCθ-/- cells. This phenotypical plasticity switch was confirmed in vivo, in the cells isolated from EAE-induced mice, and caused changes in the disease progression.

**Conclusion:** Our observation suggests that PKCθ is involved in modulation of molecular pathways underlying Th1 and Th17 lineages differentiation and their plasticity. PKCθ suppresses IFNγ – STAT1 – T-bet axis In Th17-differentiating cells. Defining mechanisms of such contribution will not only clarify the flexibility of T cell polarization, but may also shed light on the pathogenesis of autoimmunity and cancer.

## The role of Type I Interferons in alcoholic liver disease

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**Background**: Alcoholic liver disease (ALD) is very common in industrial countries, an estimated 5% prevalence in Austria, and the leading cause of liver cirrhosis and liver transplantation. Some patients develop acute alcoholic steatohepatitis, a life-threatening liver failure with a mortality rate up to 50%. This state is not necessarily associated with a significant degree of fibrosis but is characterized by neutrophil infiltration, liver steatosis and massive liver inflammation with induction of pro-inflammatory cytokines, i.e. tumour necorsis factor (TNF) alpha, Interleukin (IL) 1beta and IL-8.

Type I Interferons are long-known mediators of the innate immune system with various biological functions. Besides their well-established role in innate immune response to viral infections, they comprise various anti-inflammatory effects such as induction of IL-18BP and IL-1Ra and inhibition of IL-8. Thus, we hypothesized that type I Interferons could mitigate the devastating natural course of ASH.

**Methods**: Albumin or LyzS promoter mediated Cre-expression was used to delete interferon receptor 1 (IFNAR1) either in hepatocytes ($IFNAR1^{fl/fl}$ $AlbCr^{+/-}$) or in the myeloid compartment ($IFNAR1^{fl/fl}$ $LyzS^{tg/wt}$). 6 to 8 weeks old transgenic mice or control littermates were fed a Lieber deCarli diet containing 5% ethanol (vol/vol) for 3 weeks. Body weight was measured every other day and glutamate pyruvate transaminase (GPT) was measured before and after the experiment. After sacrificing the mice liver inflammation and steatosis was determined by histological scoring.

**Results**: We were able to successfully establish the ambitious Lieber de Carli animal model of alcoholic hepatitis. Alcohol-ingesting mice demonstrated a mean 4.5-fold increase in circulating GPT. Despite highly-efficient tissue-specific knockout of IFNAR1, we neither observed a significant difference in the weight course in the IFNAR $KO^{hep}$ nor IFNAR $KO^{myel}$ compared with WT controls receiving Lieber de Carli 5% ethanol. Accordingly, there were no differences in the GPT levels or liver body ratio. Examining liver histopathology, comparable steatosis and inflammation was observed in all three groups.

**Conclusions**: Our data are not in favour of a hypothesis that type I interferons a crucial modulators of alcoholic steatohepatitis since neither hepatocyte nor myeloid-specific type I interferon signaling does affected steatosis and inflammation in an effective in vivo model of ASH. We conclude that type I Interferons are not essential in the pathogenesis of ASH.

# Transplantation/Therapy/Diagnostics

## Evaluation of Cryopreserved and Thawed Human Ovarian Tissue after Xenotransplantation in SCID Mice

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**Background:** Ovarian tissue cryopreservation and transplantation are promising options for fertility preservation in young female cancer patients. However, the outcome of cryopreservation and thawing procedures still need to be assessed before retransplantation. Therefore the aim of this study is to establish a xenotransplantation procedure, by evaluating the function and morphology of the cryopreserved/thawed human ovarian tissue in Severe Combined Immunodeficient (SCID) mice.

**Methods:** Cryopreserved human ovarian tissue donated by one transgender patient, 41 years old, was thawed by using a rapid thawing protocol with 30 seconds of warming at room temperature, followed by 2 minutes at 37$^{o}$C in a waterbath, and 3 washing steps of sucrose gradient. Thawed tissue were cut into 3mm diameter biopsies and transplanted into 6-week-old SCID mice divided into 4 observation groups: 6 days, 4 weeks, 8 weeks, and 16 weeks (n=12 per group). Xenotransplantation procedures were performed under sterile conditions. SCID mice were first anaesthesized with 1 ml/100 mg of Ketamine and 1 ml/23.3 mg of Xylazine. Bilateral ovarectomy was performed in all mice. 1 piece of human ovarian tissue was transplanted into a subcutaneous neck pouch of each recipient mouse. During the observation period, the function of the grafts was assessed by observing daily vaginal smears to show the estrous cycle pattern. By the end of the observation period, the morphological features of the grafts were assessed. Ovarectomized (n=2) and sham operated mice (n=1) served as controls.

**Result:** From a total of 51 mice, 49 survived the operation (96,1%), and 46 mice from 49 mice (93,8%) survived through observation period. From the vaginal smears observation, all study groups firstly showed a non cycling period until the estrous cycle resumed at 2-5 weeks after transplantation. The estrous cycle was observed as regular cycle until another non-cycling period started by week 10-11. The graft recovery rate was 87.5%, as 37 grafts were found from 42 recipient mice. 7 mice are currently still in observation period. All of the recovered ovarian grafts were macroscopically comparable to pregraft thawed controls, and more abundant vascularization was found in 6-day-grafts compared to pregraft thawed controls. Histologically the human ovarian tissue grafts showed intact stroma and good vascularization.

**Conclusion:** We established a xenograft model to evaluate cryopreserved/thawed human ovarian tissue by observing estrous cycles and histological assessment of vascularization as well as stroma quality. In the future we will focus on evaluating the folliculogenesis of the graft.

# Reduction of Complement Factor H Binding to CLL Cells Improves the Induction of Rituximab-Mediated Complement-Dependent Cytotoxicity

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**Background:** The use of monoclonal antibodies like rituximab (RTX) has considerably improved the treatment of B cell chronic lymphocytic leukemia (B-CLL). The immunological efficacy of RTX in cancer therapies relies mainly on two Fc-based mechanisms, i.e. antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). However, CDC is limited, because CLL cells are protected from complement-induced damage by regulators of complement activation (RCAs). A prominent RCA in fluid phase is factor H (fH), which has not been investigated in this context yet.

**Methods:** For this study, we generated human fH-derived recombinant SCR18-20 (hSCR18-20), representing the initial binding domain of fH to cell surfaces. CDC induced by RTX in the presence or absence of fH-derived SCRs was determined by counting propidium iodide negative living cells in lysis assays.

**Results:** We here show that fH binds to CLL cells and that human recombinant fH-derived short-consensus repeat 18-20 (hSCR18-20) interferes with this binding. In complement-based lysis assays CLL cells from therapy-naive patients were differently susceptible to rituximab-induced CDC and were defined as CDC responder or CDC non-responder, respectively. In CDC responders, but notably also in non-responders, hSCR18-20 significantly boosted RTX-induced CDC. Killing of the cells was specific for $CD20^+$ cells, while $CD20^-$ cells were poorly affected. CDC resistance was independent of expression of the membrane-anchored RCAs CD55 and CD59, although blocking of these RCAs further boosted CDC.

**Conclusions:** The inhibition of fH binding by hSCR18-20 sensitizes CLL cells to CDC and may provide a novel strategy for improving rituximab-containing immunochemotherapy.

## VSV-GP Is A Potent Oncolytic Virus Platform For The Treatment Of Melanoma

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The Vesicular Stomatitis Virus (VSV) is an extremely potent oncolytic agent; however, clinical application has been limited by its devastating neurotoxicity and the rapid induction of a neutralizing antibody response. We previously reported that by substituting the normal viral glycoprotein (VSV-G) with the glycoprotein GP of the viscerotropic lymphocytic choriomeningitis virus WE-strain we can eliminate the neurotoxicity of VSV yet retain its replication fitness (Muik A et al., J Virol. 2011:5679-84; Muik A et al., J Mol Med (Berl). 2012:959-70).

Here we show that in vitro the chimeric virus VSV-GP was able to efficiently infect and lyse various human, rodent and canine melanoma cells grown as monolayer or spheroid cultures. Moreover, VSV-GP treatment was highly efficient in mouse models of malignant melanoma with dermal tumors or lung metastasis upon intratumoral or intravenous application. We demonstrated that VSV-GP does not induce neutralizing antibodies readily and therefore, repeated systemic application is feasible.

Taken together, the VSV-GP pseudotype virus is a safe and extremely potent oncolytic agent with exceptional tumor killing activity. Thus, VSV-GP is a promising novel therapeutic for the treatment of melanoma and potentially other cancers in humans.

# Liver Grafts From Anti-HBc Positive Donors Are Safe For Anti-HBc Negative Recipients In Liver Transplantation Using Combined Antiviral Prophylaxis

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**Background:** The shortage of suitable donor livers is the most prominent problem in liver transplantation today. The use of grafts from so called extended criteria donors (ECD) helps to reduce patient´s mortality on the waiting list. The number of grafts that are used from donors with antibodies against the Hepatitis B core antigen (anti-HBc), but Hepatitis B surface antigen (HBsAg) negative is rising. Herein we investigate the long term outcome of recipients of grafts from Hepatitis B core positive donors and the impact of antiviral prophylaxis.

**Methods:** This retrospective study includes 1167 liver transplantations performed at our centre between April 1977 and March 2012. Primary endpoints were patient and graft survival. Secondary endpoint was occurrence of HBV infection (positive HBV PCR). We were focussing on antiviral prophylaxis and recipient´s anti-HBs titers at time of transplantation. The median follow up of the patients was 4.4 years. Statistics were carried out by Kaplan Meier analysis, ANOVA and Mann- Whitney-test.

**Results:** 59 (5.1%) anti-HBc+ liver grafts were transplanted to anti-HBc- (56.4%) or anti-HBc+ recipients (43.6%). 5-year graft- and patient-survival were 63.2% and 74.6% respectively. 27.1% of all patients receiving anti-HBc+ grafts became HBV PCR+ post transplant (18.6% de novo, 8.5% recurrence), occurring after a mean of 2.7 years. 0% of recipients with anti-HBs >100 iU/ml at the time of transplantation became HBV PCR+ in the postoperative course compared to 25% with anti-HBs <100 iU/ml. Perioperatively Hepatitis B immunoglobulin (HBIg; 10000 iU/ day until anti-HBs titer was >500 iU/ml) was administered in 47% of HBsAg- patients. Lamivudin was administered in 46%, Tenofovir in 7% of all patients. In HBsAg- recipients, HBIg monotherapy resulted in 50%, Lamivudin monotherapy in 33%, combined (Lamivudin/Tenofovir plus HBIg) in 16.7% and no treatment at all in 35% HBV+ PCR post transplant.

**Conclusion:** Anti-HBc+ livers can be transplanted with reasonable long term patient and graft survival. These grafts should primarily be allocated to patients with anti-HBs >100 iU/ml. Antiviral prophylaxis should be carried out with Lamivudin/Tenofovir plus HBIg in HBsAg- patients.

## LCMV-GP Pseudotyped VSV for Oncolytic Virotherapy of Ovarian Cancer

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**Background:** Ovarian Cancer is one of the leading causes of death from gynecological malignancies in the western world. The prognosis of patients remains devastating since tumors are usually diagnosed at advanced stages when common therapeutic strategies such as surgery and chemotherapy have reached their limit. A promising new approach is the use of vesicular stomatitis virus (VSV)-based oncolytic virotherapy as VSV is one of the most potent oncolytic viruses and there is no pre-existing antiviral immunity in the human population. However, VSV's glycoprotein-mediated inherent neurotoxicity has hindered clinical development so far.

**Methods:** To abrogate VSV's inherent neurotoxicity, we pseudotyped VSV with the non-neurotropic envelope glycoprotein of the lymphocytic choriomeningitis virus thus rendering VSV-GP (LCMV-GP; Muik et al, J. Virol., 2011). Oncolytic potency was tested in ovarian cancer cell cultures and in a subcutaneous ovarian cancer xenograft mouse model. To improve delivery of VSV-GP to distant tumor sides and to circumvent recognition of the virus by the host immune system, we use mesenchymal stem cells (MSC) as carrier cells.

**Results:** VSV-GP exhibited a more than 10^6-fold higher LD50 compared to VSV wildtype upon intracranial injection in mouse brain. Furthermore, effective oncolytic activity of VSV-GP could be demonstrated in ovarian cancer monolayer and spheroid cell cultures. Accordingly, intratumoral injection of VSV-GP into s.c. xenografts led to tumor remission in all animals. Relapsing tumors were still susceptible to VSV-GP mediated oncolysis. In an orthotopic model, treatment with VSV(GP) led to significantly prolonged survival. VSV-GP was able to infect MSC and cells remained alive for more than 24h in vitro upon infection. Most importantly, VSV-GP efficiently replicated in MSC leading to release of high virus titers.

**Conclusion:** The results of our in vitro and in vivo studies demonstrate that LCMV GP-pseudotyped VSV exhibits a highly beneficial toxicity and efficacy profile. Thus, it represents an extremely promising candidate for oncolytic virotherapy of ovarian cancer, which can be further optimized using MSC as carrier cells.

# The Functional Influence of Activating NK Cell Receptors in Solid Allograft Rejection

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**Background:** Although it has been shown that Natural Killer (NK) cells influence allograft survival, little is known about the detailed involvement of their receptors.

**Methods:** In order to gain more insight into the functional importance of certain activating NK cell receptors on the cellular subset composition during acute rejection, we used BALB/c mice as allograft donors and NKp46- (Ncr1), NKG2D- and Ly49-deficient mice on a C57BL/6 background as graft recipients in a heterotopic heart transplantation model. Ncr1 k.o. mice possessed a green fluorescent protein (GFP) cassette knock in at the Ncr1 locus, wildtype C57BL/6 mice served as controls. Animals were either sacrificed at day 5 (d5) or at day of graft rejection (dRx) for analysis (n=5/group/time point).

**Results:** Although graft survival revealed no significant differences between wildtype and NK cell receptor deficient mice (day 7.5±1.5 day), a strong infiltration of NKp46+ (GFP+) NK cells into the allograft is already observed at d5 for all investigated groups, however, Ncr1- and NKG2D-deficient mice showed significant more frequencies of intragraft NK cells compared with wildtype mice ($p<0.01$, respectively). At dRx, NKp46+ cells are clearly diminished in the graft in all groups. In contrast, frequencies of splenic NK cells were induced at this time point, although NKG2D k.o. mice possessed significantly elevated levels of splenic NKp46+ cells already at d5 compared with wildtype, Ncr1 k.o and Ly49 k.o. mice ($p<0.001$, respectively). Moreover, these cells appeared to be less activated reflected by their reduced CD69 expression than NKp46+ cells derived from wildtype or Ly49 k.o. mice. Among T cell subsets, all groups displayed a significant intragraft induction of cytotoxic CD3+CD8+ T cells at dRx, whereas induced frequencies of CD3+CD4+ T helper cells were observed in allografts from NK cell receptor deficient mice ($p<0.01$, respectively versus wildtype). In spleen, especially NKG2D k.o. mice demonstrated elevated levels of CD3+CD4+ T cells already at d5, although these mice did not exhibit an induction of CD3+CD8+ T cells compared with wildtype, NKp46 k.o. and Ly49 k.o. mice. Strikingly, significant enhanced levels of CD8+CD122+ regulatory T cells in all NK cell receptor deficient mice were observed at dRx in both spleen and lymphnodes ($p<0.01$ versus wildtype).

**Conclusion:** In summary, our results reveal novel insights into the kinetic distribution of NK cells in allografts and secondary lymphoid organs and highlight the impact of NK cell receptor deficiency on various lymphocytes subsets during acute cellular rejection.

**Abstract Nr. 45**

# The Therapeutic Potential Of The Pharmacological Inhibition Of PBEF/NAMPT/Visfatin in Inflammatory Bowel Disease

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**Background:** Crohn's disease (CD) and Ulcerative Colitis (UC) are the two major entities of human inflammatory bowel disease (IBD). Despite recent advances in our understanding of the underlying biological mechanisms of disease, currently available therapies still often remain insufficient. In IBD patients, high circulating PBEF/NAMPT/Visfatin serum levels have been observed and elevated mRNA expression in inflamed mucosal tissue have been demonstrated. Inflammatory processes demand large amounts of energy in the form of energy-enriched substrates and enzymatic co-factors. PBEF/NAMPT/Visfatin has an important role in a central cellular energy pathway, catalyzing the rate-limiting step in the biosynthesis of $NAD^+$ from nicotinamide. Thus PBEF/NAMPT/Visfatin could impact inflammatory pathways by modulating the synthesis and bioavailability of intracellular NAD. This study aims to investigate the activation state of PBEF/NAMPT/Visfatin and components of the "NAD salvage pathway" in IBD patients. Based on this data we study the potency of FK866, a specific inhibitor of PBEF/NAMPT/Visfatin.

**Methods:** We investigated the therapeutic effect of FK866 in an acute and chronic animal model of IBD. Acute colitis was induced with 3.5% Dextran Sulfate Sodium (DSS) in C57/Bl6 wildtype mice. The therapy group was administered 20mg/kg FK866 daily compared to vehicle treated control mice. As a chronic model we used 13-week-old IL-10 knockout mice receiving either 20mg/kg FK866 or saline daily for a total of 2 weeks. Clinical and histological features as well as proinflammatory cytokines were determined. $NAD^+$ and its metabolite levels are measured by HPLC/mass-spec methods.

**Preleminary data:** Animals treated with the small molecule inhibitor FK866 show a better clinical activity index, reduced histological inflammation and significantly lowered proinflammatory cytokine levels like TNFalpha, IL-6, IL-1beta in the colon and IL-6 in serum.

**Conclusion:** The administration of FK866 and the resulting depletion in NAD results in a better clinical and histological outcomes and significantly lowers mRNA expression of various pro-inflammatory cytokines. Hence, FK866 in mice seems to be beneficial in terms of a novel therapeutic treatment strategy. Preliminary data suggests that the measurement of NAD and its metabolites is sensitive regarding the prediction of treatment response. In the next step we will investigate the transmissibility of this concept in human IBD patients using a whole tissue culture approach. Hopefully, our data will pave the way to establish a new therapeutic concept for treating inflammatory conditions, especially IBD.

# Innate Lymphocyte Activation by Targeted Manipulation of the Mevalonate Pathway: Implications for Cancer Immunotherapy

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**Background:** The mevalonate pathway is an essential metabolic pathway for all eukaryotic cells. In tumor cells increased levels of mevalonate metabolites could be found and very recently the malignant phenotype of p53 mutated breast cancer cells could be spiked to increased mevalonate pathway activity. Two widely prescribed classes of drugs, aminobisphosphonates (N-BPs) and statins, can be used to inhibit the mevalonate pathway.

**Methods:** We examined the response of innate immune cells comprising natural killer (NK) cells, γδ T cells as well as a subpopulation of $CD56^+$ inflammatory dendritic cells (DCs) to treatment with N-BPs, statins and mevalonate metabolites. Cell subsets were isolated using MACS technology. Cytokines were analyzed with multiplexed cytokine bead arrays (CBAs). Phenotypes and expansion of cell subsets were assessed by flow cytometry.

**Results:** Inhibition of farnesyl pyrophosphate (FPP) synthase, a key enzyme of the pathway, not only led to upstream accumulation of isopentenyl pyrophosphate (IPP), a cognate antigen for $Vδ2^+$ γδ T cells, but also to downstream abrogation of protein prenylation, an essential posttranslational modification of many RAS superfamily members. Inhibition with statins, which target HMG-CoA reductase - the first committed step of the pathway, likewise resulted in the suppression of protein prenylation and induced massive cellular stress. As a consequence both drugs eventually induced inflammasome activation and the release of caspase-1 processed bioactive interleukin (IL)-1β and IL-18 from $CD56^+$ inflammatory DCs. These proinflammatory cytokines led to the potent activation of both, NK and γδ T cells. Moreover, various mevalonate metabolites could be used to regulate cytokine and proliferative responses of innate lymphocytes.

**Conclusion:** Our work establishes novel immunostimulatory effects of otherwise well-established drugs and confirms the mevalonate pathway as an important therapeutic target. In addition, our work enables us to define essential requirements of NK and γδ T-cell activation, which can be harnessed for immunotherapeutic purposes.

# Treatment With Oral Active Vitamin D Is Associated With Decreased Risk Of Peritonitis And Improved Survival In Patients On Peritoneal Dialysis

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**Background:** Peritonitis is a major complication of peritoneal dialysis (PD) being associated with 5 hospitalization, catheter loss, technique failure, and increased mortality. Data on incidence rates and risk factors for peritonitis episodes vary between centers.

**Methods:** In seven Austrian PD units clinical and laboratory data on each peritonitis episode were collected from all patients (n=726) who performed PD between January 2000 and December 2009.

**Results:** The peritonitis incidence rate was 0.32 episodes/patient-year. In a multivariate analysis the risk of peritonitis 10 was decreased by 57 % in patients treated with oral active vitamin D (HR 0.43; 95 % CI 0.28-0.64). Renal disease classified as “other or unknown” (HR 1.65; 95 % CI 1.08-2.53) and serum albumin <3500 mg/dl (HR 1.49; 95 % CI 1.04-2.15) were also associated with an increased risk of peritonitis. Albumin levels <3500 mg/dl (HR 1.89; 95 % CI 1.13-3.17), age (HR 1.06 per year; 95 % CI 1.03-1.09), and cardiomyopathy (HR 3.01; 95 % CI 1.62-5.59) were associated with 15 increased mortality, whereas treatment with oral active vitamin D was associated with a significantly lower risk of death (HR 0.46; 95 % CI 0.27-0.81).

**Conclusion:** In this retrospective multi-center study we identified several factors being related to increased risk of peritonitis in PD patients. Treatment with oral active vitamin D was identified as being independently associated with decreased risk of peritonitis, and decreased all-cause mortality 20 in PD patients.

# Clinical Application of CSF PCR Testing for Diagnosis of CNS-disorders - A Retrospective 11-Years Experience

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**Background:** The PCR is the method of choice to detect viral activity in the central nervous system (CNS). Positive findings, however, do not prove an impact on the neurological disorder. This aggravates interpretation of PCR data.

**Methods:** In order to identify coherences facilitating data interpretation a retrospective analysis of CSF PCR data of 514 pediatric and 2904 adult samples was performed, focusing on Epstein-Barr virus (EBV), cytomegalovirus (CMV), herpes simplex virus (HSV), enteroviruses (ENV), human herpesvirus 6 (HHV-6), and varicella zoster virus (VZV).

**Results:** EBV was detected in 1.63%, VZV in 1.3%, HSV in 0.4%, ENV in 0.4%, CMV in 0.2%, and HHV-6 in 0.2% of the patients studied, respectively. Virus activity rates were highest among young children and patients >80 years of age. HSV, VZV, and ENV were dominant in typical infectious CNS diseases, EBV in further inflammatory diseases (bacterial CNS infections, multiple sclerosis, HIV infection) and in diseases not typically attributed to infections. HSV and EBV were frequent in further immunosuppressive conditions. Analysis of patients with repeated PCR studies revealed, that delayed viral detection (negative PCR followed by positive PCR) can occur in EBV (6 / 147) and HSV(1 / 217). Effective treatment or spontaneous amelioration (positive PCR finally followed by negative PCR) were typical for HSV, VZV, CMV, and ENV. The maximum time until viral clearance was 30 days for HSV and 15 days for VZV infections, respectively.

**Conclusion:** HSV-, VZV- and ENV-activity characterizes patients with typical infectious CNS diseases. EBV and HHV-6 tend to be active in patients with further neurological diseases which, however, does not rule out a clinical impact. In HSV infection repeated testing may be necessary to establish the diagnosis. Viral clearance from VZV or HSV may be slower than mirrored by actual treatment recommendations which supports the need for repeated CSF PCR testing.

# Rituximab Reduces Relapses and the Dose of Immunosuppressants in Steroid-Dependent and Frequently Relapsing Minimal Change Disease and Focal Segmental Glomerulosclerosis in Adults: A Systematic Review

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**Background.** Frequently relapsing and steroid-dependent nephrotic syndrome due to minimal change disease (MCD) and focal segmental glomerulosclerosis (FSGS) remains a therapeutic challenge, since steroids and other immunosuppressive agents exhibit an unfavorable adverse event spectrum. Rituximab has shown promising effects in childhood and adult MCD and FSGS. The aim of this review was to systematically summarize and analyze data from preexisting studies including case reports and case series reporting the outcome of RTX treatment in these patients.

**Methods.** Study data was identified by a PubMed and Embase search, and the authors were contacted for additional clinical data if necessary. The number of relapses was calculated and the use of immunosuppressive co-medication prior to and after RTX treatment was quantified. Factors associated with relapsing disease were analyzed by logistic regression analysis.

**Results.** We identified twelve studies including 45 patients with frequently relapsing and steroid-dependent MCD or FSGS. Treatment with RTX significantly reduced the number of relapses per year from 1 [0-6] relapse prior treatment compared to 0 [0-2] after therapy ($p<0.001$) during a median follow up of 20 (5.1-82.2) months. In univariate analysis patients with a relapse rate $\geq$ 1 per year showed a higher probability for remission following RTX therapy when compared to patients with < 1 relapse per year ($p=0.017$). The use of immunosuppressive co-medication was also significantly reduced after RTX therapy ($p<0.001$).

**Conclusion.** The data published suggest that RTX seems to be effective in reducing the number of relapses and sparing immunosuppressive medication in frequently relapsing and steroid-dependent nephrotic syndrome due to MCD and FSGS. These promising findings have to be confirmed in prospective studies.

# Metallothioneins as Markers for Biological Organ Age in Preimplantation Kidney Biopsies

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**Background:** Structure and function of the kidney deteriorate with age and age-related diseases contribute to this process, leading to the high frequency of end-stage renal disease in the elderly. The difference between chronological and biological age is a main problem in determining donor kidneys for transplantation. The ideal situation would be to match the functional capacity of a donor kidney to the patient's renal requirements. Therefore the identification of markers for biological organ age is a strong clinical need.

**Methods:** Age-regulated gene expression changes in 37 zero hour kidney biopsies from donors with no clinical signs of AKI at the time of explantation were determined using microarray technology followed by ANOVA and SAM analysis. Expression changes of selected genes were confirmed by quantitative real-time PCR. In situ hybridization was used to localize mRNA expression in zero hour biopsies. Functional aspects were examined comparing cell lines (HK-2, htert-RPTEC) grown in normoxic and hypoxic conditions.

**Results:** Donors were classified into 3 age groups (<40, 40-59, >60 years). In the given Microarray data age-associated transcriptional changes were identified: 16 transcripts were found to be significantly upregulated in age group 3 as compared to age group 1. These transcripts were dominated by genes encoding for metallothionein (MT) isoforms. In situ hybridization demonstrated localization of MT mRNA in renal proximal tubular cells. Cell lines overexpressing MT2A were less sensitive towards hypoxia-induced apoptosis.

**Conclusion:** Metallothionein expression might serve as a marker for biological age in zero hour biopsies. Furthermore our data support the idea that a reduced expression of MT isoforms in elderly kidney predisposes to a reduced anti stress response capacity.

# The Effect of Preservation Solutions HTK, HTK-N and TiProtec on Various Tissues after Rat Hind-Limb Allotransplantation

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**Background:** Ischemia/reperfusion (I/R) injury is an early factor damaging grafts in solid organ and composite tissue transplantation. We herein investigate the effect of the novel preservation solutions HTK-N and TiProtec on tissue preservation and damage in a vascularized composite allotransplantation (VCA) model.

**Methods:** Orthopic hind-limb transplantations were performed in male Lewis-rats following 10h of CI. Limbs were flushed and stored in HTK-N, TiProtec, HTK or NaCl-solution. Skin, muscle, nerve, vessel and bone-samples were taken at the 10th post-operative day (POD) for histology, confocal and electron-microscopy.

**Results:** In the NaCl treated group signs of muscle atrophy were observed at POD 10, which were not found in the other groups. The confocal microscopy of the muscle revealed no significant difference of muscle-cell viability on POD 10 between HTK-N (82,2%), HTK (80,6%) and NaCl (85,4%), whereas TiProtec (61,2%) was inferior. Histology showed a superiority (p=0,08) of HTK in muscle preservation displaying a diffuse inflammatory infiltrate and only localized necrosis contrary to mainly major necrosis in the HTK-N, TiProtec and NaCl groups. In all other tissues no significant differences concerning tissue damage were observed. The majority of skin alterations included a mild inflammatory infiltrate in the dermis and rarely interface reactions, infiltration of the epidermis and sporadic epithelial necrosis. Nerve samples revealed mostly severe perineural inflammatory infiltrate, vacuolization and mucoid degeneration. Vessels showed intact endothelial cells and only a mild infiltrate. Electron microscopy revealed that vessel-preservation was equaly good in all groups.

**Conclusion:** Nerve and muscle are most susceptible to I/R injury in a VCA model. Skin and vessels on the other hand are relatively unaffected by I/R. HTK has the best preservation ability for muscle tissue, which is a major component of a VCA and crucial to gain function after limb transplantation.

# Tetrahydrobiopterin Preconditioning Saves Murine Pancreatic Isografts from Brain Death Exacerbated Ischemia Reperfusion Injury

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**Background:** Brain death (BD) has been associated with an immunological priming of donor organs and is thought to further aggravate ischemia reperfusion injury (IRI). Recently we were able to show that the essential nitric oxide synthase co-factor tetrahydrobiopterin (BH4) prevents IRI following murine pancreas transplantation.

Herein we assessed the impact of donor BD on IRI in a murine model of syngeneic pancreas transplantation and tested the therapeutic potential of BH4 in this clinically relevant setting.

**Methods:** Male C57BL/6 (H-2b) mice were used as size-matched donors and recipients. Cervical heterotopic pancreas transplantation was performed using a modified no-touch technique. Animals were followed for 3h after BD induction, continuously ventilated trough a tracheostomy.

Experimental groups included (n=5 per group): non-treated BD donors (1), pre-treatment of BD donors with 50mg/kg BH4 before organ retrieval (2), ventilated non-treated donors (no BD, sham group) (3), non brain death non-treated donors (4). Following 2 hours of reperfusion, microcirculation (functional capillary density, FCD; capillary diameter, CD) as well as cell viability was assessed by intravital confocal fluorescence microscopy. Parenchymal graft damage was assessed by histology, BH4 levels were determined by HPLC and mRNA expression of inflammatory candidate markers was measured by real-time RT-PCR.

**Results:** BD had dramatic impact on pancreatic microcirculation 2h after reperfusion as highlighted by significantly reduced FCD as well as CD values when compared to controls non brain death ($p<0.05$). Moreover BD induced intragraft mRNA expression levels of IL-1ß, TNFa, IL-6 and ICAM-1. In contrast BH4 treated pancreatic grafts showed significantly improved microcirculation after reperfusion as reflected by significantly higher FCD and CD values ($p<0.001$, respectively). BD significantly impacted cell viability 2h following reperfusion, whereas BH4 treated grafts displayed similar percentages of viable cells in graft biopsies as non brain death controls ($p<0.001$). Early parenchymal damage in pancreatic grafts was significantly more pronounced in organs from BD donors when compared to sham or non brain death donors ($p<0.05$). Pre-treatment with BH4 however significantly ameliorated parenchymal damage in organs from BD donors ($p<0.05$).

**Conclusion:** This study provides in vivo evidence that donor brain death aggravates ischemia reperfusion injury after experimental pancreas transplantation. Donor pre-treatment with BH4 offers a novel therapeutic option in preventing BD exacerbated ischemia reperfusion injury.

# Donor brain death results in differentially modulated immune activation in solid organs

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Background: Donor brain death (BD) and its pathophysiological changes have been shown to influence graft quality, therefore accelerating the immune response post transplantation. However, detailed information regarding immune activation of distinct lymphocyte subsets in the periphery and in BD donor organs is still missing in order to explain enhanced immunogenicity.

Methods: For this purpose, C57BL/6 mice underwent BD induction and were followed for 3 hrs under continuous ventilation, whereas ventilated mice were used as sham group (SH) (n=5).

Results: By cell isolation and flow cytometry, we observed a strong induction of activated CD25+CD3+CD4+ T cells in BD donor derived hearts and kidneys compared with SH ($p<0.05$). Contrarily, an infiltration of cytotoxic CD3+CD8+ T cells was exclusively induced in hearts as a consequence of BD ($p<0.01$). Moreover, we detected enhanced levels of CD3-NKp46+ NK cells in BD donor hearts, livers and kidneys which appeared significantly activated reflected by their CD69 expression. The latter observation is in sharp contrast to CD3+CD4+ T helper or CD3+CD8+ cytotoxic T cells, which did not show induction of CD69 in all investigated organs (spleen, liver, heart, kidney, peripheral blood). Interestingly, an induction of co-stimulatory molecules including CTLA-4 and CD28 on all lymphocytes isolated from peripheral blood and kidneys as a consequence of BD was obvious ($p<0.01$). In addition, all investigated organs displayed higher frequencies of CTLA-4+CD11c+ conventional dendritic (mDCs) cells compared with the SH, whereas the kidney displayed the highest level of MHC class II+ mDCs ($p<0.05$ versus spleen, lymphnodes and liver, respectively). Strikingly, CTLA-4+MHC class II+ PDCA1+ plasmacytoid cells were highly induced indicating their massive infiltration especially in liver, kidney and lymphnodes due to BD ($p<0.05$ compared to SH, respectively). Among B cells, CD19+CD220 mature B cells were clearly diminished, whereas BD results in an induction of CD19+CD220- immature B cells in all organs.

Conclusion: In summary, our results gain novel insights into the pathophysiology of BD induced immune activation revealing significant differences between various organs and the periphery. This indicates distinct mechanisms of activation which needs consideration for future treatment strategies.

# HIV-1 Gene Therapy using Zinc -Finger Nucleases (ZFN) and Transcription Activator Like Effector Nucleases (TALENs)

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**Background**: A broad panel of antiviral drugs has become available for the therapy of HIV infection; however, treatment is not curative. The only patient considered to be cured so far is the “Berlin patient”, who received a bone marrow transplant for leukemia from a donor that was homozygous for a disrupting mutation in the CCR5 gene. As CCR5-defective donors are rare, designer nucleases such as zinc finger nuclease (ZFN) and transcription activator like effector nucleases (TALENs) that disrupt the CCR5 gene were developed. In addition to the targeted knock out of the CCR5 receptor a knock in of a gene encoding the fusion inhibitory C peptide (maC46) would contribute to an overall antiviral effect and help to prevent development of resistant viruses. Here, we tested the efficacy of this approach in vitro as well as the general genotoxicity of ZFNs in a mouse model.

**Methods:** In cell lines, knock out of the CCR5 receptor was determined by T7E1 assay and targeted integration of maC46 was analyzed by PCR. In a worst case scenario, genotoxicity of constitutively expressed ZFNs was studied in a syngenic mouse model. Hematopoietic stem cells from C57BL/6 were isolated and transduced with a lentiviral vector expressing the CCR5-ZFNs. The cells were then transplanted into lethally irradiated recipient mice. Mice were followed for repopulation, ZFN expression and development of Leukemia for several months.

**Results:** CCR5 disruption and targeted transgene integration was shown for different cell lines. Cells transduced with a lentiviral vector constitutively expressing the CCR5-ZFN enriched over time during HIV-1 $^{JR\text{-}CSF}$ infection. Lin -/- bm cells from C57BL/6 expressing constitutively CCR5-ZFN repopulate well and constant expression of ZFN was detected in blood for several months.

**Conclusion:** Our approach described here will help to minimize the risks of stem cell-based gene therapy by targeted integration of an antiviral transgene into a safe harbor. Further studies should allow us to analyze the conditions that determine efficacy and safety of ZFN-based immune/gene therapy for HIV-infection.

# The Viral Vector Vaccine VSV-GP Boosts Immune Response Upon Repeated Applications

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**Background:** Vesicular stomatitis virus (VSV) is a potent candidate vaccine vector for various diseases. However, VSV's inherent neurotoxicity has limited its clinical application. Additionally, VSV induces neutralizing antibodies rapidly and is thus ineffective upon repeated applications. Our group has recently shown that VSV pseudotyped with the glycoprotein (GP) of the lymphocytic choriomeningitis virus, VSV-GP, is not neurotoxic. Here, we evaluated the potential of VSV-GP as a vaccine vector.

**Methods:** We used Ovalbumin (OVA) as a model antigen and analyzed immunogenicity of GP-pseudotyped and wild-type VSV expressing OVA (VSV-GP-OVA and VSV-OVA) in vitro and in vivo in mouse models.

**Results:** Both vectors infected murine bone marrow-derived dendritic cells (bmDCs) in vitro. These bmDCs were able to activate OVA specific $CD8^+$ and $CD4^+$ T cells. Mouse experiments revealed that both VSV-OVA and VSV-GP-OVA induced functional OVA-specific CTLs and anti-OVA antibodies upon single immunization. However, boosting with the same vector was only possible for the GP-pseudotype but not for wild-type VSV. The efficacy of repeated immunization with VSV-OVA was most likely limited by the high levels of neutralizing antibodies, which we detected after the first immunization. In contrast, no neutralizing antibodies against VSV-GP were induced even after boosting. CTL responses induced by VSV-GP-OVA were as potent as those induced by an adenoviral state-of-the-art vaccine vector. Additionally, immunization with both vectors completely protected mice from infection with Listeria monocytogenes expressing OVA.

**Conclusion:** Taken together, VSV-GP is non-neurotoxic, induces potent immune responses, enables boosting and thus is a promising novel vaccine vector.

## LCMV-GP Pseudotyped Oncolytic Vesicular Stomatitis Virus for the Treatment of Prostate Cancer

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**Background:** Prostate cancer is the most common non-skin cancer in men and the second leading cause of cancer death in U.S. and Europe. Diagnosed at early stages, prostate cancer can be surgically removed. However, when it is diagnosed at advanced stage or in case of relapse or metastasis, long-term effective therapies are not available and median survival ranges between 9 and 21 months. Despite many research efforts, treatment and cure of advanced prostate cancer still is an unmet medical need. Oncolytic viruses that preferentially replicate in and kill tumor cells are a potent novel treatment option for cancer patients where conventional therapies have failed. Our group previously reported that oncolytic Vesicular Stomatitis Virus (VSV) pseudotyped with the LCMV glycoprotein (VSV-GP) is an especially promising, highly efficient and safe oncolytic virus. Here, we propose the use of the oncolytic VSV-GP for treatment of the prostate cancer.
**Methods:** We used established prostate cancer cell lines (PC3, Du145, DuCaP, VCaP, LNCaP, 22Rv1) to test the efficacy of oncolytic virus therapy for prostate cancer, using VSV-GP or wildtype VSV. We analyzed both, efficacy of virus-mediated cell killing and production of viral progeny by the infected cells. Mechanisms which might induce resistance to VSV-GP, such as sensitivity to interferon (IFN) or impaired expression of the viral receptor were also studied using these cell lines.
**Results:** The efficacy of VSV-GP treatment was highly variable among the prostate cancer cell lines tested. In PC3 and Du145, high viral titers were produced after 24h of infection. At the same time, almost 100% of the Du145 culture was killed. However, 10% of the PC3 cell culture survived even after 72h of treatment with the oncolytic virus. Analysis of the possible resistance mechanism revealed that PC3 cells develop a more efficient IFN-α induced antiviral response than the other cell lines tested, thus preventing VSV-GP replication and spread. On the other hand, LNCaP, VCaP and DuCaP cells where more resistant to VSV-GP than to wildtype VSV, suggesting a virus entry-related resistance mechanism. However, the observed resistance did not correlate with the expression profile of functional LCMV-GP viral receptor.
**Conclusion:** VSV-GP is a highly potent novel therapeutic for the treatment of prostate cancer. Our results indicate that VSV-GP might have a narrower tropism than wildtype VSV in prostate cancer, although the reason is still not clear. The next reasonable and necessary step is testing VSV-GP using both in vivo models and primary prostate cancer samples.

# Recall responses to tetanus and diphtheria vaccination in elderly persons depend on adequate B cell memory and plasma cells generated earlier in life

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**Background:** Demographic changes and a more active life-style in older age have contributed to an increasing public awareness of the need for lifelong vaccination. However, many older persons have been vaccinated against selected pathogens during childhood but lack regular booster immunizations.

**Methods:** In order to evaluate the impact of regular vaccinations when started late in life the immune response against tetanus and diphtheria following two immunizations with a combination vaccine (Boostrix®) administered at a 5-years interval was analyzed in 87 healthy elderly persons (≥ 60 years).

Results: Protection against tetanus and diphtheria differed substantially at the time of enrollment with 12% and 65% of the participants not being protected against tetanus or diphtheria, respectively. Most vaccinees developed protective antibody concentrations against both antigens 4 weeks after the first vaccination. However, 5 years later antibody concentrations had again dropped below protective levels in 10% (tetanus) and 45% (diphtheria) of the cohort. Protection could be restored in almost 100% after the second vaccination. No correlation between tetanus- and diphtheria-specific responses was observed, and antibody concentrations were not associated with age-related changes in the T cell repertoire, inflammatory parameters, or CMV-seropositivity suggesting that there was no general biological “non-responder type”. Post-vaccination antibody concentrations depended on pre-existing plasma cells and B cell memory as indicated by a strong positive relationship between post- and pre-vaccination antibody concentrations and between post-vaccination antibodies and the number of antibody-secreting cells. In contrast, antigen-specific T cell responses were not or only weakly associated with antibody concentrations.

**Conclusion:** In conclusion, our findings demonstrate that single shot vaccinations against tetanus and/or diphtheria do not lead to long-lasting immunity in many elderly persons even when administered at relatively short intervals. A large enough antigen-specific memory B cell pool and specific plasma cells generated by adequate priming and consecutive booster vaccinations is a prerequisite for long-term protection.

## Austrian Drug Prescription Report 2006-2011: Antibiotics

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**Background:** Antibiotics belong to the most frequently prescribed groups of drugs in outpatient care. This study analyzes the data from all prescriptions at the expense of the Austrian National Health Service down to ATC level 5 (compound) from the years 2006-2011.

**Methods:** Prescription data was obtained from the Austrian National Health Services (Hauptverband der österreichischen Sozialversicherungsträger) with permission from the Austrian Federal Chancellery and was analyzed for numbers of prescriptions, costs, daily drug doses, generics quote and savings potential.

**Results:** The number of prescribed defined daily doses (DDDs) has – after a steady rise – been falling from 2009 to 2011 and halts at 44.2m. Expenses fell from € 78m in 2009 to € 71.9m in 2011, the generics quote rose from 32.6% of the DDDs in 2006 to 39% in 2009 and fell to 37.8% in 2011 – a relevant increase of the generics quote could have saved up to € 15m in 2011. There has been hardly any change in the prescription habits at ATC level 4 (chemical/pharmacological subgroup) from 2006 to 2011 with sometimes major changes among substances within a subgroup in 5-year and 1-year trends. The “Top 5” subgroups by DDD in 2011 were aminopenicillins (17m, 13.6m of which accounted for amoxicilline/clavulanic acid), cephalosporins (10.4m), chinolones (5.2 m) and tetracyclines (3.5m).

**Conclusion:** Our data give a detailed insight into prescription practice of antibiotics in Austrian ambulatory care down to ATC level 5 and demonstrate a trend reversal toward declining costs and prescriptions, but also display a relevant savings potential.

# Effect of Storage Temperature and Antibiotic Impregnation on the Quantity of Bone Morphogenetic Protein-7 in Human Bone Grafts

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**Background:** Human bone grafts are widely used to surgically rebuild skeletal defects. Transmission of infectious diseases can be prevented by performing safety tests routinely applied in bone banks before grafting. Other highly important qualities for human tissue grafts are their biocompatibility and biological functionality. Bone morphogenetic protein-7 (BMP-7), also known as osteogenic protein-1(OP-1), is related to the ability of bone tissue to induce bone formation during remodeling processes. However, no test to quantify the BMP-7 content in bone grafts is carried out in bone banks. The aim of this study was to quantify the amount of BMP-7 in bone samples in different storage conditions used in bone banks and thereby evaluate the benefit of this test as a routine measure before bone grafting.

**Methods:** Fresh as well as frozen bone chips each with and without antibiotic coating were screened for their content in BMP-7. Human bone chips were produced from femoral heads of two female donors who had undergone total hip replacement surgery. The amount of BMP-7 was detected using a commercially available ELISA-test.

**Results:** There were no significant differences between samples of the four groups obtained from the first femoral head. Bone chip samples derived from the second femoral head showed significant differences between the four groups. The actual amount of these differences in BMP-7 however is small enough to make the variance biologically irrelevant. There also were significant differences in the amount of BMP-7 comparing both donors. It is important to note that there was a significant difference comparing the groups between both femoral heads reflecting donor to donor variability. Further, the results showed that antibiotic coating as well as freezing temperature (-80°C) did not significantly influence the amount of BMP-7.

**Conclusion:** ELISA-testing for BMP-7 as a measure to characterize the osteoinductive ability of bone grafts should be considered as a routine quality control test for bone banks.

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