

# **39th Annual Meeting of the Arbeitsgemeinschaft Dermatologische Forschung (ADF)**

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P001 (V01)

**Administration of CCL18 in humanized mice prevents allergic airway inflammation and hyperresponsiveness**

I. Bellinghausen<sup>1</sup>, S. Reuter<sup>2</sup>, H. Martin<sup>2</sup>, J. Maxeiner<sup>2</sup>, S. Grabbe<sup>1</sup>, C. Taube<sup>2</sup> and J. Saloga<sup>1</sup> <sup>1</sup>Department of Dermatology, University Medical Center, 55131 Mainz, Germany; <sup>2</sup>Department of Pulmonary Medicine of the III, Medical Clinic, University Medical Center, 55131 Mainz, Germany. The dendritic cell (DC)-derived chemokine CCL18 is constitutively expressed in human serum and up-regulated in several inflammatory diseases including allergic asthma. However, in absence of a counterpart, *in vivo* studies regarding the effects of CCL18 in mouse asthma models are not possible. This study was set out to investigate the role of CCL18 not only *in vitro* but also *in vivo* using a recently developed humanized mouse model of allergic asthma. For *in vitro* experiments, CCL18 was added to co-cultures of CD4+ T cells from grass pollen, birch pollen or house dust mite allergic donors and autologous monocyte-derived allergen-pulsed mature DC. For *in vivo* studies, human PBMC from allergic donors together with the respective allergen in the presence or absence of CCL18 were injected intraperitoneally in NOD-Scid- $\gamma$ - $\gamma$ - mice. Addition of CCL18 inhibited the allergen-specific production of IL-4 and IL-5 by DC-stimulated CD4+ T cells, while IFN- $\gamma$ , IL-10 and T cell proliferation were not affected. *In vivo*, CCL18 inhibited airway hyperresponsiveness and lung inflammation measured 48 h after intranasal allergen challenge, while addition of another Th2-associated chemokine, CCL17, had no effect. Chemotaxis assays revealed that CCL18 preferentially attracted Treg and less efficiently T effector cells. These data demonstrate that CCL18 may represent a molecule of significant importance in immunoregulation with therapeutic potential in allergic inflammation.

P002 (V13)

**Fc epsilon R1-mediated mast cell reactivity is amplified through prolonged Toll-like receptor-ligand treatment**

R. Saluja<sup>1,2</sup>, I. Delin<sup>3</sup>, G. Nilsson<sup>1</sup> and M. Adner<sup>3</sup> <sup>1</sup>Clinical Immunology and Allergy Unit, Department of Medicine, Karolinska Institutet, 171 76 Stockholm, Sweden; <sup>2</sup>Department of Dermatology and Allergy, Charité – Universitätsmedizin, 10117 Berlin, Germany; <sup>3</sup>Karolinska Institutet, Experimental Asthma and Allergy Research, The Institute of Environmental Medicine, 17176 Stockholm, Sweden. Mast cells play a central role in allergic asthma, contributing to both bronchoconstriction and airway inflammation. Microbial infections induce asthma exacerbations in which the contribution of mast cells remains obscure. Here we have investigated the effect of Toll-like receptor ligand treatment on IgE-receptor mediated mast cell reactivity. For the studies we employed *in vitro* differentiated connective tissue like mast cells (CTLMC) and mucosal like mast cells (MLMC) from C57BL/6 mice. Both phenotypes were treated for 24 h or 96 h with specific TLR ligands; LPS (TLR4), poly(I:C) (TLR3), Pam3CSK4 (VPGVG)4VPGKG (TLR1/2) and FSL-1 (TLR2/6), before sensitization with IgE and activation with antigen. We found that prolonged exposure with TLR-ligands promotes mast cell reactivity following IgE-receptor activation, assessed as increased release of beta-hexosaminidase, cysteinyl-leukotrienes, leukotriene B4, interleukin-6, monocyte chemoattractant protein-1 and macrophage inflammatory protein-1. The effect was obtained in both CTLMC and MLMC, with the latter releasing the highest amount of mediators. The effect of LPS, the TLR ligand that induced the most pronounced effect, was mediated through a MyD88- and JNK-dependent pathway. Thus, prolonged exposure of mast cells to pathogens/TLR-ligands modulates their effector responses by priming them for increased release of several inflammatory mediators when activated by IgE-receptors. These data suggest that infections might exaggerate the severity of allergic reactions by enhancing mediator release from mast cells.

P003

**Adjuvant activity of Gram-positive bacteria on grass pollen**

B. Heydenreich<sup>1</sup>, I. Bellinghausen<sup>1</sup>, W. Becker<sup>2</sup>, S. Grabbe<sup>1</sup>, A. Petersen<sup>2</sup> and J. Saloga<sup>1</sup> <sup>1</sup>University Medical Center, Department of Dermatology, Mainz, Germany; <sup>2</sup>Research Center Borstel, Clinical and Molecular Allergology, Borstel, Germany. Recently, it has been established that pollen grains contain Th2-enhancing activities besides allergens. The aim of this study was to analyze whether pollen carry additional adjuvant factors like microbes and which immunologic effects they may exert. Timothy pollen grains were collected, disseminated on agar plates, and the growing microorganisms were cultivated and defined. Furthermore the immunologic effects of microbial products on DC and T cell responses were analyzed. A complex mixture of bacteria and moulds was detected on grass pollen. Besides Gram-negative bacteria that are known to favour Th1-directed immune responses, moulds were identified being sources of allergens themselves. Here, we focused on Gram-positive bacteria which were found in high numbers, e.g. *Bacillus cereus* and *Bacillus subtilis*. Contact of immature dendritic cells (DC) from grass pollen allergic donors with supernatants of homogenized Gram-positive bacteria induced maturation of DC as measured by up-regulation of CD80, CD83, and CD86 and by enhanced production of IL-6, IL-12p40, and TNF- $\alpha$ , which was less pronounced compared to effects induced by LPS. Consequently, stimulation of autologous CD4+ T cells with supernatants of homogenized Gram-positive bacteria plus grass pollen allergen-pulsed DC led to an enhanced proliferation and production of IL-4, IL-13, IL-10, IL-17, IL-22, and IFN- $\gamma$  production compared to T cells which were stimulated with allergen-pulsed immature DC alone, while production of the transcription factor for regulatory T cells, FoxP3, was not significantly affected. These data indicate that grass pollen are colonized by several microorganisms which influence the immune response differently. Similar to LPS, supernatants of homogenized Gram-positive bacteria may serve as adjuvants by augmenting DC maturation and inflammatory Th1, Th2, and Th17 responses helping to initiate allergic immune reactions.

P004

**T cell killing by tolerogenic dendritic cells protects from allergy in mice**

T. Schmidt<sup>1</sup>, U. Luckey<sup>1</sup>, M. Maurer<sup>2</sup>, N. Lorenz<sup>1</sup>, B. de Jongh<sup>2</sup>, M. Metz<sup>2</sup> and K. Steinbrink<sup>1</sup> <sup>1</sup>Department of Dermatology, University Medical Center, Johannes Gutenberg University Mainz, Mainz, Germany; <sup>2</sup>Allergie-Centrum-Charité, Department of Dermatology and Allergy, Charité – Universitätsmedizin Berlin, Berlin, Germany. Low zone tolerance (LZT) to contact allergens is concerned as a physiological mechanism for the regulation and curbing of allergies. The tolerance reaction is induced by epicutaneous applications of subimmunogenic doses of haptens resulting in the generation of CD8+ suppressor T cells, which in turn prevent the development of Tc1-mediated contact hypersensitivity (CHS). Previous studies revealed significantly elevated levels of the tumour necrosis factor (TNF) during the course of LZT. Thus, in the present study, the functional relevance of TNF in LZT was analyzed using

TNF-deficient (TNF- $\alpha$ ) as well as TNF receptor-deficient (p55- $\alpha$  / -; p75- $\alpha$  / -; p55- $\alpha$  / -p75- $\alpha$  / -) mice. We observed that all knockout strains, except for p55- $\alpha$  / -, failed to develop a LZT reaction, indicating that TNF and its receptor p75 are critical for the development of LZT. Surprisingly, TNF- $\alpha$  and TNF receptor p75 signaling is not required for the induction and function of CD8+ suppressor T cells of LZT. In order to identify the major source of LZT promoting TNF, flow cytometry analysis identified CD11+CD8+ dendritic cells (DCs) located in the skin-draining lymph nodes as TNF-producers in LZT. Subsequent experiments showed that TNF induced signalling via TNF receptor p75 is essential for the effector phase of LZT and that CHS effector CD8+ T cells exhibited a high expression of the TNF receptor p75. As TNF is a well-known inducer of apoptosis, the role of apoptotic signalling was analyzed. Importantly, adoptive transfer experiments combined with the use of different congenic mice strains revealed a significant increase in the percentage of apoptotic CD8+ CHS effector T cells during the effector phase of LZT as compared to CHS. In addition, DC-derived TNF- $\alpha$  induced apoptosis via TNF receptor p75 in hapten-specific CD8+ effector T cells of CHS is critical for LZT and inhibition of the T cell-dependent allergic inflammation. Furthermore, activation of tolerogenic DCs by CD8+ suppressor T cells of LZT and enhanced TNF receptor 2 expression by contact allergy effector CD8+ T cells are required for LZT.

In conclusion, CD8+CD11c+ killer DCs activated by LZT suppressor CD8+ T cells induce TNF-driven apoptosis in haptenVspecific CHS effector CD8+ T cells via p75, thereby preventing the development of allergic skin inflammation.

P005

**Role of CD4+CD25+FOXP3+ regulatory T cells and CD8+ suppressor T cells in low zone tolerance to allergens**

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The development and mechanisms of tolerance to allergens are poorly understood. Low zone tolerance (LZT) to contact allergens, induced by topical application of subimmunogenic doses of haptens, results in the generation of CD8+ suppressor T cells, which prevent the development of contact hypersensitivity (CHS). However, the precise mechanisms of LZT are not yet understood. In this study, the role of CD4+CD25+Foxp3+ regulatory T cells (Tregs) and their interactions with CD8+ T cells in LZT were analyzed.

We demonstrated that depletion of Tregs during tolerization (by use of anti-CD25-Ab and in DEREG mice) completely abolished the development of LZT resulting in a pronounced CHS response (significant ear swelling, hapten-specific T cell proliferation, Tc1 cytokine pattern). Adoptive transfer experiments of CD8+ T cells generated in the absence of Tregs during induction of LZT revealed that activation of Tregs is required for the generation of CD8+ suppressor T cell which prevent the development of the allergic skin inflammation (CHS). In order to investigate Tregs in more detail, flow cytometry analysis demonstrated a significantly higher percentage of CCR7+ Tregs and increased expression of CD103 on Tregs during the induction phase of LZT as compared to CHS. In addition, a flow cytometry-based method was used to analyze T cell proliferation and to identify particular activated T cell subpopulations. Labelling of T cells with the fluorescent CellTrace™ Violet enabled us to investigate the hapten-specific proliferation of CD4+, activated CD4+CD25+ T cell populations and CD4+CD25+Foxp3+ Tregs, respectively. After hapten-specific restimulation, a higher percentage of proliferating CD4+ and CD4+CD25+, effector T cells as well as of Tregs, characterized as CD4+CD25+ high or CD4+CD25+Foxp3+ cells, was observed in CHS as compared to LZT. Thus, activated and proliferating CD4+CD25+Foxp3+ Tregs are involved in both, LZT and CHS. Subsequent studies which combine this assessment of T cell proliferation with analysis of surface molecules and cytokines will allow for characterization of defined T cell subpopulations in hapten-specific immune responses.

P006 (V16)

**CD13 is a negative regulator of Fc $\epsilon$ R1 dependent mast cell activation *in vitro* and anaphylaxis *in vivo***

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Anaphylaxis is caused by degranulation of mast cells (MCs) following antigen dependent cross linking of specific IgE antibodies bound to the high affinity receptor for IgE (Fc $\epsilon$ R1). Receptors for type I allergic reactions today focus on mediator induced symptoms rather than the process of degranulation. Moreover, targeting the signaling pathways downstream of Fc $\epsilon$ R1 may provoke severe side effects as constituents of these pathways are often promiscuous and indispensable for other processes. Thus, the most specific therapeutic strategies for type I allergic reactions should aim to intervene with the process of the activation of the Fc $\epsilon$ R1 receptor at the cell membrane. Plasma membrane receptors like Fc $\epsilon$ R1 are frequently associated with partner proteins (co-receptors) that fine-tune the main receptors signaling strength and specificity. Using a proteomic approach (immunoprecipitation/mass spectrometry) we identified an interaction between Fc $\epsilon$ R1 and CD13 (aminopeptidase N) on murine MCs. We confirmed that CD13 is expressed on the surface of MCs as well as on intracellular compartments and antigen-triggered activation results in co-capping and co-internalization of Fc $\epsilon$ R1 and CD13. Moreover, antibody-mediated crosslinking of CD13 causes IL-6 production in an Fc $\epsilon$ R1-dependent manner. These data are indicative of a functional interaction between Fc $\epsilon$ R1 and CD13 on MCs. Accordingly, antigen stimulation of CD13-deficient MCs results in increased degranulation and proinflammatory cytokine production compared to wild-type cells. To investigate if these observations are functionally relevant *in vivo*, mice were passively sensitized with anti Dinitrophenol (DNP)-IgE and challenged with titrated doses of DNP to determine the threshold dose of DNP eliciting strong systemic anaphylaxis. Anaphylaxis was measured by detecting the correlating core body temperature decrease. Challenge with the DNP dose just below threshold results in a temperature drop of -2.51,21°C. In contrast, pretreatment of sensitized mice with Bestatin, a specific inhibitor of CD13, followed by challenge with DNP, leads to a highly significant, dose dependent, strong temperature drop of -3,761,28°C (Bestatin 2 mg) and -5,281,07°C (Bestatin 4 mg). CD13-deficient mice develop an even stronger anaphylactic reaction with a temperature drop of -9,451,33°C. In conclusion, we have identified a novel negative regulator of MC activation *in vitro* and of anaphylaxis *in vivo* and further analyses will focus on CD13 mediated inhibition of Fc $\epsilon$ R1 induced degranulation as novel therapeutic strategy.

## P007 (V24)

**In vivo analysis of adjuvant effects of a low molecular-weight, non-protein fraction of aqueous birch pollen extracts**

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Background: A recent study revealed that a non-allergen-containing fraction of aqueous birch pollen extracts (APE) contains micromolar concentrations of adenosine. This was shown to differentially impact on the potential of dendritic cells of atopic and non-atopic donors to induce regulatory T cells. Using skin prick testing as model system, we aimed at analyzing the effect of pollen-derived adenosine on the allergic elicitation phase.

Methods: A protein-free, low molecular weight fraction of APE (APE < 3 kDa) was generated by ultra-filtration using a 3 kDa cutoff centrifugal device. Birch pollen-allergic patients were pricked on the forearm with an allergen-containing fraction of APE, supplemented with either PBS or the low molecular weight fraction of APE. Wheal and erythema size were measured. In the same patients, skin prick testing with allergen plus different concentrations of adenosine were performed. Additionally, basophil activation tests and release of beta-hexosaminidase and histamine from the human mast cell line LAD2 were performed using APE fractions and adenosine.

Results: The non-allergic fraction of APE alone did not induce any skin reaction in healthy or birch pollen-allergic patients (n = 4). Pricking of patients with the allergen fraction of APE resulted in wheals and erythemas of variable size. When prick testing was performed with the allergen fraction of APE supplemented with the non-allergic fraction, wheal and erythema sizes were significantly larger than with the allergen fraction alone. This result was reproduced for different pollen specimens as well as for two different time-points (repetitive prick testing). Similarly, adenosine (100 M) showed an aggravating effect on wheal size. In cellular assays, neither APE (0.03–10 mg/ml) nor adenosine alone induced any activation. However, high concentrations of APE (30 mg/ml) lead to antigen-independent activation of basophils. Pre-stimulation of basophils with 0.1 g/ml rBet v 1 and subsequent stimulation with APE < 3 kDa or adenosine resulted in enhanced basophil activation. Concurrently, LAD2 mast cells did not respond to stimulation with APE or adenosine in the absence of FcεRI cross-linking. However, LAD2 stimulated with IgE/anti-IgE in the presence of APE < 3 kDa released slightly increased amounts of beta-hexosaminidase than control-stimulated LAD2. Furthermore, APE < 3 kDa and adenosine differentially modulated the histamine release of LAD2, depending on concentration and time-point of stimulation.

Conclusion: Both low molecular-weight APE and adenosine exert an aggravating effect on the cutaneous allergic response to pollen allergen. Our results imply that this might be due to enhanced degranulation of cutaneous mast cells/basophils triggered by pollen-derived adenosine.

## P008

**Specific immunotherapy in the house dust mite mouse model of allergen induced airway inflammation**

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Allergen-specific immunotherapy (SIT) represents the only causal treatment for immediate type allergies. While highly effective for some allergens, SIT for perennial allergens such as HDM is less effective and still offers room for improvement.

Dermatophagoides pteronyssinus und D. farinae are two house dust mite species being the main causative agent for HDM based allergic diseases. The group I allergens are cysteine proteases, showing more than 80% sequence identity between Der p 1 and Der f 1. Due to the protease activity and its impact on several innate activation/inflammation mechanisms natural Der p 1 was considered to be less suitable for tolerance induction, as compared to enzymatically inactive Der p 1. By using recombinant pro Der p 1, an inactive pro-form lacking the protease activity, the effect of high dose specific immunotherapy was examined in a preventative and a therapeutic setting. Repeated intranasal allergen administration prior sensitization led to a significant reduction of airway inflammation upon allergen challenge and to a reduction of Th2 cytokines after allergen-specific restimulation of mediastinal lymph node cells. Similarly, tolerance induction was obtained in the therapeutic setting (i.e. after sensitization) after repeated subcutaneous allergen application. Furthermore we asked if pro Der f 1 administration can induce tolerance in Der p 1 induced allergic lung inflammation. First results indicate that – despite of high sequence homology – pro Der f 1 fails to induce tolerance and even partial tolerance can not be achieved in the therapeutic setting in C57BL/6 mice. Sequence analysis of Der p 1 and Der f 1 revealed four different amino acids in the main CD4 T cell epitope (aa 113–127; Harris et al.) A) demonstrating that this epitope is the dominant T cell epitope in C57BL/6 mice and B) suggesting that it is crucial for tolerance induction in this setting.

## P009

**A high-throughput screening assay for detection of auto-IgE**

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IgE-mediated atopic diseases [hay fever, allergic asthma and atopic eczema (AE)] affect about 25% of the German population. Recently, autoreactive IgE has been detected in patients with AE and is suspected to play a role in disease aggravation and chronicity. To date, the prevalence of auto-IgE has not been determined on epidemiological level and was mainly studied in keratinocytes or the A431 carcinoma cell line, but far less in airway cells. It is still unclear, whether auto-sensitization occurs as an immunological complication due to chronic allergic inflammation in adults, or whether it is already present in children. Aim of this study was to establish a rapid screening assay that allows the analysis of autoreactive IgE against primary keratinocytes and airway epithelial cells in sera from well-characterized birth-cohorts.

Methods: To compare protein yield and the impact of the extraction process on IgE-epitopes, HaCaT cells (a human keratinocyte cell line), as well as primary keratinocytes (pKc) from healthy and AE-donors, were grown to 70–80% confluence and whole protein was extracted using different buffers (RIPA/Laemmli). Moreover, we compared the impact of the protein amount bound to 96-well plates on the detectability of auto-IgE. Patient sera were incubated overnight. After washing with different washing buffers, bound IgE was detected by a peroxidase-conjugated secondary goat-anti human IgE mAb at 450 nm. We tested 185 AE patients plus controls (10 month–70 years.) with optimized assay conditions.

Results: Thirty-one percent of all patients showed IgE-activity towards HaCaT cells. Interestingly, the highest OD was detected in children at low age (1–10 years.). Also, in 17% of adults, auto-IgE was detected. Human pKc from healthy donors and AE-patients bound more auto-IgE than HaCaT cells.

Conclusion: In this study we identified auto-IgE at a similar percentage as reported previously. Furthermore, auto-IgE might be an early immunological feature of AE in young children. Outlook: Current studies in birth-cohorts address the kinetics of auto-IgE-levels against primary keratinocytes and airway epithelial cells in relation to age and disease severity.

## P010

**Evaluation of the sensitizing potential of antibiotics in vitro using the human cell lines THP-1 and MUTZ-LC and primary monocyte – derived dendritic cells**

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Since the 7th amendment to the EU cosmetics directive foresees a complete ban on animal testing, alternative *in vitro* methods have been established to evaluate the sensitizing potential of cosmetics ingredients. To find out whether these novel *in vitro* assays are also capable to predict the sensitizing potential of drugs, model compounds such as beta-lactams and sulphonamides – which are the most frequent cause of adverse drug reactions – were co-incubated with THP-1, MUTZ-LC, or primary monocyte-derived dendritic cells for 48 h and subsequently expression of selected marker genes (IL-8, IL-1β, CCL20, NQO1, GCLM, PIR and TRIM16) was studied by real time PCR. We found that benzylpenicillin and phenoxymethylpenicillin are capable to induce the mRNA expression of these genes in mDCs and except for IL-8 in THP-1 cells but not in MUTZ-LC. Ampicillin stimulated the expression of some marker genes in mDCs and THP-1 cells. SMX did not affect the expression of these genes both in THP-1 and mDCs except for PIR. These data reveals that novel *in vitro* DC based assays might play a role also in the evaluation of the allergenic potential of novel drug compounds but these systems seem to lack the ability to detect the sensitizing potential of prohapten that require metabolic activation prior to sensitization.

## P011

**IL-31 abrogates terminal cell differentiation and skin barrier function in human epidermal structures**

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Atopic dermatitis (AD) is an inflammatory skin disease affecting 10–20% of children and 1–3% of adults in industrialized countries. Enhanced expression of IL-31 is detected in skin samples of AD patients, but its functional role in the pathogenesis of AD is not completely understood. In this study we analyzed the effect of IL-31 on keratinocyte differentiation and barrier function in 3D organotypic skin models using NHEK or HaCaT cells with inducible IL-31RA. IL-31 treated 3D skin equivalents elicited a differentiation defect, associated with statistically significant reduced epidermal thickness, disturbed epidermal constitution and stratum basale, and poor development of the stratum granulosum. The differentiation defect was associated with a profound repression of terminal differentiation markers, including filaggrin, an essential factor for skin barrier formation. Functional studies in IL-31 treated 3D skin models therefore revealed a highly increased uptake of fluorescence labeled recombinant allergens (Pnl p1, Fel d1) and an increased release of IL-12 or IL-8 after topical challenge with 0.2% SDS in comparison to untreated controls.

We identified IL20 and IL24 as well as EGR1, IRF1, GADD45B, JUNB and DUSP1 as target genes of IL-31 and could demonstrate that release of IL-20 and IL-24 in keratinocytes is responsible for part of the effect on FLG expression and thus for terminal differentiation.

Our study defines a novel function of IL-31 as an important regulator of keratinocyte differentiation and demonstrates a link between the presence of IL-31 in skin, as found in patients with AD, and filaggrin expression and disturbed barrier function.

## P012

**Meta-analysis of genome-wide association studies on atopic dermatitis identifies three novel risk loci**

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Atopic dermatitis (AD) is a common chronic skin disease with high heritability and complex mode of inheritance. Apart from filaggrin (FLG), the genes influencing AD are largely unknown. We conducted a genome-wide association meta-analysis of 5606 cases and 20 565 controls from 16 population-based cohorts and followed up the ten most strongly associated novel markers in a further 5419 cases and 19 833 controls from 14 replication studies. Three SNPs among the 10 were significant in the replication cohorts combined analysis, and met genome-wide significance in the discovery and replication cohorts combined. Two of these variants are located in genes which have been implicated in epidermal proliferation and differentiation, while one is located within the cytokine cluster on 5q31.1. Fine-mapping and conditional analyses indicated that there are two distinct signals at this locus, both of which were associated with transcript levels of IL13. We also replicated the FLG locus and two recently identified association signals at 11q13.5 and at 20q13.3. Our results underline the importance of both epidermal barrier function and immune dysregulation in AD pathogenesis.

P013

### Tolerance induction in hymenoptera venom-allergic patients undergoing ultra-rush immunotherapy and sting-challenge

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While the clinical efficacy of allergen-specific immunotherapy (SIT) is well documented, the underlying immune mechanisms are not yet fully understood. In this study we determined B- and T-cellular immune responses in patients suffering from immediate type allergy against hymenoptera venom (HV) who were treated by SIT with either wasp or bee venom extract. Peripheral blood was drawn on 10 different time-points: the day before initiation of SIT (t1), during the 3 days of build-up phase (t2–4), after 2, 4 and 12 weeks of maintenance phase (t5–7), directly before (t8) and one (t9) as well as 14–21 (t10) days after sting challenge. To analyze the humoral immune response, specific IgE and IgG4 antibodies (abs) against the recombinant major allergen Ves v 5 (common wasp) or Api m 1 (honey bee) were measured. In addition, CD4+CD25+CD127low T cells representing Foxp3+ regulatory T (Treg) cells were quantified using FACS analysis. While there was no significant change of allergen-specific IgE abs during build-up and maintenance phase of SIT, an early increase of IgG4 abs was noticed. Interestingly, during the weeks after sting challenge SIT-treated patients tolerating natural allergen exposure did not show a decrease of allergen-specific IgE, which is often seen in HV allergic patients, but rather an increase of IgG4 abs. On the cellular level, CD4+CD25+CD127low Treg cell numbers initially diminished after initiation of SIT but started to reappear by t7. Similarly, a temporary drop of Treg cells was noticed directly after sting challenge. Our data thus show that tolerance induction in HV allergic patients is influenced by a dynamic interplay of both B and T cells in which allergen-specific IgG4 abs and Treg cells might play a major role.

P014

### Long-term efficacy of specific immunotherapy: functional *in vitro* characterization of IgG 'blocking' antibodies

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Allergen-specific immunotherapy (SIT) is still the only causal therapy to treat immediate type allergies. Furthermore, several studies have shown that allergic patients treated by SIT experience an ongoing therapeutic effect for at least up to five years after completion of therapy. To better understand the role of immunological changes induced by SIT, we followed the humoral response of birch pollen allergic patients up to 66 months after initiation of therapy. SIT-induced alterations in allergen-specific serum immunoglobulin concentrations were determined by both ImmunoCap (IgE, IgG, IgG4) and western blotting (IgG1, IgG4). Compared to pre-treatment time points birch pollen-specific IgE levels did not change significantly during the entire observation period. In contrast, a continued increase of birch pollen-specific IgG and IgG4 antibody synthesis was noticed during SIT. However, although antibody concentrations of both, IgG and IgG4, started to decline after termination of SIT they still remained above pre-treatment levels. Similarly, ratios of birch pollen-specific IgE/IgG and IgE/IgG4 decreased significantly during SIT, while showing opposite tendencies in the follow-up period. Monitoring the patients' sera by western blotting using birch pollen extract, natural and recombinant Bet v 1 revealed that increases of IgG were not only due to IgG4 but also to the IgG1 isotype fraction. Again, levels of each of the immunoglobulins decreased in parallel in a time-depending manner after discontinuation of SIT. Since the role of 'blocking' IgG antibodies in promoting allergen tolerance by SIT is still a matter of debate, we analyzed potential functional properties by determining the influence of SIT-induced allergen-specific IgG antibodies on IgE binding to allergen and allergen uptake mediated by the low affinity IgE-receptor CD23 on APC. Using facilitated allergen presentation (FAP) and the IgE blocking factor assay we could demonstrate that SIT-induced increases of IgG antibodies in patients' sera coincided with enhanced inhibition of allergen binding to IgE and reduced allergen uptake by B cells. Of note, after terminating SIT the decrease of IgG antibody titers was accompanied by diminished IgE-blocking effects. To elucidate solely the role of IgG in blocking IgE-allergen interactions, allergen-specific IgG antibodies were depleted in plasma of SIT-treated patients. Depletion of IgG antibodies led to an abrogation of the inhibitory capacity in the plasma samples as determined by analysis of the IgE blocking factor. These data point to a potential role of IgG antibodies in establishing allergen tolerance in patients treated by SIT. Thus, maintenance of elevated titers of allergen-specific IgG antibodies induced by SIT might support long-term tolerance by blocking allergen-IgE-interactions and inhibiting IgE-mediated allergic reactions.

P015

### Anti-inflammatory effects of a non-anticoagulant polyglycerolsulfate in cutaneous inflammation

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Heparin, a classical anticoagulant and antithrombotic agent, has been shown to exert anti-inflammatory effects in animal models and clinical trials. However, the use of the polysulfate heparin as an anti-inflammatory drug is limited due to its anticoagulant properties. Here, we investigated the effect of a non-anticoagulant dendritic polyglycerolsulfate (dPGS) on inflammatory responses using passive systemic anaphylaxis (PSA), passive cutaneous anaphylaxis (PCA) and contact hypersensitivity (CHS) in mice as models. To first test whether dPGS affects type I allergic reactions, mice were subcutaneously (s.c.) injected with dPGS (30 mg/kg body weight) 10 min prior to the elicitation of PSA. Vehicle-treated mice had a mean temperature drop of 3.5, 0.2°C 20 min after the induction of PSA, while that of dPGS-treated mice was only 1.6, 0.3°C ( $P < 0.005$ ). Subsequent measurements of serum mouse mast cell (MC)-protease-1 (mMCP-1) levels confirmed that MC degranulation was markedly reduced in dPGS-treated mice when compared to vehicle-treated animals (6.01.1 pg/ml vs 11.82.2 pg/ml,  $P < 0.05$ ). Interestingly, we also found that MC incorporate dPGS as assessed by confocal microscopy and by FACS analysis (21.96%, 0.67% dPGS-Cy3+ cells after 30 min). To determine the effects of MN8001 on cutaneous inflammation, mice were s.c. injected with dPGS at different time points prior to the elicitation of PCA or CHS. Mice injected with dPGS 48 h and subsequently 24 h, but not 10 min, before challenge showed markedly reduced ear swelling (66% reduction,  $P < 0.05$ ) 1 h after challenge when compared to vehicle-treated mice. Also mice that had received daily s.c. injections of heparin or dPGS showed reduced CHS-associated ear swelling (50% reduction,  $P < 0.05$ , and 40% reduction,  $P < 0.02$ ) when compared to vehicle-treated mice. Though T cells were shown by confocal microscopy to incorporate dPGS *in vitro*, hapten-specific T cell proliferation was not impaired *ex vivo*, and a single dose of dPGS prior to sensitization did not affect ear swelling responses, both indicating that dPGS has no significant impact on sensitization. In contrast, multiple-dose injections after sensitization resulted in significantly reduced ear swelling ( $P < 0.5$ ), pointing towards effects in the effector phase of CHS. Finally, we tested the role of dPGS in a non-allergic, non-immunological model of neurogenic skin inflammation. Mice were s.c. injected with dPGS 10 min prior to the topical application of capsaicin. Ear swelling responses in dPGS-treated mice were reduced by 62% ( $P = 0.0002$ ) compared to vehicle. Taken together, dPGS potently reduces allergic as well as non-immunological skin inflammation. Additional investigations are needed to further identify the exact mechanisms of action and to characterize the treatment potential of the substance for cutaneous inflammation in humans.

P016

### Wasp venom immunotherapy: analysing effector mechanisms of CD4+CD25hiCD127low regulatory T cells by differential gene expression

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Background: There is a broad body of evidence that the induction of regulatory T cells (Treg) in the course of hymenoptera venom immunotherapy (VIT) plays a major role in leading to long-lasting immune tolerance to venom allergens.

Objective: We seeked to unravel Treg effector mechanisms induced by wasp VIT on a molecular level by means of differential gene expression.

Materials and Methods: CD4+CD25hiCD127low Treg of five age-matched wasp venom allergic patients were electromagnetically isolated from peripheral blood mononuclear cells (PBMC) before and 1 month after the onset of wasp VIT. To screen for the genes specifically induced or repressed in Treg by VIT, RNA was isolated and reverse transcribed into cDNA, followed by one-color hybridization on Agilent Whole Human Genome Microarrays (Miltenyi Biotec).

Results: Three thousand two hundred and fifty-nine genes were differentially regulated in the Foxp3+ Treg population ( $P < 0.05$ ). Among those, 406 were  $\geq 1.3$ -fold higher and 334 were  $\geq 1.3$ -fold weaker expressed after 1 month VIT as compared to the pre-immune status. Functional and pathway analysis of the differentially expressed genes revealed biological processes involved in T cell immunity as well as cell proliferation and differentiation. Genes upregulated included e.g. protein kinase C gamma (PKC- $\gamma$ ), cytokine inducible SH2-containing protein (CISH), receptor-type tyrosine-protein phosphatase U (PTPRU), and cyclin-dependent kinase 1 (CDK1) in conjunction with cell cycle checkpoint cyclins B1, B3 and A2. Interestingly, activators of the WNT/ $\beta$ -catenin signalling pathway, wingless-type MMTV integration site family member 3 and 9B (WNT3, WNT9B), were also induced. Since the activity of the central WNT pathway molecule  $\beta$ -catenin has been shown to prolong Treg survival and in consequence maintained suppression, it is tempting to speculate that the WNT signalling pathway might contribute to the VIT-mediated induction of Treg suppressive activity.

Conclusions: RNA Microarray data hints to an enhanced effector phenotype of CD4+CD25hi-CD127low Treg in the course of VIT.

P017

### Prompt mobilization of high TNF- $\alpha$ producing slan (6-sulfo LacNAc) dendritic cells by psychosocial stress in patients with extrinsic atopic dermatitis

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Psychosocial stress is one of the main trigger factors of atopic Dermatitis (AD) in children and adults. Inflammatory dendritic cells (DCs) are regarded as important drivers of the T cell mediated immune mediated inflammation in AD. However, it is not known, how psychosocial stress can potentially lead to flares of the eczema. We recently described slanDCs as strong inducers of pro-inflammatory T cell responses (Th1 and Th17) and identified these cells at high frequency in lesional psoriatic skin. In this study we asked, for the relevance of slanDCs in AD in general and with specific reference to psychosocial stress. Within the dermal inflammatory infiltrate of lesional AD we identified increased numbers of proinflammatory DCs selectively expressing 6-sulfo LacNAc (slan). Investigating the patients blood, slanDC showed a significantly higher expression of CD86 in comparison to healthy donors. SlanDCs produce high amounts of IL-12 and TNF- $\alpha$  in patients and controls, while monocytes appeared suppressed in patients with AD. In addition, we asked whether the frequency and the function of slanDCs can be modulated by the highly standardized Trier stress test (TSST). The TSST is a psychological procedure that allows experimenters to induce stress under laboratory conditions. We therefore performed the TSST in thirteen patients with AD, who have house dust mite specific IgE-levels and were prick test positive for the respective antigen. The highest cortisol levels were determined ten minutes after the TSST which thereafter gradually decreased to almost normal levels. The overall frequency of slanDCs in the blood of patients with steady state AD was similar to that of healthy controls. Following the TSST the frequency of slanDCs rapidly increased from 1.8% to 2.4%, with a peak one minute after TSST. The high frequency of slanDCs was short lived and values significantly decreased within the following 10 min but were still increased 1 h after the TSST. Studying the TNF- $\alpha$  production of slanDCs among PBMC isolated before and after TSST we observed that the mobilized slanDCs retained their particularly high capacity to produce TNF- $\alpha$  expression, as measured on the single cell level by FACS-analysis. Taken together, slanDCs are a novel population of inflammatory dermal DCs in psoriasis, express increased levels of CD86 in blood, are promptly mobilized by experimental stress and retain their high level production of TNF- $\alpha$ . Therefore, slanDCs may well contribute to the pro-inflammatory skin immune reaction observed following psychosocial stress in AD.

P018 (V25)

### Contact allergens cobalt and nickel induce proinflammatory gene expression by facilitating TLR4 receptor dimerisation

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Development of contact allergies requires cooperation of adaptive and innate immunity. Besides a hapten-specific T cell response an innate immune signal is obligatory for mounting a contact hypersensitivity reaction. We recently demonstrated that nickel (Ni<sup>2+</sup>), the clinically most relevant contact hapten, directly interacts with the human Toll-like receptor TLR4 (hTLR4), resulting in the generation of a proinflammatory response. Based on structural modelling and mutagenesis data we proposed a dimerisation-dependent mechanism of hTLR4 activation by metal haptens. Yet, direct experimental proof for the mechanism of hapten-induced hTLR4 activation is still missing and the involvement of the hTLR4 co-receptor MD2 in this process is presently unclear. Moreover, it is currently unknown if related metal allergens, such as cobalt (Co<sup>2+</sup>) likewise require hTLR4 binding to elicit innate immune activation.

To further elucidate the molecular basis of hapten-induced proinflammatory signalling we generated various soluble hTLR4 (sTLR4) mutants covering the extracellular domain of hTLR4 fused to a signal sequence from the murine Ig chain, which targets the resulting fusion protein to the secretory pathway. Upon transfection into HEK293 cells these proteins are secreted into the culture supernatant and can be used for functional inhibition experiments. Of note, we observed that sTLR4 secretion occurred in absence of MD2 as sTLR4 was readily detected in supernatants of both sTLR4-transfected HEK293 wildtype cells and HEK293 cells stably expressing MD2. Remarkably, addition of sTLR4 produced in absence of MD2 was sufficient to inhibit Ni<sup>2+</sup>- and Co<sup>2+</sup>-induced proinflammatory gene expression in hTLR4/MD2-expressing HEK293 cells whereas responses to the classical TLR4 agonist lipopolysaccharide (LPS), which critically depend on combined interaction with hTLR4 and MD2, were unaffected. Blockade of hapten-induced hTLR4 activation was sensitive to mutation of the metal-binding histidines H456 and H458 in sTLR4 and did not occur upon introduction of a point mutation interfering with hTLR4 dimerisation, suggesting that inhibition occurred via hapten-induced homodimerisation of sTLR4. Preliminary co-immunoprecipitation experiments using HEK cells co-transfected with differentially tagged hTLR4 variants, i. e. hTLR4-flag and hTLR4-HA, support this concept and confirm that hTLR4 dimerisation is facilitated by stimulation with Ni<sup>2+</sup> or Co<sup>2+</sup> and occurs independently of MD2. Yet, presence of MD2 was strictly required for Ni<sup>2+</sup>- and Co<sup>2+</sup>-induced proinflammatory gene

expression, suggesting that subsequent MD2-binding is still necessary for efficient downstream signaling of the hapten-induced hTLR4 dimer. Altogether, our data demonstrate that Ni<sup>2+</sup> and Co<sup>2+</sup> bind to H456 and H458 of hTLR4 resulting in receptor dimerisation. While dimerisation itself does not require MD2, subsequent activation of the TLR4 signalling cascade depends on its presence. Our data describe a first approach to selectively block TLR4 activation by haptens without interfering with LPS responses, which is a prerequisite for development of safe TLR4 inhibitors for prophylaxis and treatment of contact allergies to Ni<sup>2+</sup> and Co<sup>2+</sup>.

#### P019 (V27)

##### Rapid induction of systemic Th2-responses by intranasal instillation of ragweed pollen extract

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Pollen grains are able to induce allergic sensitization by the release of allergens after contact with the mucosa of the respiratory tract. Furthermore, it has been shown that pollen grains contain non-protein substances with proinflammatory or immunomodulatory properties that exert adjuvant activity on the induction of Th2-sensitization. A major obstacle in deciphering the non-specific and antigen-specific effects of allergenic pollen species *in vivo* has been the lack of physiological animal models for allergic sensitization and inflammation. Aim of this project was to establish an *in vivo* system to study non-specific proinflammatory/immunomodulatory and specific sensitizing properties of ragweed pollen (an important neo-allergen recently imported to Europe from northern America).

Methods: To examine whether protein-containing or not-protein-containing aqueous pollen extracts (APE) exert proinflammatory effects *in vivo*, we instilled female, 10 weeks old BALB/c mice intranasally with extracts from ragweed pollen (RWE), protein- and not-protein-containing fractions (>3 kDa and <3 kDa respectively) or Amb a 1 (the major ragweed allergen) for eleven consecutive days 24 h. After the last instillation, a bronchoalveolar lavage (BAL) was performed and cell counts and differentiation obtained. Control mice for Th2-sensitization were i.p. sensitized with RWE/aluminiumhydroxide (Alum) and subsequently exposed with PBS intranasally. For evaluation of systemic T cell responses, nose-draining lymph nodes and splenocytes were ex vivo-restimulated with RWE, medium or chicken ovalbumin (OVA) as a protein control. After 6 days of culture, IL-4, -5, -13 and IFN-gamma secretion were analyzed by ELISA. B cell responses were analyzed on the level of total IgE and RWE-specific-IgG1 at the time point of BAL.

Results: Instillation of total RWE, the protein-containing >3 kDa fraction, Amb a 1, but also the protein-free <3 kDa fraction induced the influx of neutrophils into the BAL-fluid. In contrast, only the protein-containing total RWE and the >3 kDa fraction induced the influx of eosinophils and lymphocytes into the BAL-fluid, together with a significant increase of total IgE and RWE-specific IgG1. Mice sensitized with RWE/Alum i.p., but also when instilled with RWE intranasally only, showed a significant increase of IL-4, IL-5 and IL-13 secretion into cell culture supernatant and a concomitant decrease of IFN-gamma compared to controls. To our surprise, Th1/Th2-cytokines were not detected in cultures from nose-draining lymph nodes.

Conclusion: We have established a murine model for exposure of the airways with environmentally relevant pollen that allows the further analysis of their proinflammatory and sensitizing properties. While the protein-free <3 kDa fraction showed a sole proinflammatory effect, the protein-containing RWE and >3 kDa fraction induced an allergic sensitization on T- and B cell level with an inflammatory BAL-infiltrate and systemic response in the spleen within just 11 days. Outlook: We are currently using this model to analyze the early events of allergic sensitization and inflammation in pulmonary tissue and primary/secondary lymphoid organs, which are elicited by different pollen fractions or pollen-derived single substances.

#### P020

##### SDC4 mediated cell migration affects the severity of induced asthma in mice

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Dendritic Cells (DC) play an important role in the induction of an immune-response and the maintenance of immunological tolerance. Syndecans (SDC) are transmembrane proteoglycans with heparin sulfate side chains. SDC act as integrin co-receptors and sequester extracellular signals like cytokines, thereby affecting cell migration. We could show that SDC4<sup>-/-</sup> DC migrate slower than wildtype DC and therefore alter contact hypersensitivity responses in mice. We now investigated SDC 4-knockout mice compared to wildtype mice in the asthma model. Wildtype-mice and SDC4<sup>-/-</sup> mice were sensitized twice by 20 g ovalbumin plus the adjuvant alum by intraperitoneal application. Over the next days several doses of 20 g Alexa 647 stained ovalbumin each were administered once a day in 0.9% NaCl via the nose of the mice to induce pulmonary inflammation and dyspnoea. The resulting asthmatic response was measured by plethysmography and bronchoalveolar lavage. The transport of stained ovalbumin into the local lymph nodes by the DC was measured by microscopic imaging. The alleviated asthmatic response of the SDC4<sup>-/-</sup> mice was then correlated with the count of ovalbumin-positive DC in the tracheobronchial lymph nodes.

#### P021

##### Artificial extracellular matrix composed of collagen I and sulfated glycosaminoglycans prevents TGFβ1-induced differentiation of human dermal fibroblasts to myofibroblasts

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Cutaneous wound healing is a multistep process that involves several cell types and events. During these process dermal fibroblasts (dFb) have to differentiate to myofibroblasts (MfB) to gain the contractile, extracellular matrix (ECM) synthesizing phenotype necessary for wound closure. This differentiation is stimulated by transforming growth factor beta 1 (TGFβ1) and results in the expression of high amounts of the MfB differentiation markers collagen I (coll), alpha smooth muscle actin (αSMA) that mediates the contractile activity when integrated into stress fibers, and ED-A FN – a splice variant of fibronectin.

Functional biomaterials are considered to support healing in patients with disturbed wound healing. Modern biomaterials based on native ECM components may be superior to solely synthetic biomaterials for clinical applications. Here, we investigated artificial ECM (aECM) consisting of coll and the glycosaminoglycans (GAGs) hyaluronan (HA) or chondroitin sulfate (CS). Additionally, GAGs were

chemically modified by the introduction of sulfate groups to obtain high-sulfated GAG derivatives. Sulfate groups in these aECMs are expected to bind and concentrate growth factors and improve their bioactivity. Previously, we have shown that the introduction of sulfated GAGs into aECM promotes proliferation of dFb and suppresses matrix synthesis of these cells (1).

In this study we analyzed the effect of aECM on the differentiation of human dFb to MfB in the presence of soluble or aECM-adsorbed recombinant, active TGFβ1 within 72 h. Myofibroblast differentiation was evaluated by the mRNA expression levels of the MfB-differentiation markers coll, αSMA and ED-A FN determined by qRT-PCR and fluorescence microscopy for αSMA and ED-A FN.

Our qRT-PCR data generally show a two to three-fold up-regulation of MfB markers αSMA, coll and ED-A FN after stimulation with soluble or adsorbed TGFβ1. This up-regulation was observed for polystyrene control and all aECMs. Artificial aECMs with high proportions of sulfate like coll/HA3.0 and coll/CS3.1 show less pronounced expression of the MfB markers in non-stimulated controls as well as after TGFβ1 stimulation compared to non- or low-sulfated derivatives. Although the same amount of TGFβ1 was added to all aECMs, high-sulfated GAG containing aECMs hindered the differentiation of myofibroblasts within 72 h.

Consequently, we show that the introduction of sulfate groups in GAGs of the aECM may interfere with the MfB differentiation by TGFβ1. We suppose that the degree of sulfation can be used to fine-tune the properties of aECM with regard to fibroblast differentiation. This is important since by regulating the MfB differentiation aECMs also could modify the outcome of the wound and prevent hypertrophic scar formation due to persisting MfB stimulation.

Reference: (1) van der Smissen et al. Biomaterials (2011) in press.

#### P022

##### Integrin-mediated leukocyte recruitment leads to UVB-induced skin hemorrhage during thrombocytopenia

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Platelets have been recognised as important mediators not only of hemostasis but also of vascular integrity during inflammation. In the present project we investigate the role of platelets in UVB-induced cutaneous inflammation. Previously, we observed that UVB-irradiation under thrombocytopenic conditions leads to skin bleeding (Purpura solaris) that occurs time- and dose-dependent and is strictly limited to the sites of irradiation. In current experiments shaved C57BL/6 wildtype mice were treated with a platelet depleting antibody and exposed to a single dose of UVB (100 mJ/cm). Twenty-four hour later UVB-treated thrombocytopenic animals showed Purpura solaris which was not observed in non-depleted mice. Petechial bleeding spots were quantified and punch biopsies were taken. Wet weight analysis of skin biopsies indicated less edema formation after UVB-irradiation if mice were platelet depleted. However, UVB-irradiation induced a significant influx of neutrophils as confirmed by measuring the neutrophil-specific enzyme myeloperoxidase (MPO). To further understand the role of neutrophil recruitment in Purpura solaris, mice were treated with a neutrophil depleting antibody prior to platelet depletion and UVB-irradiation. Interestingly, under leukocytopenic conditions skin bleeding was virtually absent. To exclude a direct role of adaptive immunity during the development of Purpura solaris, irradiation experiments were performed additionally in NOD scid IL2-R gamma deficient (NSG) mice. Of note, the absence of B- and T-cells did not prevent UVB-induced bleeding under thrombocytopenia. These observations led us to the hypothesis that leukocyte extravasation might be required for the development of Purpura solaris. To further clarify the pathogenic mechanism, CD18<sup>-/-</sup> mice that are characterised by leukocyte adhesion deficiency were challenged with UVB under thrombocytopenic conditions. We observed reduced cutaneous damage regarding both skin bleeding and neutrophil infiltration in comparison to wildtype mice. To further elucidate the occurrence of skin hemorrhage during thrombocytopenia, intravital microscopy in the dorsal skinfold chamber was performed. Visualising the UVB-mediated inflammatory processes in the dermal microcirculation indicates a local and temporal coherence between leukocyte adhesion and the onset of bleeding. Taken together, these data indicate that platelets are required to prevent Purpura solaris which depends on integrin-mediated leukocyte recruitment.

#### P023

##### Cross-linked regulation of protease activity in the epidermis by transglutaminated SPINK6

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Kallikrein-related peptidases (KLKs) are a group of serine proteases, expressed in several tissues. In the epidermis some KLKs are localized in the stratum granulosum and stratum corneum, where they participate in the desquamation process. Their activity is controlled by KAZAL-type inhibitors of the SPINK-family. Recently, we identified SPINK6 as a potent epidermal inhibitor of distinct KLKs members. Transglutaminases (TGases) 1, 3 and 5 are mostly expressed in the upper layers of the epidermis and are responsible for the cross-linking formation of the cornified envelope in calcium depend manner during keratinocyte differentiation.

In this study we investigated if SPINK6 is a substrate for transglutaminases. First we detected co-localization of SPINK6 and TGase 1 and 3 in human skin biopsies. As demonstrated by western blot analyses SPINK6 is crosslinked by TGase 1, TGase 3 to fibronectin. Transglutaminated SPINK6 exhibited proteolytic inhibitory activity as demonstrated by fluorogenic tetrapeptide substrates. Incubation of recombinant SPINK6 and TGase 1 and 3 together with epidermis extracts resulted in diverse higher molecular bands in western blot experiments indicating different substrate specificities of TGases with SPINK6. A similar pattern of immunobands was observed in extracts of differentiated primary keratinocytes without TGase treatment. These signals were enhanced by preincubating recombinant SPINK6 with primary keratinocytes. Trypsin-like proteolytic activity was significantly decreased in cultured keratinocytes, which were first treated with SPINK6 suggesting proteolytic inhibition of transglutaminated SPINK6.

In summary, we identified SPINK6 as a substrate for TGase 1 and 3. Ongoing experiments are on their way to find interaction partners for transglutamination.

#### P024 (V11)

##### Non invasive *in vivo* positron emission tomography (PET) and optical imaging (OI) analysis of the different mast cells (MC) migration properties due to the phenotype, administration route and mouse strain

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Local or systemic MC-engraftment of genetically MC deficient mice (Kit<sup>w/sh</sup> or Kit<sup>w/v-v</sup>) or knock-out mice (e.g. TNF<sup>-/-</sup>) is a powerful tool to analyze the specific impact of MC or MC-derived mediators. The aim of our study was to analyse differences in MC migration patterns due to the phenotype, mouse strains and mode of administration [intra-peritoneal (i.p.), intra-venous (i.v.), intra-articular (i.a.), sub-cutaneous (s.c.), or intra-cutaneous (i.c.)] non-invasively by *in vivo* small animal PET and OI.

Bone marrow derived MC (BMMC) or fetal skin MC (FSMC) were labeled with 0.7 MBq [<sup>64</sup>Cu]PTSM or Cy5 vibrant dye solution. [<sup>64</sup>Cu]PTSM-BMMC were tracked *in vivo* for up to 48 h and Cy5-BMMC or FSMC for up to 4 weeks. We injected 5 × 10<sup>6</sup> [<sup>64</sup>Cu]PTSM- or Cy5-BMMC (i.p., i.v., i.c., s.c. or i.a.) in C57BL/6 (Bl/6), KitW-sh or Kit w/w-v mice and performed PET/CT and OI investigations. Additionally, we analyzed biodistribution of [<sup>64</sup>Cu]PTSM-BMMC, Cy5-labeled BMMC and FSMC *ex vivo* by  $\gamma$ -counting, autoradiography and OI.

We detected impressive differences between the various local and systemic routes of MC-reconstitution in BL/6 and MC-deficient mice. Locally i.a. (ankle and paw) reconstituted Cy5-BMMC as well as Cy5-FSMC resided at the site of administration but did not migrate into the draining lymph nodes (LN) or any other organ, while s.c. (shoulder) or i.c. (ear) reconstituted Cy5-BMMC resided in the skin and migrated exclusively into the draining LN. Examination of systemically i.p. reconstituted Cy5-BMMC revealed predominant accumulation into the perithymic LN, peripancreatic LN, liver and all other LN in BL/6, KitW-sh, and Kit w/w-v mice. Systemically i.v. engrafted Cy5-BMMC accumulated mainly in the liver, spleen, lung, pancreatic- and mesenteric LN of BL/6 mice while they migrated in KitW-sh and Kitw/wv mice predominantly into inguinal and axillary LN, liver, spleen and slightly into the lung but not into mesenteric LN. Interestingly, after i.v. but not, or only slightly after i.p. administration we detected an impressive migration of Cy5-BMMC into the bone marrow in all examined mouse strains 4 weeks after MC-engraftment. Systemically i.v. engrafted Cy5-FSMC migrated into the spleen and only marginally into the liver but in contrast to BMMC additionally into the lung. Using [<sup>64</sup>Cu]PTSM-labelling and PET imaging we detected homing of i.p. engrafted BMMC mainly into the perithymic LN and the liver within 30 min. Systemically i.v. engrafted [<sup>64</sup>Cu]PTSM-BMMC were predominantly detectable in the spleen, lung and the liver but not in the perithymic LN.

[<sup>64</sup>Cu]PTSM-labelling and PET-imaging is most suitable for quantitative high resolution imaging up to 48 h due to <sup>64</sup>Cu and impairment of MC viability while Cy5-labelling and OI is very well suitable for long time investigations of MC migration for up to 4 weeks. Our data clearly exhibit impressive differences in MC homing patterns due to the mode of MC engraftment, the mouse strain, and MC phenotype and are of great importance for local and systemic MC engraftment studies.

### P025 Bovine milk-supplemented medium induces proliferation in human fibroblasts *in vitro*

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Products derived from bovine milk are an integral component of human nutrition in the Western population. As milk contains many hormones, cytokines, and growth factors there is debate about the benefit or risk of milk consumption to human health. In the present study we investigated the impact of commercial bovine milk on human fibroblasts *in vitro* in comparison to regular fetal calf serum (FCS). Our data show that induction by 10% bovine milk led to a 6-fold increase in proliferation as measured by BrdU incorporation in serum-deprived fibroblasts. The effect of FCS on proliferation was only slightly higher (7.5-fold). In order to study the underlying molecular pathways we tested the effect of bovine milk and FCS on a set of kinases involved in proliferation. By Western blot analysis we found an activation of PKB/Akt, p44/42, JNK and p38 in a compound specific pattern. From these observations we hypothesize that one or more milk-derived factors contribute to kinase activation and subsequently to cell proliferation. This issue will be addressed by further studies.

### P026 Targeting IL-1 $\beta$ for the treatment of EBS-DM

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Epidermolysis bullosa simplex type Dowling-Meara (EBS-DM) is one of the most severe subtypes of EBS. Characteristically, keratin 5 or 14 filaments harboring dominant mutations aggregate in the cytoplasm, leading to a collapse of the intermediate filament network and the EBS-DM associated skin blistering phenotype. Patient keratinocytes are much more susceptible to stress, which was shown by the onset of major signaling pathways upon osmotic shock, temperature shock or scratch assays. We found that the inflammatory cytokine IL-1 $\beta$  is significantly upregulated in patient keratinocyte cell lines. Besides IL-1 $\beta$  being involved in wound healing, it also plays a major role in proteasome activation for the clearance of cytotoxic protein aggregates (e.g. Huntington disease). Here we show that EBS-DM specific overexpression of K14 and subsequent aggregation leads to an increase of mature IL-1 $\beta$  levels, which in turn activates K14 expression via the JNK/SAPK pathway. In view of a possible treatment we showed that K14 expression is inducible by IL-1 $\beta$  and that a downregulation of K14 expression levels and JNK phosphorylation can be achieved by the treatment of patient keratinocytes with neutralizing IL-1 $\beta$  antibody or the IL-1 $\beta$  inhibiting small molecule diacerein.

### P027 Primary human epidermal keratinocytes and porcine *ex vivo* wound healing models under high glucose conditions show reduced susceptibility to Cx43 targeting

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Connexin 43 (Cx43) is downregulated during early wound healing (WH) at wound margins. Several mouse models and the dysregulation of Cx43 in diabetic chronic wounds indicate that this downregulation is important for WH. Therefore, Cx43 is a promising target to accelerate WH.

We have previously shown that Cx43 targeting is beneficial in porcine *ex vivo* wound healing models as well as in infant and adult human keratinocytes to improve wound healing. However, cells isolated from diabetic patients, although showing no difference in Cx43 expression and localisation compared to non-diabetic cells, exhibit a reduced susceptibility to Cx43 targeting by Cx-mimetic peptide Gap27. Additionally, diabetic keratinocytes migrate significantly slower than non-diabetic keratinocytes and show a reduced Cx-hemichannel activity. One possible reason for this "diabetic phenotype" might be the hyperglycaemia in diabetic donors. To investigate this hypothesis, we determined the behaviour of non-diabetic cells as well as the healing properties of *ex vivo* wound healing models under hyperglycaemic conditions.

We show here that cells under hyperglycaemia exhibit strong similarities to cells from diabetic origin. They exhibit higher fibronectin protein levels whereas there are only minor influences on Cx43 protein level. Hyperglycaemia in scratch wound assays leads to decreased migration rates. Application of Gap27 or Cx43-specific antisense oligonucleotides under these conditions does not result in significantly accelerated migration although it does under physiologic glucose concentrations. Cultivation under hyperglycaemic conditions also leads to a decreased Cx-hemichannel functionality. Comparable to the *in vitro* findings, cultivation of porcine *ex vivo* wound healing models under hyperglycaemic conditions result in a delayed wound closure and impaired response to Gap27 and Cx43-antisense ODNs. These data show that the altered phenotype of cells of diabetic origin is at least partly due to the hyperglycaemic conditions of the diabetic donors. This is also supported by the loss of "diabetic phenotype" during long-term culture of cells of diabetic origin in euglycaemic conditions.

### P028

#### Impact of physical plasma on T lymphocytes

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Physical plasma is defined as the fourth state of matter and consists of partially ionized gas. Since non-thermal plasma exhibit temperatures below thermal cell damage and has disinfectant abilities there are numerous applications in medicine e.g. sterilization of medical instruments or implants that are heat sensitive. It has been shown that plasma treatment has lethal effects on bacteria, whereas it stimulates growth of investigated eukaryotic cells. Because of that plasma treatment has the potential to heal wounds.

The aim of this study was to investigate the impact of non-thermal atmospheric-pressure plasma on human immune cells, in particular on Jurkat cells (T lymphocyte cell line). Proliferation and apoptosis of T lymphocytes after argon plasma treatment with a plasma jet (kINPen 09) were analyzed by different assays. Growth of Jurkat cells was inhibited after plasma treatment. There was also a correlation between plasma dose and percentage of apoptotic cells (Annexin V and Caspase 3 positive cells). Fewer cells survived with prolonged treatment times. Additionally, it could be shown that argon plasma treatment activated not only apoptotic signaling proteins like p38MAPK (p38 mitogen-activated protein kinases) and JNK (c-Jun N-terminal kinases) but also induced phosphorylation of ERK1 (extracellular signal-regulated kinases) and MEK1 (MAPK/ERK kinase1), which are known to promote proliferation.

### P029

#### Anti-inflammatory effects of a collagen-containing wound dressing in a cell-based inflammation model *in vitro*

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Introduction: Chronic wounds contain elevated levels of inflammatory cytokines leading to tissue damage and impairing healing. Hence, reduction of these mediators is a suitable way to promote normal healing. Studies have shown that collagen can bind significant amounts of cytokines. We investigated the influence of a collagen-dressing\* on the concentration of IL-8 and IL-6 in a cell-based inflammation model.

Materials and Methods: The cell-based inflammation model comprised of HaCaT-keratinocytes stimulated with TNF- $\alpha$  to mimic chronic inflammation. Cells were cultured with or without addition of collagen-dressing\*. Cell viability was investigated by luminometric measurement of the cellular ATP content. Concentrations of IL-6 and IL-8 in the supernatants were determined by specific ELISA.

Results: TNF- $\alpha$  had no significant effect on keratinocyte viability or proliferation but led to a distinct increase in the release of the inflammatory cytokines IL-6 and IL-8 *in vitro*. The collagen-dressing\* alone neither had an effect on cell viability and proliferation nor did it induce the expression of IL-6 or IL-8. However, treatment on the TNF- $\alpha$ -stimulated cells with the collagen-dressing\* led to a significant decrease in unbound IL-8 in the supernatant and a minor reduction in the IL-6-concentration. Conclusions: Collagen dressings should be able to improve the healing outcome of chronic wounds by decreasing the excessive concentrations of inflammatory mediators. Using a cell-based inflammation model it could be shown that collagen\* directly influences the amount of IL-6 and IL-8 released by TNF- $\alpha$ -stimulated HaCaT-cells most likely by binding these mediators as well as acting directly on the TNF- $\alpha$  present by reducing its concentration.

\*Suprasorb® C, Lohmann & Rauscher.

### P030

#### Comparison of two analytical methods for the detection of cell viability, cytotoxicity, and apoptosis induction in human HaCaT keratinocytes by cyclodextrins and cyclodextrin-antiseptica-complexes

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Introduction: Cyclodextrins (CD) are ring-shaped degradation products of starch. They are used in food, pharmaceutical, and chemical industries. For antimicrobial substances such as chlohexidine diacetate (CHX), polihexanide (PHMB) and iodine (IOD) the packaging into CDs could achieve a better skin compatibility, higher antimicrobial activity, and increased storage stability. We have examined the effect of  $\beta$ -CD and  $\beta$ -CD-antiseptica complexes on the viability, toxicity and the induction of apoptosis in human cells using a HaCaT keratinocyte model. The verification of biocompatibility is of vital importance for the application of pharmaceuticals and medical devices. Reliable analytical methods are necessary for the detection of cytotoxic and apoptotic effects on human cells during application of these products. We have evaluated two independent analytical methods with regard to comparability of the results.

Materials and Methods: The flow cytometric (FC) method determines the concentration dependent apoptotic and toxic effects after 24 h on HaCaT keratinocytes by annexin-V/7-amino-actinomycin D (7AAD) staining. Apoptotic cell death is accompanied by a change in plasma membrane structure by surface exposure of phosphatidylserine. This can be detected by its affinity for annexin V, a phospholipid binding protein. 7-AAD is a fluorescent DNA-binding agent that penetrates dead cells only. The double staining used in this assay allows differentiation between living, apoptotic and necrotic cells. In addition, the viability, cytotoxicity and apoptosis events of HaCaT keratinocytes after 24 h were detected by the cell based ApoTox-GloTM triplex assay (Promega GmbH). In this assay, all three parameters are detected in one approach by fluorescent and chemiluminescent measurement of live/dead cell protease activity and caspase-3/7 activation.

Results: In the FC assay of  $\beta$ -CD cells were viable at concentrations up to 5.0 mg/ml. At a concentration of 10 mg/ml toxic effects of  $\beta$ -CD were measured and apoptosis was detected within 20% of the cell population. In the ApoTox-GloTM triplex assay cells were viable at  $\beta$ -CD concentrations of up to 5 mg/ml. At 10 mg/ml cytotoxicity and apoptosis was detected.

Toxic effects of the  $\beta$ -CD-CHX complex were detectable at a concentration of 5.0 mg/ml in the FC assay and at 2.5 mg/ml in the triplex assay. In both assays no apoptosis was detected. The  $\beta$ -CD-PHMB complex had toxic effects at a concentration of 0.5 mg/ml in the FC assay and 0.1 mg/ml in the triplex assay. In both assays no apoptosis was detected. The  $\beta$ -CD-IOD complex had toxic effects at a concentration of 7.5 mg/ml in the FC assay and 5.0 mg/ml in the triplex assay. In both assays apoptosis was detectable at a concentration of 7.5 mg/ml. All complexes have a higher cytotoxicity compared to  $\beta$ -CD alone due to the toxicity of the individual antiseptica.

Conclusions: The results observed for cell viability, cytotoxicity, and induction of apoptosis in human HaCaT keratinocytes demonstrated a high comparability of both analytical methods. By the detection of phosphatidylserine exposure on the cell surface in the flow cytometric assay and caspase-3/7 activation in the ApoTox-GloTM triplex assay two independent marker of apoptosis were analyzed. This is a useful tool for the validation of the test results.

### P031 (V23)

#### Insulin resistance at the endothelial cell level represents a pathomechanism for psoriasis and its co-morbidities

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Psoriasis, a chronic inflammatory disease, appears mainly on the skin, but is associated with severe co-morbidities such as diabetes or coronary atherosclerosis. The common denominator of these

co-morbidities is insulin resistance. We have previously shown that the psoriatic cytokine milieu not only induces systemic insulin resistance, but epidermal insulin resistance represents a pathomechanism during the plaque development.

Pathomechanisms in endothelial cells leading to the manifestation of the cutaneous phenotype on one hand and to the co-morbidities on the other hand are not sufficiently investigated. Due to the similarities between a psoriatic and an atherosclerotic plaque, we hypothesize that the underlying mechanism and pathways that are known to play a role in the development of atherosclerosis also contribute to the pathogenesis of psoriasis and its co-morbidities.

By measuring insulin-dependent PKB phosphorylation in primary endothelial cells (HUVEC and HDBEC [human dermal blood endothelial cells]), we could show that interleukin (IL)-17, IL-22, IL-23 and TNF $\alpha$  induce insulin resistance. Moreover we could elucidate which signaling components are involved in mediating insulin resistance by using chemical inhibitors or siRNA knockdown. The expression of adhesion molecules such as E-Selectin and ICAM-1 is repressed by insulin and altered under conditions of insulin resistance.

Therefore we suggest that insulin is not only cardio protective, but as well anti-inflammatory and that under conditions of systemic inflammation as in psoriasis the disturbed insulin response contributes to the pathogenesis of psoriasis and its co-morbidities. Thus therapeutic approaches interfering with the altered insulin response in psoriasis might be very effective by targeting both the dermal as well as the cardiovascular dimension of psoriasis.

### P032

#### Profilaggrin peptide fragments as contributors to the antimicrobial barrier of the skin

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Human skin and especially the stratum corneum as the outermost barrier of the body contain antimicrobially active peptides to control growth of microorganisms and to prevent infection and inflammation. Recently peptide fragments of the N-terminal B-domain region of profilaggrin (PFLG) were identified in antimicrobially active HPLC-purified fractions of stratum corneum extracts. Peptides derived from these sequences were previously recombinantly expressed and some of them were found to be antimicrobially active. Now more detailed ESI-MS- and MS/MS-analyses of stratum corneum extracts revealed the presence of two additional PFLG peptides (FLG764-903 and FLG2849-2900) in antimicrobially active HPLC fractions. These peptides, originated from the PFLG repeat region, may also act as antimicrobial peptides (AMPs). Therefore they were recombinantly expressed in *E. coli* as fusion proteins and subsequently cleaved and purified by HPLC. The analyses of antimicrobial activity in an agarose radial diffusion assay system are currently underway. Results will show, whether at least some defined peptides originating from the PFLG repeat region are AMPs and thus may contribute, in addition to N-terminal PFLG peptides, to the "antimicrobial barrier" of healthy skin.

### P033

#### Investigating the influence of mitochondrial DNA deletions on skin developmental and ageing processes by a conditional deleter twinkle mouse model

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Deletions of the mitochondrial DNA (mtDNA) are observed in a wide range of human pathologies and in ageing, but their physiological impact on these processes as well as development has still to be determined. In particular, as witnessed by their accumulation in photo-aged skin, mtDNA deletions have been postulated to play a role in skin ageing, but little is known about the mechanisms involved.

We have generated a conditional mouse model allowing the expression of a severe mutant of the mitochondrial helicase Twinkle (K320E) via Cre-lox recombination. This helicase is involved in mtDNA replication and patients harboring mutations in its gene accumulate deletions in various post-mitotic tissues. Thus, crossing these conditional twinkle K320E floxed mice with appropriate mouse cre-lines will allow us to accelerate the accumulation of deletions in any desired cell compartment of the skin.

To investigate the influence of deletions on skin developmental and ageing processes, we chose to target respectively early epidermal progenitor cells with their progeny (crossing with keratin 14-cre line) and non-dividing dermal fibroblasts (crossing with collagen 1 alpha 2-cre line). Animals expressing the mutated twinkle in keratin 14 positive (K14+) cells displayed a short lifespan, growth retardation and hairless skin. Back skin analysis showed that hair follicle morphogenesis was impaired, but the overall differentiation of the epidermal compartment was not affected. As this phenotype was intriguingly close to the one displayed by mice completely lacking mtDNA in K14+ cells (our previously described epidermal mitochondrial Transcription factor A (TFAM) knockout mice), we determined the mtDNA copy number in epidermal sheets from wild-type and mutant mice. As suspected, we observed a massive mtDNA depletion in the mutant epidermis. However, no deletions could be found, neither by Southern blot nor Long Range Amplification PCR. Our model further strengthens the hypothesis that mtDNA deletions are not accumulating to detectable levels in highly proliferating tissues since the generation of deletions is a slow process likely to be incompatible with high cellular proliferation rate.

In contrast, due to the low proliferative rate of fibroblasts, the dermis specific mouse model we are currently generating should allow us to study the long-term effects of an accumulation of mtDNA deletions and in particular their putative influence on skin ageing.

### P034

#### Dual role of platelets in cutaneous inflammation

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Platelets are recognized as effector cells of primary hemostasis. Beyond that, more recent studies describe a pivotal role in the process of vascular integrity during inflammation. In the present study we investigate the role of platelets in the context of inflammation and hemorrhage in two models of cutaneous inflammation. Reverse passive Arthus reaction (rpA) and croton oil-induced irritative contact dermatitis (ICD) provoked petechial skin bleeding at the inflammatory site in thrombocytopenic mice but not in control animals. In addition, neutrophil infiltration was reduced in the analysis of the neutrophil-specific myeloperoxidase (MPO) activity (reduction of MPO: rpA: 41%; ICD: 69%). Furthermore reduced edema formation was observed in skin biopsies of thrombocytopenic mice in both models (reduction of biopsy weight: rpA: 41%; ICD: 50%). To investigate the role of neutrophils in rpA- and ICD-induced skin bleeding in thrombocytopenic mice, neutrophils were depleted by injecting anti-Gr1 antibodies 24 h before skin inflammation was initiated. In the absence of leukocytes thrombotic hemorrhage was prevented in rpA- and ICD-induced skin inflammation. To confirm these findings and further investigate the relevance of leukocyte extravasation during skin inflammation thrombocytopenia was induced in beta2-integrin-deficient mice (CD18 $^{-/-}$ ), that are incapable of leukocyte extravasation. As expected, platelet-depleted CD18 $^{-/-}$  mice showed reduced levels of infiltrating neutrophils and edema formation in the inflamed tissue. Additionally a protective effect against thrombocytopenia-induced skin bleeding was observed in these mice. In analogue experiments with NOD scid IL2-R gamma deficient (NSG) mice, which lack mature B-, T- and NK-cells, rpA- and ICD-induced skin bleeding was not prevented under thrombocytopenic conditions, indicating that tissue hemorrhage is independent of the adaptive immunity. To further identify underlying mechanisms of inflammatory bleeding, leukocyte interactions with the vessel wall were visualized in the inflamed skin

of platelet-depleted mice by intravital microscopy in the dorsal skinfold chamber. Confirming that leukocyte adhesion is a prerequisite for thrombocytopenic hemorrhage, *in vivo* imaging revealed a local and temporal coincidence of leukocyte vessel wall interaction and initial bleeding. In summary, our findings demonstrate a dual role of platelets during inflammation. Platelets prevent inflammatory hemorrhage while supporting the extravasation of leukocytes. Anti-inflammatory treatment targeting the proinflammatory effect of platelets should consider that the procoagulatory potential remains unaffected.

### P035

#### Molecular pathomechanisms in Dowling-Meara type epidermolysis bullosa simplex cell lines

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Dominantly inherited Dowling-Meara type epidermolysis bullosa simplex (EBS-DM) is caused by mutations in either the keratin-5 (KRT5) or the keratin-14 (KRT14) gene. In EBS-DM K5/K14 heterodimers disintegrate under conditions of mechanical stress, leading to cell lysis followed by severe blistering of the epidermis. In this study we investigated EBS-DM cell lines, carrying the mutations K14 R125P or K14 R125H. We performed microarray analysis, semi-quantitative realtime PCR and western blot analysis to determine gene and protein expression levels within the mutant cell lines compared to wildtype keratinocytes. Here we report that these cell lines are highly invasive in Matrigel (TM) invasion assays associated with increased expression of kallikrein related peptidases and matrix metalloproteinases. Furthermore, we observed atypical expression of cytokinins, junction proteins and chemokines. On RNA and protein levels a deregulation of the Cdc42 pathway was found as well as a higher susceptibility to anoikis, when cultured under anchorage independent conditions. Our investigations shed new light on the pathomechanism of EBS-DM thereby providing the necessary basis for the development of therapies.

### P036

#### The role of occludin in differentiation and apoptosis of keratinocytes – implications for squamous cell carcinomas

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Besides their paracellular barrier function, tight junction (TJ) proteins are known to be implicated in several cellular pathways, including the regulation of differentiation and proliferation. Likewise an altered TJ expression has been observed in various epithelial tumors. We analyzed expression and localization of the TJ proteins occludin (Ocln), ZO-1, claudin-1 (Cldn-1), Cldn-4, and JAM-A in cutaneous squamous cell carcinoma (SCC) in comparison to its precursors as well as sun-exposed and non-sun-exposed skin.

Our studies exhibit that a broader localization of ZO-1 and Cldn-4 as well as a down-regulation of Cldn-1 in the lowermost layers is frequently found in all tumor entities investigated as well as in sun-exposed skin. In contrast, a complete loss of Ocln is specifically found in a high proportion of SCC while this is not the case in precursor lesions and sun-exposed skin. To further investigate the role of Ocln we studied the effect of siRNA mediated suppression of Ocln on differentiation and apoptosis in human keratinocytes of monolayer cultures and of 3-D skin models. We observed altered expression of the differentiation marker proteins filaggrin and involucrin as well as decreased susceptibility for induction of apoptosis by the death ligand TRAIL (TNF-related apoptosis-inducing ligand). Downstream-regulators of these Ocln-dependent alterations are elucidated at the moment. Our observations suggest for the first time a role of Ocln in epidermal differentiation processes and for implication of Ocln in apoptosis in keratinocytes. Because down regulation of Ocln is also observed in SCC from other origins, as well as in several other carcinomas, we propose that this may be a common principle in tumor pathogenesis and could turn out to be a promising target for therapeutically treatment.

### P037

#### Influence of atmospheric pressure plasma on keratinocytes

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In physics, plasma is known as the fourth state of aggregation and denotes a partially or fully ionized gas. Recently, cold atmospheric pressure plasma sources gain attention as a possible tool for biomedical applications, emitting UV radiation and creating reactive oxygen and nitrogen species. It is well known that prokaryotes die during a plasma treatment whereas eukaryotes are able to protect themselves and survive the same duration of treatment. Therefore plasma could be very interesting for chronically wound healing.

The keratinocyte cell line HaCaT was treated with the atmospheric pressure plasma jet kINPen 09. It was shown that the impacts on the HaCaT cells were dose dependent. The higher the plasma dose the higher the intensity for both Annexin V, an early apoptosis, and Caspase 3, an late apoptosis signal. Furthermore DNA-strand-breaks were shown with comet assay. And even though cells were dying after long plasma treatments, short plasma doses were not lethal. After a short duration of plasma treatment HaCaT cells even seemed to be stimulated. The increased cell growth and proliferation was measured with different experiments.

### P038

#### The chick embryo as experimental system for melanoma cell invasion

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A primary melanoma will not kill the patient, but its metastases. The current lack of drugs specifically inhibiting melanoma cell migration is in part due to the lack of suitable *in vivo* models able to mimic the complex 3D-*in vivo* situation that melanoma cells have to cope with in the patient for successfully driving the disease. Therefore, melanoma research requires adequate model systems resembling the situation in the patient to study cancer initiation and progression. Here, the technical aspects of the chick embryo model are presented in detail including step-by-step instructions and pitfalls. The capabilities are exemplified by a summary of our original experiments supplemented by new data on transplantation of non-transformed melanocytes into the neural crest and on malignant invasive growth of melanoma cells in the hindbrain. After transplantation into the neural crest melanoma cells (but not primary human melanocytes) spontaneously resume embryonic neural crest cell migration along both the medial and lateral pathways and finally undergo apoptosis in the specific target areas. Upon transplantation into ectopic areas such as the hindbrain (as novel model for brain metastasis and liquor seeding) or the optic cup, malignant invasion, haematogenous spreading and local tissue destruction

occurs. Neural crest cell migration and malignant invasion depend on embryonic morphogens (e.g. BMP and Wnt signalling) and are inhibited by specific antagonists. The chick embryo model allows the distinction and scrutiny of physiological and invasive migration of melanoma cells in designated niches *in vivo* and the manipulation thereof either via pre-conditioning of the transplanted melanoma cells (e.g. pre-treatment with morphogens, up- or down-regulation of specific genes, etc.) or via treatment of the embryo itself. The easy handling, low-budget prize and outstanding susceptibility to manipulation with good reproducibility render the chick embryo an excellent model for melanoma research.

Our approach to turn malignant melanoma cells back to their embryonic origin discloses two novel principles for the therapy of malignancy: (i) The inhibition of EMT as prerequisite of metastasis; and (ii) the shutoff of the migratory phase via the restrictions imposed on neural crest cells after reaching their target areas. Induction of differentiation in this niche leads on non-compliance to apoptosis.

### P039 Non-inflammatory pathogenic effects of collagen VII-specific autoantibodies

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Epidermolysis bullosa acquisita is a subepidermal autoimmune blistering disease associated with autoimmunity against collagen VII of the anchoring fibrils. In previous studies, we have shown that collagen VII-specific antibodies induce dermal-epidermal separation *ex vivo* and in experimental animals. In further studies, we and others showed the development of the full-blown experimental disease in animals requires complement activation and recruitment of granulocytes. However, the contribution of Fc-independent tissue damage by collagen VII-specific autoantibodies has not yet been characterized. Therefore, in our present work, we studied the pathogenic relevance of the direct interference of autoantibodies with the interaction between collagen VII and its ligands and with physiological processes. For this purpose, we have initially cloned mouse collagen VII and expressed different recombinant forms of this antigen, including its full-length form and the non-collagenous I domain. In further experiments, we have shown that collagen VII-specific antibodies inhibit the binding of collagen VII to laminin 332, but not to collagen IV, and fibronectin. Subsequently, collagen VII-specific antibodies were shown to inhibit the gel contraction and keratinocyte migration in the corresponding *ex vivo* assays. Prolonged administration of lower doses of collagen VII-specific antibodies to mice resulted in predominant mucous disease and weight loss of the animals rather than widespread inflammatory skin blistering. In conclusion, for the first time, our results demonstrate granulocyte-independent pathogenic effects of collagen VII-specific antibodies and suggest an important effect of autoantibodies on wound healing and cell migration as well as non-inflammatory mechanisms to tissue damage in EBA by the direct inhibition of collagen VII function by the autoantibodies. In addition, our findings reveal pathogenic events, which should be considered as therapeutic targets in diseases associated with collagen VII-specific autoimmunity.

### P040 Behaviour of primary human keratinocytes and fibroblasts on soft poly (n-butyl acrylate) networks with tailored mechanical properties

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Biomaterials are explored in regenerative medicine to induce and support the regeneration of functional tissue. A still unsolved clinical problem is the hypertrophic scar formation during wound healing. Recently, completely amorphous poly(n-butyl acrylate) networks (cPnBAs) were synthesized with elastic moduli (250 and 1100 kPa) adjusted to tissue elasticities. These cPnBAs would be sufficiently flexible when applied as wound dressing and the transparency of the material would allow the observation of the granulation process.

On both cPnBAs, which differ in their elastic modulus, adherence and viability of primary human keratinocytes and fibroblasts were examined 48 h after seeding, as well as the deposited extracellular matrix components fibronectin, collagen and elastin and the secreted factors IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, IL-8, IL-10, IL-12, TNF $\alpha$ , GM-CSF, VEGF, HGF and KGF in mono- and cocultures. Both cell types were seeded with 30 000 cells/cm<sup>2</sup> in monocultures on 13 mm diameter cPnBA disks in 24 well plates, while in coculture 10 000 cells/cm<sup>2</sup> each cell type were used.

Both cell types adhered and were viable to a large extend (85–95%) on both materials. On cPnBA1100 a higher ratio (5.32.0) of keratinocytes to fibroblasts was found, in contrast to cPnBA0250 (2.60.9). Furthermore a lower deposition of collagen, fibronectin and elastin by cocultured cells was detected on cPnBA1100 compared to cPnBA0250 whereas fibroblasts cultured alone showed only a lower deposition of collagen on cPnBA1100. The secretion of IL-1 $\alpha$ , IL-8, IL-12, GM-CSF and VEGF was lower for keratinocytes on cPnBA1100 than on cPnBA0250. For fibroblasts IL-6 was lower on cPnBA1100 compared to cPnBA0250. For secreted factors in the coculture no differences were found between cPnBA0250 and cPnBA1100.

These results give a first hint that the use of cPnBA1100 as wound dressing might be associated with a reduction of scar formation. To confirm this hypothesis, further *in vitro* and *in vivo* experiments are required.

### P041 Claudins are key regulators for TJ barrier function in keratinocytes

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Skin barrier function is indispensable to prevent the uncontrolled loss of water and solutes and to protect the body from external assaults. To fulfill this function keratinocytes undergo a complex pathway of differentiation and form the stratum corneum. In addition, tight junctions (TJs) – cell-cell junctions that are well known from simple epithelia to form a selective paracellular barrier – have been shown to form a paracellular barrier in the epidermis and to be involved in skin barrier function. Primary and secondary alterations of TJ proteins have been shown to be involved in skin diseases, e.g. atopic dermatitis, psoriasis vulgaris, and ichthyosis. However, detailed molecular analysis of the contribution of individual TJ proteins to TJ barrier function in keratinocytes is still missing. Therefore, we investigated the TJ transmembrane proteins Claudin-1 and -4 and Occludin as well as the TJ plaque protein Zonula occludens protein 1 (ZO-1) by siRNA knockdown experiments. We show for the first time that TJs form a barrier for sodium, chloride, and calcium ions in keratinocytes. In addition, they are likely to contribute to water barrier function. Further, we confirm their barrier properties for small and large solutes. Significant changes in the permeability of ions, small and large solutes are found by Claudin-4 knockdown, while knockdown of other TJ components show only minor effects concerning permeability, but are involved in other important cellular functions. These analyses of single TJ proteins identified members of the Claudin family as key regulators of TJ barrier function for ions as well as small and large solutes in keratinocytes.

### P042 mTOR signalling plays a role in the development of psoriatic lesions

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Psoriasis is a chronic inflammatory skin disease, characterized by red scaly plaques which may occur on any site of the body. Although biologics directed against certain cytokines, show promising results in the therapy of the disease, a comprehensive analysis of deregulated signalling components that might represent novel therapeutic targets are still missing.

In an analysis of different signalling pathways, we especially found the PI-3K/PKB-Akt pathway to be deregulated in psoriatic skin lesions compared to non-lesional skin. Using HaCaT cells as well as primary keratinocytes we could show that insulin induced activation of the PI-3K/PKB-Akt pathway plays a crucial role in maintaining skin homeostasis. Under conditions of systemic inflammation such as psoriasis, high levels of IL-1 $\beta$  in the skin lead to blockade of differentiation by means of insulin resistance and activation of proliferative pathways. Both mechanisms contribute to the formation of the psoriatic plaque. Furthermore we could show that the mTOR signalling cascade is hyperactivated in psoriatic lesions. While the mTOR complex is highly activated in the basal layer, downstream signalling components such as the ribosomal protein S6 and 4E-BP1 are preferentially activated in suprabasal layers. We could show that IL-1 $\beta$  activates the mTOR signalling cascade *in vitro*. We provide evidence that mTOR signalling also interferes with the balance between keratinocyte differentiation and proliferation. These findings are especially interesting as mTOR is efficiently inhibited by rapamycin (sirolimus), which is a pharmacologically well established drug. Thus, controlling correct PKB-Akt/mTOR signalling in the skin might represent a novel anti-psoriatic strategy.

### P043 Agonistic VEGF-variants engineered to simultaneously bind to and stimulate VEGFR-2 and alphaVbeta3 integrin

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The interplay between vascular endothelial growth factor-A (VEGF) and the extracellular matrix is of fundamental importance for the formation of functional vascular networks. Yet, the molecular mechanism by which the VEGF-matrix crosstalk regulates angiogenesis is not completely understood. This study aims at a better understanding how VEGF-matrix interactions control and potentiate blood vessel formation. To investigate the simultaneous activation of VEGF receptor-2 (VEGFR-2) and alphaVbeta3 integrin and a potential synergism in the downstream signaling pathways, a novel bi-functional protein consisting of the fibronectin type III domain 10 (FNIII10) and VEGF (FNIII10-VEGF) was generated. Structural and functional integrity of this bi-functional protein were confirmed by circular dichroism spectroscopy, surface plasmon resonance analysis and VEGFR-2 phosphorylation. Chemical crosslinking of ligand-receptor complexes on human umbilical vein endothelial cells (HUVE cells) demonstrated, that FNIII10-VEGF was able to bind simultaneously to VEGFR-2 and integrin alphaVbeta3. Intriguingly, attachment and spreading of HUVE cells to immobilized FNIII10-VEGF was significantly enhanced over attachment and spreading observed on FNIII10 or VEGF proteins alone. These *in vitro* findings suggest, that the bi-functional protein FNIII10-VEGF can mediate synergistic effects on endothelial cells. The signaling pathways regulating these effects are currently under investigation. In addition, the potency of FNIII10-VEGF to induce angiogenesis was assessed *in vivo* using wound healing as a model. Diabetic mice characterized by an attenuated angiogenic response were used for these studies. Our *in vitro* findings with FNIII10-VEGF and previous investigations with other growth factors strongly suggest, that the presentation of a growth factor in an immobilized state is beneficial for the induction of functional vessel networks. Therefore, for *in vivo* studies, we immobilized either FNIII10-VEGF or VEGF (as control) proteins to a fibrin matrix. To this end, both molecules were expressed as fusion proteins comprising the factor XIIIa transglutaminase substrate sequence (TG), which allows covalent cross-linking of the VEGF-variants into a fibrin matrix during the natural polymerization process. Single treatment of full-thickness punch biopsy wounds with fibrin gels containing either soluble VEGF or matrix bound TG-VEGF or TG-FNIII10-VEGF, accelerated wound closure over treatment with fibrin only. More importantly, blood vessel growth was significantly increased in wounds treated with covalently immobilized proteins TG VEGF and TG-FNIII10-VEGF when compared to soluble VEGF. Collectively, the findings of this study corroborate a critical role for the interplay between VEGF-A and extracellular matrix during wound angiogenesis, and suggest that protein engineering provides a potent molecular approach to use these interactions for therapeutic angiogenesis.

### P044 Genetic ablation of mast cells does not impact skin wound healing and fails to prevent tissue fibrosis in mice

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Impaired wound healing, defective regeneration as well as fibrosis of diverse tissues are leading causes of morbidity and mortality. A detailed understanding of the complex molecular and cellular events underlying these processes is required to design new therapeutic approaches. Studies examining the role of altered mast cell (MC) function in the pathology of defective tissue regeneration and fibrosis have obtained contradictory results, most likely due to the use of different model systems. Therefore, a conclusive evidence for the functional impact of MC in tissue remodeling is still lacking. The aim of this study is to examine whether wound healing defects or tissue fibrosis are due to dysregulated MC activity. To investigate the consequences of MC depletion in murine skin with respect to the quality and kinetics of tissue repair we developed a novel mouse model of Cre-inducible diphtheria toxin receptor-mediated cell lineage ablation. In this mouse model Cre recombinase is expressed cell-type specific in MC under control of the Mep25-promoter. Efficient systemic ablation of connective tissue MC is achieved by repetitive intraperitoneal injections of diphtheria toxin. In response to mechanically-induced tissue damage genetic ablation of MC did not impact the kinetics of reepithelialisation, the formation of vascularized granulation tissue and the deposition of collagen in scar tissue. However, MC ablation resulted in an impaired wound contraction during the early phase of repair. The recruitment of polymorphonuclear cells but not macrophages during the inflammatory phase of repair was significantly attenuated. In a model of bleomycin-induced skin fibrosis genetic ablation of MC failed to prevent the development of skin fibrosis. Dermal thickness, the amount of deposited collagen and the formation of collagen crosslinks within fibrotic lesions were comparable in MC depleted and control mice. Collectively we conclude that the absence of MC does not impact skin wound healing and fails to prevent tissue fibrosis in mice.

### P045 Premature aging: unrepaired DNA damage or a defective DNA-damage sensor?

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Accumulating DNA damage accompanies the aging process in multiple organs and is thought to be responsible for premature aging in DNA-repair disorders like Cockayne syndrome. Cockayne syndrome mutations impair one branch of nucleotide excision repair that heals helix-distorting lesions like UV-products in the DNA. Total failure of nucleotide excision repair is not necessarily followed by premature aging but by severely elevated skin cancer susceptibility. Thus alternative hypothesis explaining premature aging in Cockayne syndrome are intensively discussed. Here we present evidence that all genes that can cause Cockayne syndrome are, beside their role in DNA repair, involved in RNA polymerase I transcription. This structural catenation of DNA repair and ribosomal transcription must have a functional impact. Thus we can show that DNA damage by UV is followed by a long-lasting stimulation of initiation of RNA polymerase I transcription. As transcription through the rDNA is hampered by transcription-blocking lesions, full-length rRNA synthesis is repressed and shorter transcripts are detectable by northern blot analysis. Transcription of ribosomal proteins by RNA polymerase II is not disturbed by UV light and ribosomal proteins are accumulating. As ribosomal proteins interact with HDM2 and stabilize p53, rRNA transcription dictates the amount and duration of the p53 response after UV irradiation. We can show that RNA polymerase I transcription serves as a DNA damage sensor for acute helix-distorting lesions. As RNA polymerase I transcription is impaired in Cockayne syndrome this might be sensed by the cell as massive DNA damage and is followed by senescence and apoptosis. These data imply that transcription of the long high-copy rDNA by RNA polymerase I serves as DNA damage sensor of the cell and might also sense accumulating DNA damage that is typical for the aging cell.

#### P046 Signalling pathway analysis in psoriatic tissue analysed by MELC

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Keratinocytes undergo a defined differentiation process as they migrate from the basal layer to the skin surface. This process is disturbed in psoriasis, a chronic inflammatory disease. Cell differentiation in keratinocytes is regulated by the coordinated action of the Notch family of receptors and ligands. Their role in psoriasis has not been analysed yet. We have used the Multi Epitope Ligand Cartography (MELC), a high-dimensional fluorescence microscopy, to assess potential changes of Notch expression in psoriatic skin as well as signalling pathways that affect Notch regulation. The MELC Technology is based upon repeated staining cycles of tissue sections through (i) incubation with a fluorophore-labelled antibody, (ii) fluorescence imaging and (iii) soft bleaching. The technique is capable of mapping hundred of different fluorescent markers in one tissue section and assessment of their combinatorial expression. We suspected the NF- $\kappa$ B signalling pathway to be crucial in controlling Notch function and therefore analysed signalling effectors of that pathway. Our results indicate that (i) Notch expression is fundamentally disturbed in Psoriasis possibly because of an (ii) aberrantly activated NF- $\kappa$ B pathway. Our results also demonstrate that the MELC Technology may be particularly helpful to delineate signalling events in diseased tissue.

#### P047 (V33) Cell-based regenerative medicine by nestin+ progenitors: stimulation of reepithelialization, angiogenesis, and granulation tissue formation in human skin organ culture

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The healing of chronic human skin ulcers and severe burn wounds presents a major clinical problem and agents/strategies that accelerate granulation tissue formation, angiogenesis, and reepithelialization are urgently needed. Since there is a shortage of preclinical assays to analyze how candidate wound healing promoters act on normal human skin, we have recently developed a simple, quantifiable, full-thickness human skin wound healing assay in which it is possible to analyze key wound healing parameters, and the contribution of skin appendages to it, under serum-free conditions. Here, we report that this assay is very suitable for testing cell-based regenerative medicine strategies *in situ*. Moreover, we provide the first evidence that the transplantation of heterologous nestin+, adult human progenitor cells, isolated from human sweat gland stroma, promotes reepithelialization, angiogenesis and granulation tissue formation *in situ* compared to untreated controls. Accelerated macroscopic wound closure *in vitro* was accompanied by a significantly increased length and area of the newly generated epithelial tongues at wound edges, as well as by enhanced involucrin expression (terminal differentiation marker) keratinocyte proliferation and epithelial energy metabolism (MTCO). Interestingly, the epithelial stem cell marker CK15 was strongly up-regulated within the transplanted, nano-particle-demarcated nestin+ progenitor cell aggregate. This could suggest that some of these stem cells had differentiated into CK15+ epithelial progenitors. Nestin+ progenitor cells also stimulated granulation tissue formation *in situ*, since the number of CD90+ fibroblasts, MHC class II+ macrophages/dendritic cells and immunohistochemically detectable (c-Kit+) mast cells was significantly increased. Furthermore, the number of CD31+ endothelial cells and of CD31+ vessel cross-sections was significantly higher in wounded human skin transplanted with primary human nestin+ progenitors. These preclinical data suggest that the transplantation of autologous nestin+ cells derived from a patient's own sweat glands could stimulate key components of human skin wound healing. This novel cell-based regenerative medicine strategy deserves to be fully explored in the future management of chronic human skin ulcers and burn wounds.

#### P048 Sensitization of melanoma cells for death ligand-induced apoptosis by the potassium channel IK-1 inhibitor TRAM-34 correlates with the intrinsic pathway and SMAC release

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Melanoma only poorly responds to chemotherapy, and the death ligand TRAIL, which may trigger apoptosis in melanoma cells via TRAIL-R1/DR4 and TRAIL-R2/DR5, appears as a promising therapeutic strategy. However, prevalent and inducible TRAIL resistance may limit its clinical use, as previously shown in melanoma cells. Potassium channels as IK1 may play significant roles in tumor progression and may serve as therapeutic targets. Functional expression of IK1 in melanoma cells is demonstrated by quantitative RT-PCR analysis and patch clamp recordings. We prove that TRAM-34, a selective IK1 inhibitor, strongly enhanced TRAIL sensitivity of melanoma cells and overcomes prevalent and inducible TRAIL resistance. Unraveling the signaling pathways revealed that TRAM-34 was able to overcome the lack of caspase-3 processing in selected TRAIL-resistant cells. Disruption of the mitochondrial membrane potential and release of proapoptotic mitochondrial factors, such as cytochrome c, AIF and SMAC, clearly indicated the involvement of mitochondrial apoptosis pathways. Importantly, TRAM-34 mediated enhancement of the TRAIL-induced apoptosis was critically dependent on the expression of either Bax or Bak, and apoptosis was abrogated in Bax/Bak double knockout cells as well as by overexpression of antiapoptotic Bcl-2.

Taking into account the physiological role of death ligands in immune surveillance, sensitization of melanoma cells for death ligands may be supportive for an anti-tumor immune response. This data prove the critical role of mitochondria in TRAIL resistance of melanoma cells and present a new strategy for TRAIL sensitization based on the targeting of ion homeostasis. Furthermore, combinations with the potassium channel inhibitor TRAM-34 may help for a breakthrough of TRAIL-mediated strategies in melanoma.

#### P049 The ripoptosome – a novel molecular complex differentially regulated by cFLIP isoforms and a critical switch between apoptotic and necroptotic cell death in skin tumor cells

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The intracellular control of different forms of cell death is of paramount importance during tumorigenesis, cancer therapy, and immune surveillance of skin cancer. In this context the differential regulation of apoptosis and necroptosis, a more recently described programmed form of necrosis, is critical. In this report we have identified and further characterized the Ripoptosome, a novel intracellular signalling complex that consists of caspases-8/10, RIP1, FADD and cFLIP isoforms. Using gel filtration, combined with immunoprecipitation, we could estimate the molecular size of this complex at about 2 MDa. The stoichiometry and the posttranslational modification of the proteins within the Ripoptosome differentially regulate cell death and cell survival. The formation of the complex can be induced by genotoxic stress or loss of inhibitor-of-apoptosis proteins (IAPs). Moreover we show that the Ripoptosome is negatively regulated by cFLIPL and the 26S proteasome and regulates the intracellular fate of cell death responses by serving as an intracellular switch to control the respective triggers. The Ripoptosome can be activated by stimulation of different membrane bound receptors e.g. death receptors (CD95) or Toll-like receptors (TLRs), such as TLR3. Upon TLR3 stimulation, we show that this complex is recruited to the intracellular adaptor protein TRIF. We demonstrate that loss of cIAPs sensitizes to poly (I:C) induced cell death in both a caspase-8- and RIP3-kinase-dependent manner, as shown by overexpression studies with HeLa cells that endogenously express low levels of RIP3. Whereas loss of cFLIP proteins promote Ripoptosome formation and cell death, expression of the long isoform cFLIPL, but not its short variant cFLIPS blocks the Ripoptosome. Rather cFLIPS promotes Ripoptosome formation in the absence of cIAPs and induce spontaneous necroptotic RIP1/RIP3 kinase-dependent cell death. Interestingly inhibition of the 26S proteasome (using the inhibitor MG-132) in cells that express high levels of cFLIPS lead to increased Ripoptosome formation even in the presence of cIAPs. These data suggest that proteasomal degradation is a negative regulator of Ripoptosome formation. We conclude that the differential composition of Caspase-8/cFLIP isoform dimers in the Ripoptosome is critical for the assembly and/or stability of the Ripoptosome. Surprisingly and in contrast to data in tumor cells, primary human keratinocytes do not form the Ripoptosome. These data point to a decreased susceptibility to form this signalling platform in primary cells.

Our studies thus show that cancer cells are prone to form the Ripoptosome, leading to increased cell death as a result of genotoxic stress or triggering of a number of membrane-bound receptors. Understanding this complex in more detail will thus allow for better approaches for the elimination of tumor cells. Therefore therapeutics that promote Ripoptosome formation and activity, such as IAP antagonists, might be important novel candidates as anti-cancer therapy.

#### P050 Monocyte/macrophage specific expression of matrix metalloproteinase-14 is dispensable for induced contact dermatitis but essential for monocyte/macrophage migration

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Monocyte infiltration and differentiation into macrophages has been shown to be crucial in the inflammatory phase of contact dermatitis. Metalloproteinases have been described as key enzymes in this process, however the role of matrix metalloproteinase-14 (MMP-14) in this context remains largely unknown. To answer this question, we generated animals with conditional ablation of MMP-14 in the monocyte/macrophage lineage by crossing mice carrying the floxed exons of the MMP-14 gene with mice expressing Cre recombinase under the control of the lysyzyme M (LysM) promoter. LysM-Cre+/MT1-MMPfl/fl (knock out) animals were healthy and fertile; skin architecture and differentiation were normal. To analyze the inflammatory process during contact dermatitis, we challenged mouse ears by croton oil treatment and measured the ear swelling over time. No macroscopic differences in knock out compared to wt mice were observed. However, challenge of the skin resulted in a significantly reduced number of infiltrating monocytes/macrophages in the knock out tissue, even though the number of infiltrating neutrophils was similar in both genotypes. Furthermore, a reduction in the number of a specific subpopulation of CD8+ T-cells was observed, whereas the number of B-cells was not altered in both animals. *In vitro* analysis of bone marrow derived macrophages revealed an impaired migratory capacity of the knock out cells on fibronectin pointing to a role of MMP-14 in mediating specific cellular functions such as transendothelial migration of monocytes and T-cell attraction.

#### P051 Effect of TRAILR on homeostasis of murine mast cells

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Background: Mast cells are crucial effector cells in allergy, host defense, inflammation and tissue remodeling. TNF-related apoptosis-inducing ligand receptor (TRAILR) takes part in the extrinsic pathway of apoptosis and represents a promising target for cancer therapy. However, limited information exists regarding the role of TRAILR in homeostasis of mast cells.  
Objective: To investigate the functional role of TRAILR in mast cell biology *in vivo*.  
Design and Methods: We generated transgenic mouse models of mast cell-specific and ubiquitous knockout of TRAILR. Using these mouse models, the effect of TRAILR on numbers of tissue mast cells and passive systemic anaphylaxis was investigated. Moreover, in cultured mast cells, the role of TRAILR on apoptosis of mast cells, survival after deprivation of growth factors, degranulation and mediator release was characterized.  
Results: Wildtype bone marrow-derived mast cells (BMMC) and peritoneal cell-derived mast cells (PCMC) were found to constitutively express TRAILR. Interestingly, stimulation of KIT with SCF significantly enhanced the expression of TRAILR and the sensitivity towards TRAIL-induced apoptosis. *In vitro* assays performed with knockout BMMC and PCMC, however, did not reveal any role for TRAILR in survival of mast cells after deprivation of growth factors or mediator release in response to stimulation with IgE/Ag, LPS, MALP-2 and calcium ionophore. Consistently, in knockout mice, the number of mast cells in back and ear skin, stomach, mesenterium and peritoneal cavity was compara-

ble with controls under physiologic conditions. Also, knockout of TRAILR failed to affect passive systemic anaphylaxis.  
Conclusion: We conclude that TRAILR plays a minor role in physiologic homeostasis of mast cells, but may control survival of mast cells under pathologic conditions that involve KIT activation.

#### P052

##### Cell cycle arrest of human fibroblasts induced collagen type I synthesis

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It is well described that the morphology of postmitotic fibroblast and their metabolic profile differs from mitotic fibroblasts and that *in vivo* e.g. collagen content and therefore also collagen synthesis decreases age dependently. This *in vitro* study focused on investigating differences between mitotic, postmitotic and physiologically postmitotic fibroblasts.

Human fibroblasts were isolated from foreskin of donors younger than 5 years. In preliminary tests a suitable mitomycin C concentration for optimal cell cycle arrest was determined by treating fibroblasts for 2–4 h with different mitomycin C concentrations. Cell proliferation and toxicity of the treatment was evaluated 24 h after the treatment. Subsequently collagen type I synthesis of mitotic, postmitotic and physiologically postmitotic fibroblasts was monitored for 24–96 h. To obtain the relative amount of collagen type I the measured concentration had to be correlated to the cell number of fibroblasts. The cell number was determined by lysing the cells with Triton X, determining the LDH concentration in the cell free supernatant and comparing it to a standard curve.

Morphologic differences between physiologically young, postmitotic young fibroblasts and physiologically postmitotic fibroblasts were documented. It could be shown that the relative amount of collagen type I of chemically postmitotic young fibroblasts was considerably higher than that of mitotic young fibroblasts. The relative concentration of collagen type I of the physiologically postmitotic fibroblasts was also significantly higher than the relative collagen type I concentration of young mitotic fibroblasts. This effect could be monitored for fibroblasts up to passage 32.

In conclusion we assume that induction of chemical as well as physiological cell cycle arrest of fibroblast cultures *in vitro* induces differentiation to fibrocytes. In consequence the collagen type I synthesis was significantly higher than in mitotic fibroblasts.

#### P053

##### Activation of mitochondrial apoptosis pathways via Bax through BMS-345541 in melanoma cells leads to sensitization for TRAIL-induced apoptosis

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Background: New therapies are needed for treatment of metastatic melanoma. The death ligand TRAIL (TNF-related apoptosis-inducing ligand) is known to trigger apoptosis in a variety of cancer cells, while normal cells are largely spared. However, prevalent as well as inducible resistance prevented its efficient use in cancer therapy. The transcription factor NFκB (nuclear factor-kappaB) plays an essential role in the immune response and has also been implicated in chemoresistance in cancer. In the present study, we investigate the effects of the small molecule BMS-345541, described as NFκB inhibitor, on death ligand resistance in melanoma.

Methods: TRAIL-sensitive melanoma cell lines A-375 and Mel-HO were compared to permanently resistant MeWo and Mel-2a as well as to cell lines selected for death ligand resistance A-375-TS, Mel-HO-TS (TRAIL-selected) and A-375-CS, Mel-HO-CS (selected with an agonistic CD95 antibody, CH-11, for resistance to the death ligand CD95L).

Results: BMS-345541 was found to strongly enhance death ligand sensitivity of melanoma cells and to help to overcome prevalent and inducible death ligand resistance. Unraveling of the proapoptotic signaling pathways in A-375-TS revealed an enhancement of the processing of effector caspase-3 and initiator caspase-9 in the combination BMS + TRAIL. Significant loss of the mitochondrial membrane potential, release of the proapoptotic mitochondrial factors cytochrome c, SMAC and AIF were evident for the activation of intrinsic mitochondrial apoptosis pathways. BMS-345541 mediated enhancement of TRAIL-induced apoptosis was critically dependent on the proapoptotic Bcl-2 protein Bax but not on Bak, and apoptosis was abrogated in Bax knockout cells as well as by overexpression of Bcl-2. Importantly, treatment with BMS alone resulted in translocation of Bax to the mitochondria already after 2 h, thus clearly proving the BMS effect on Bax as the critical step for its proapoptotic activity in melanoma cells and the sensitization for TRAIL. This new role of BMS-345541 appeared as largely independent of NFκB.

Conclusions: This data prove the critical role of mitochondria in TRAIL resistance of melanoma cells and present a new strategy for TRAIL sensitization. Furthermore, a new activity is described for BMS-345541, which may be useful in future for therapeutic approaches.

#### P054

##### Is lorricin a Trojan Horse antimicrobial peptide mimicking a bacterial microcin B17 precursor?

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Lorricin is the most abundant protein of the cornified cell envelope in human differentiated keratinocytes. This 25.6 kDa protein has unique structural characteristics, consisting of 47% glycine, 23% serine and 6% cysteine, with a high proportion of Gly-Ser and Gly-Cys motifs within the N-terminal glycine loop domain. Interestingly both, Gly-Ser- and Gly-Cys-motifs within a glycine loop domain represent a prerequisite of the E. coli microcin B17 (mccB17). Microcins are antimicrobial peptides produced by Gram-negative bacteria, presumably against closely related species. Due to posttranscriptional modifications of the mccB17 the Gly-Ser and Gly-Cys-motifs are converted into oxazols and thiazols, respectively, via bacterial enzymes, thus generating the antimicrobial active peptide. We therefore hypothesized that lorricin and/or peptide fragments could represent 'Trojan Horse Antimicrobial Peptides', mimicking the precursor of a mccB17-like microcin that is possibly further processed by bacteria at the skin surface upon contact with stratum corneum proteins. To test this hypothesis we generated recombinant lorricin peptides: This was achieved by generating a fusion protein (to avoid possible suicidal activity of a putative E. coli-cidal antimicrobial peptide) consisting in a His-SUMO-tag and the lorricin peptides or a Profinity eXact tag and the lorricin peptides. The SUMO-fusion proteins were trapped by a Ni-affinity column, digested with SUMO-protease into the tag and the lorricin peptides, and finally separated by RP-HPLC. The Profinity eXact fusion proteins were trapped by Profinity eXact cartridges and digested directly on the column. ESI-MS-analyses of a purified N-terminal lorricin peptide revealed identity with the mass predicted from its sequence. This lorricin peptide is currently under investigation for antimicrobial activity against several Gram-negative as well as Gram-positive bacteria using the radial diffusion assay system. When lorricin peptides represent Gram-negative bacteria killing AMPs via formation of a 'Trojan Horse AMP', this would support the hypothesis that human skin uses a yet unknown strategy of host defense.

#### P055

##### Regulation of sebocyte proliferation and lipogenesis by the EGFR ligand epigen

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We have previously shown that ubiquitous overexpression of epigen, a ligand of the epidermal growth factor receptor (EGFR), results in significantly enlarged sebaceous glands in transgenic mice. To avoid possible unspecific effects derived from the expression of the transgene in several other organs, an inducible, tissue-specific expression system was employed.

To study the effects of epigen in the interfollicular epidermis, hair follicle, and sebaceous glands, we generated a transgenic mouse line with doxycycline-inducible expression of the growth factor under the control of the keratin 14 promoter. Expression was induced by addition of doxycycline to the drinking water, and the effects were monitored by measuring epidermal sebum levels with a sebometer and by histological analysis. We also employed qRT-PCR and Western blot to evaluate the expression of the EGFR/ERBB family in SZ95 sebocytes during differentiation. Finally, chemical inhibitors and siRNA-mediated downregulation of the EGFR and ERBB2 were employed for functional studies.

Induction of epigen expression in transgenic mice before epidermal stratification and hair follicle morphogenesis (embryonic day 11.5) resulted in a thickened epidermis, enlarged sebaceous glands, and increased sebum levels from the third postnatal week. The sebaceous gland phenotype fully regressed within a few weeks following doxycycline removal but renewed administration of doxycycline resulted in its re-emergence. Surprisingly, postponing the induction of epigen expression to later developmental stages resulted in significantly weaker effects, while induction in adult mice failed to evoke any phenotypical alteration. When crossed into the EgrfWa5/+ background, a mouse line carrying a dominant negative EGFR, the sebum levels of epigen transgenic animals returned to the levels of control animals, indicating that the phenotype is largely EGFR-dependent. Both RT-PCR and Western blot analysis revealed that EGFR, ERBB2, and ERBB3 are expressed by sebocytes, the receptor levels being significantly reduced during sebocyte differentiation. In addition, inhibition of ERBB2 phosphorylation or siRNA-mediated downregulation of ERBB2 reduced the proliferation of SZ95 cells and enhanced their lipogenic activity. Our data indicate that the EGFR/ERBB system is an important regulator of sebaceous gland physiology. Signaling via these receptors appears to support the proliferation of undifferentiated sebocytes and to avoid their premature differentiation.

#### P056

##### Unbalanced SOD2 overexpression in senescent fibroblasts triggers altered redox signalling and skin ageing

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The free radical theory of ageing postulating increased concentrations of reactive oxygen species (ROS) to drive ageing is still controversially discussed. We here addressed the question whether unbalanced expression of antioxidant enzymes is responsible for the alterations in redox signaling that trigger the senescent phenotype in fibroblasts eventually resulting in skin ageing. A proteomic approach with 2D fluorescence difference gel electrophoresis and mass spectrometry identified manganese superoxide dismutase (SOD<sub>2</sub>) to be 13-fold increased in senescent fibroblasts (CPD 70) compared to young fibroblasts (CPD10), while hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>)-detoxifying enzymes (peroxiredoxin1,2,6) showed only minor changes. Increased SOD<sub>2</sub> expression and activity were confirmed *in vitro* in three independent fibroblast strains and *in vivo* in the skin of six old (61 years) compared to six young (U 25 years) individuals. Expression and activity of the H<sub>2</sub>O<sub>2</sub>-detoxifying enzymes catalase and glutathione peroxidase did not reveal any or only a slight increase finally leading to enhanced intracellular H<sub>2</sub>O<sub>2</sub> concentrations in senescent fibroblasts as confirmed by adenoviral transduction of the highly H<sub>2</sub>O<sub>2</sub> specific biosensor HyPer. Using *in situ* DHE staining in the presence and absence of distinct ROS scavengers on skin cryosections, H<sub>2</sub>O<sub>2</sub> was also confirmed *in vivo* to be increased in skin sections from old compared to young individuals. H<sub>2</sub>O<sub>2</sub> was identified to be responsible for the increase in the activity of Matrix-Metalloproteinase 1 (MMP-1) which promotes connective tissue degradation in senescent fibroblasts and skin ageing as shown with inhibitors of H<sub>2</sub>O<sub>2</sub> C detoxifying enzymes or H<sub>2</sub>O<sub>2</sub> scavengers. This was further confirmed by lentiviral transduction of senescent fibroblasts with a vector specifically overexpressing catalase in the mitochondria with reduction in H<sub>2</sub>O<sub>2</sub> concentrations and a significant decrease in MMP-1 activity in senescent fibroblasts. Using lentiviral transduction of a reporter gene construct with luciferase under the control of the TRE element in the MMP-1 promoter, transactivation of AP1 (activating protein 1) was detected in both senescent and H<sub>2</sub>O<sub>2</sub>-treated young fibroblasts. These data were confirmed using Western Blot analysis and a specific Transcription Factor ELISA for phosphorylated cJUN representing the major constituent for active AP-1 in senescent fibroblasts. Downregulation of H<sub>2</sub>O<sub>2</sub> - induced cJUN by specific siRNAs or its phosphorylation by inhibition of the stress-activated JNK resulted in a marked decrease in MMP-1 activity, indicating that the increase in AP-1 transactivation is cJUN-dependent in senescent fibroblasts. Finally, SOD<sub>2</sub> overexpression was distinctly confirmed to cause MMP-1 activation in senescent fibroblasts as shown by a marked decrease in MMP-1 activity after silencing SOD<sub>2</sub> via targeted siRNA in senescent fibroblasts, and vice versa, lentiviral-mediated SOD<sub>2</sub> overexpression in young fibroblasts resulted in enhanced MMP-1 activation. In conclusion, we have identified SOD<sub>2</sub> overexpression with imbalanced increase in H<sub>2</sub>O<sub>2</sub> in senescent fibroblasts which C via enhanced c-JUN phosphorylation C activates AP1 and induces target genes including MMP-1 eventually leading to skin ageing. Rebalancing antioxidant homeostasis is a promising strategy to prevent connective tissue degradation in skin ageing.

#### P057

##### Oxidative stress mediated IGF-1 resistance contribute to skin atrophy in connective tissue specific manganese superoxide dismutase deficient mice

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Earlier, we have shown impaired IGF-1 signalling in the connective tissue specific Manganese Superoxide Dismutase (MnSOD) deficient mice which may at least in part be responsible for the observed skin atrophy and accelerated ageing phenotype. The reason behind this impairment may be due to less serum and/or local (tissue) IGF-1 synthesis and secretion along with reduced basal IGF-1 receptor expression. *In vitro* studies using murine dermal fibroblast and keratinocytes, demonstrate that induction of mitochondrial superoxide anion generation by rotenone and subsequent oxidative stress inhibits the expression of IGF-1, an effect probably mediated through the activation of certain repressive type of transcription factor(s). Treatment with N-acetyl cysteine, a potent scavenger of reactive radicals, partially rescues the inhibitory effect of oxidative stress on IGF-1 expression. In addition, superoxide anion mediated oxidative stress also induces IGF-1 resistance in murine dermal fibroblasts, as revealed from reduced levels of phosphorylated (activated) AKT/PKB (Ser478), p70S6 kinase, ribosomal protein S6 and Cyclin D1. This resistance was dependent on the superoxide anion concentrations in the mitochondria of fibroblasts treated with rotenone. Moreover, IGF-1 resistance was partially rescued with N-acetyl cysteine treatment. Oxidative stress mediated IGF-1 resistance in the dermal fibroblasts is assumed to be due to increased stability of lipid phosphatase, viz. Phosphatase and tensin homolog (PTEN), which by dephosphorylating Phosphatidylinositol (3,4,5)-trisphosphate

inhibits the phosphorylation and activation of AKT/PKB and thereby, inhibits the transmission of signal from the receptor to the terminal effectors. Studies are being continued to identify the repressive transcription factor(s), which may be involved in the oxidative stress mediated reduction of IGF-1 transcription as well as the exact role of PTEN on IGF-1 resistance.

#### P058 Cartilage oligomeric matrix protein (COMP) – a modifier of skin matrix suprastructure

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The extracellular matrix (ECM) is a key regulator of cellular functions controlling a multitude of cellular activities in different target tissues. It is composed of a large variety of proteins, glycoproteins and proteoglycans, which form an interconnected network by binding of individual constituents to each other. Cells are embedded in this environment, which, depending upon composition can assume a pliable (healthy skin) or rather stiff nature (bone, fibrotic skin). Work by our and many other groups has provided clear evidence that cellular activities are profoundly regulated by the biochemical composition of ECM as well as by its mechanical properties.

Here we concentrated on the role of Cartilage Oligomeric Matrix Protein (COMP) for matrix assembly in skin. COMP is composed of five identical monomers, which are pentamerized at their N termini via coiled-coil domains. Electron microscopy revealed that this gives rise to a flower bouquet-like structure, in which the C termini are free in space to interact with other ECM proteins.

We raised antibodies to COMP, which detected the protein in a characteristic spatial distribution in healthy human skin from various anatomical sites as a continuous line in the papillary dermis immediately subjacent to but not overlapping with the dermo-epidermal basement membrane. We demonstrate that it is expressed and released exclusively by dermal fibroblasts. Binding studies using surface plasmon resonance and ELISA of recombinantly produced proteins revealed that COMP binds with high affinity to interstitial collagen I and to the fibril-associated (FACIT) collagens XII and XIV, which decorate the surface of major interstitial collagen fibrils in the dermis. Ultrastructural analysis using immunoelectron microscopy with gold-labeled antibodies confirmed these interactions. They further suggested that COMP localizes to anchoring plaques in the papillary dermis, which are specific basement membrane patches interspersed into the banded fibril network of the upper dermis and involved in the cohesion of the basement membrane to the dermis. Experiments are currently in progress analyzing potential binding of COMP to anchoring fibril collagen VII.

Our findings show for the first time expression of COMP in healthy human skin. We propose that COMP is an important component of anchoring plaques and a critical ECM protein, which by virtue of its five-armed shape and binding to collagens with each single arm is responsible for the spatial organization of collagen fibers and yet undefined ECM constituents in a functional dermal suprastructure.

#### P059 Progressive decrease in number, differentiation potential and accumulation of DNA damage of ABCB5+ mesenchymal stem cells in the skin during aging

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Recently, the expression of ABCB5, a novel P-glycoprotein of the ABC superfamily of active transporters, was found in a newly defined mesenchymal stem cell subpopulation in the skin. Even though its decrease in number and/or function may result in impaired regenerative capacity and aging, robust *in vivo* data are currently not available. Here we studied the cell surface expression pattern, the cell number and the specific localization of ABCB5+ mesenchymal stem cells (MSC) in human skin in young (0–20 years), middle aged (21–70 years) and old healthy individuals (>71 years). Dermal ABCB5+ cells did not express the hematopoietic progenitor cell antigen CD34 nor CD133 which constitute another stem cell subset in human skin. By contrast ABCB5+ MSC expressed a panel of previously established mesenchymal stem cell markers (CD29, CD90, CD59, CD44, CD105, CD73), revealed plastic adherence and were able to undergo chondrogenic, adipogenic and osteogenic differentiation. Notably, ABCB5+ MSC of old individuals revealed enhanced adipogenic and markedly decreased osteogenic and chondrogenic differentiation capacity as compared to ABCB5+ MSC of young individuals. ABCB5+ MSC were found in close association of CD31+ vessels in younger individuals, while this perivascular localization was lost in the old age group. This age-dependent change in the niche preference was combined with a significant decline in the percentage of ABCB5+ cells in the old age group compared to the younger age groups ( $P < 0.0001$ ), while the total number of resident cells in the dermis per high power field remained identical in all age groups. In a first attempt to understand the mechanisms underlying the ABCB5+ stem cell decline in old individuals, we studied the expression of  $\gamma$ H2AX, a phosphorylated histone protein which detects DNA double strand breaks and is considered to be a robust *in vivo* aging marker. Interestingly, we found an age-dependent increase in  $\gamma$ H2AX in ABCB5+ MSC. Further analysis of DNA-damage-associated pathways by mRNA profiling identified several genes in the nucleotide-excision repair pathway to be highly expressed in ABCB5+ MSC of old donors indicating that beside double-strand breaks ( $\gamma$ H2AX), helix-distorting lesions represent a significant challenge to ABCB5+ MSC of old individuals. The DNA damage did not result in enhanced p53 expression/activation, while enhanced expression of pro-apoptotic genes, among them p73, was found in ABCB5+ MSC of old individuals. Collectively, a robust decrease in ABCB5+ MSC most likely due to enhanced apoptosis and reduced stress resistance with distinct changes in niche preference and differentiation potential may contribute to a reduced regenerative capacity in skin aging.

#### P060 Mesenchymal stromal cells for the treatment of inherited skin fragility disorders

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Inherited skin fragility disorders are characterized by the formation of blisters in the skin and mucosa. Over the past few years, several reports proposed different cell based therapies like hematopoietic stem cell transplantation (HSC) and mesenchymal stromal cells (MSCs) as new therapeutic options for the management of Epidermolysis Bullosa (EB). In this study, we analyzed in a well-described mouse model with a desmoglein-3 (Dsg-3) knockout, the therapeutic potential of MSC application for the treatment of intraepidermal skin defects. Murine transgenic EGFP or DsRed expressing MSCs were applied systemically or by local intradermal injections into the skin. Systemic application of MSCs was performed without irradiation or in a combined transplantation with hematopoietic stem cells (HSCs)

including a myeloablative irradiation of the recipient mice. We found by Realtime PCR, Western Blot analysis and immunostainings that neither systemic application, nor local injection of MSCs into the dorsal skin of Dsg-3 knockout mice restored Dsg-3 expression. Furthermore, flow cytometry analysis of epidermal cell suspensions showed no EGFP or DsRed positive keratinocytes. Therefore, a transdifferentiation of MSCs into keratinocytes or a cell fusion of EGFP or DsRed positive donor cells with keratinocytes of the recipient seems to be unlikely. From this study, we conclude that MSCs possess a restricted transdifferentiation potential into epidermal cells albeit their importance for the treatment of dermal defects (like in the case of recessive dystrophic EB with mutations in the collagen 7 gene) seems to be promising since we found that MSCs express collagen 7 to similar amounts like fibroblasts.

#### P061 Partial characterization of epidermal RNase activities

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The stratum corneum is an efficient barrier to the passage of nucleic acids. It contains DNases and RNases that degrade DNA and RNA, respectively, originating from either the living cells of the skin or from the environment. Previously, we identified DNase1L2 and DNase 2 as DNA-degrading enzymes in the stratum corneum. Here we extended our investigations from DNases to RNases. Zymographic analyses showed that the main RNA-degrading enzyme of murine and human stratum corneum had an apparent molecular weight of approximately 15 kD. Additional minor RNase activities were detected at higher molecular weights. The main RNase activity increased during differentiation of human keratinocytes *in vitro* and was partially secreted into the culture supernatant. Proteomic analysis of the 15 kD RNase band identified peptides corresponding to RNase 7. Microarray analysis of mRNA expression suggested that RNASE7 is the most strongly upregulated RNase gene in differentiated human keratinocytes. This study provides a basis for unraveling the catabolism of RNA in terminally differentiated keratinocytes and on the skin surface.

#### P062 (V08) A crucial role of granulocyte-macrophage colony-stimulating factor in the pathogenesis of experimental epidermolysis bullosa acquisita

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Cytokines such as colony-stimulating factors (CSF) are proteins regulating immune functions. While an integral part of the host response, aberrant cytokine responses are linked to pathogenesis of chronic inflammatory diseases. Consequently, cytokines were successfully identified as therapeutic targets for several chronic inflammatory diseases. However, and despite noted aberrant cytokine expression, a cytokine-targeting therapy has not been established in autoimmune skin blistering diseases (ASBD). ASBD are collectively characterized by muco-cutaneous tissue injury, autoantibodies against structural proteins of the skin, limited therapeutic options and an increased mortality. From different disease models, we know that neutrophilic granulocytes are indispensable for manifestation of epidermolysis bullosa acquisita (EBA). Therefore, we analyzed the contribution of granulocyte (G)- and granulocyte-macrophage (GM)-CSF, two key cytokines required for neutrophil release from bone marrow and recruitment to areas of inflammation. Induction of EBA in C57Bl/6 or BALB/c mice by repetitive injection of rabbit anti-murine-type VII collagen (COL7) IgG led to elevated serum concentrations of GM-CSF but not of G-CSF. In C67Bl/6 mice, serum GM-CSF concentrations strongly correlated with clinical disease severity. Furthermore, while GM-CSF expression was absent in control mice injected with pre-immune rabbit IgG, GM-CSF was regularly observed in the skin of mice with EBA disease. Compared to controls, extent of skin disease was significantly reduced in mice lacking GM-CSF expression, despite unaltered IgG and complement (C3) deposition at the dermal-epidermal junction. On the molecular level, GM-CSF controlled neutrophil migration from bone marrow into the blood and from blood into skin. Furthermore, GM-CSF enhanced reactive oxygen species release from immune-complex activated neutrophils *in vitro*. In summary, GM-CSF modulates different pathways in the pathogenesis of experimental EBA. Inhibition of GM-CSF may therefore be a promising novel therapeutic strategy in EBA and other ASBD.

#### P063 (V20) Tyrosine kinase inhibitor SU6668 blocks the nucleic-acid-induced IFN $\lambda$ -expression of keratinocytes: TBK1 as a potential drug-target for the treatment of cutaneous lupus erythematosus

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Background: Cutaneous lupus erythematosus (CLE) is characterized by high levels of type III IFNs in skin lesions and blood. *In vitro* studies have shown that the synthetic RNA analogon poly(I:C) is a strong inducer of the IFN $\lambda$ -expression in keratinocytes, and endogenous nucleic acids are supposed to drive this IFN-expression *in vivo*. Despite the obvious role of III IFNs in CLE, therapeutic drugs which focus on this IFN-system are lacking. This prompted us to analyze the impact of specific drugs, which could interfere with the IFN $\lambda$ -pathway.

Methods: Cultured normal human epidermal keratinocytes (NEHK) were stimulated with different synthetic nucleic acids (polyAU->TLR3, R848->TLR7/8, CpG->TLR9, polyI:C->TLR3, MDA5, RIG-I). Two drugs, Chloroquine (inhibits nucleic acids in the endosome) and SU6668 (TBK1-inhibitor) were tested of their capability to suppress type III IFN-induction. In addition to IFN $\lambda$ , IL6, TNF $\alpha$ , CXCL9 and CXCL10 were measured in the culture supernatants by ELISA.

Results: PolyI:C, but not the specific TLR3 ligand polyAU or other TLR-ligands tested stimulate the IFN $\lambda$ -expression of keratinocytes. Chloroquine diminishes this expression-level only slightly, while the TBK1-inhibitor SU6668 significantly blocks the polyI:C-induced IFN $\lambda$  expression, and also reduces the expression of IL6 and CXCL9.

Conclusion: This study demonstrates that blocking of the IRF3/7 pathway using the TBK1-inhibitor SU6668 strongly reduces the expression of IFN and other proinflammatory cytokines, which are involved in the pathogenesis of CLE. TBK1 might therefore represent a potential target for future therapeutic strategies in this disease. The fact, that polyI:C but not polyAU induces the IFN-expression and the finding that SU6668 is more effective than chloroquine in the inhibition of this cytokine implicates that particularly cytosolic but not endosomal receptor pathways are involved in the nucleic-acid-induced IFN $\lambda$ -expression in keratinocytes.

#### P064 IL-27 regulates IL-18 binding protein in skin resident cells

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IL-18 is an important mediator involved in chronic inflammatory conditions such as cutaneous lupus erythematosus, psoriasis and chronic eczema. An imbalance between IL-18 and its endogenous antagonist IL-18 binding protein (BP) may account for increased IL-18 activity.

IL-27 is a cytokine with dual function displaying pro- and anti-inflammatory properties. Here we provide evidence for a yet not described anti-inflammatory mode of action on skin resident cells. Human keratinocytes and surprisingly also fibroblasts (which do not produce any IL-18) show a robust, dose-dependent and highly inducible mRNA expression and secretion of IL-18BP upon IL-27 stimulation. Other IL-12 family members failed to induce IL-18BP. The production of IL-18BP peaked between 48 and 72 h after stimulation and was sustained for up to 96 h. Investigation of the signalling pathway showed that IL-27 activates STAT1 in human keratinocytes and that a proximal GAS site at the IL-18BP promoter is of importance for the functional activity of IL-27.

The data are in support of a significant anti-inflammatory effect of IL-27 on skin resident cells. An important novel property of IL-27 in skin pathobiology may be to counter-regulate IL-18 activities by acting on keratinocytes and importantly also on dermal fibroblasts.

#### P065

##### Non-invasive repetitive *in vivo* analysis of inflammatory skin diseases by intravital multiphoton tomography

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Increasing incidence of inflammatory skin diseases such as Atopic Dermatitis (AD) has been noted in the past years. According to recent estimations around 15% of newborn subjects are affected with a disease severity that requires medical treatment. Although its pathogenesis is multifactorial, recent reports indicate that an impaired physical skin barrier predisposes for the development of AD. The major part of this barrier is formed by the stratum corneum (SC) wherein corneocytes are embedded in a complex matrix of proteins and lipids. Its components are synthesised in the stratum granulosum (SG) and secreted via lamellar bodies at the SC/SG interface.

Multiphoton tomography of endogenous fluorophores is a powerful tool for non-invasive 'optical biopsies', i.e. high resolution *in vivo* examination of human skin. Several biomolecules like NADH, melanin, collagen or elastin, showing autofluorescence or second harmonic generation, can be visualised *in vivo*. These molecules provide information about the subcellular morphology, epidermal architecture and physiological condition of the skin and can indicate changes in cell metabolism. Additional parameters like fluorescence decay times (FLIM) or spectral shift of the emitted fluorescence could be used for objective diagnosis and a therapy follow-up in inflammatory skin diseases.

Due to the application of intravital multiphoton tomographic skin analysis we facilitate the non-invasive investigation of human epidermis in the longitudinal course of AD therapy. Within a clinical *in vivo* study we focused on the skin metabolism at the SC/SG interface in AD affected patients in comparison to healthy subjects. In this study, FLIM of NADH provides access to the metabolic state of human skin. We could ascertain by blinded analysis of 40 skin areas of 20 patients in a 3 month follow-up that the metabolic status at the SC/SG interface was altered in AD compromised skin even in non-lesional, apparently healthy skin regions. This illustrates an impaired skin barrier formation even at non-affected skin of AD subjects appearing promotive for the development of acute skin inflammation. To further evaluate the morphological *in vivo* data skin biopsies for histological examination and alignment were performed.

To the best of our knowledge, multiphoton tomography is the first *in vivo* non-invasive high resolution diagnostic approach. Therefore, our findings allow a deeper understanding of the individual disease development and the improved management of the therapeutic intervention in clinical application.

#### P066

##### Health-related quality of life in hand eczema patients: international development and validation of a new questionnaire

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Background: Hand eczema leads to a substantial loss of Health-related Quality of Life (HrQoL) in those affected. Previously, these impairments were observed in the HrQoL research using generic instruments (e.g. Short Form 36), or skin-specific instruments (e.g. Dermatology Life Quality Index (DLQI) or Skin-dex-29). The generic instruments ask irrelevant questions for hand eczema (e.g. 'problems in climbing stairs'), therefore the interpretation and comparison of results in relation to hand eczema is problematic. Similar problems arise in the skin-specific instruments: they can either be applied to hand eczema patients in a limited fashion, or their psychometric properties may not be adequate.

We used an exploratory factor analysis to explore the dimensional structure of the DLQI in a sample of 920 hand eczema patients (mean age 46.9 years, SD 13.7; 54.9% females, 44.1% males). The analysis failed to reproduce the original multidimensional structure proposed by Finlay and colleagues. Those results limit the application of the DLQI for hand eczema patients to a one-dimensional construct of HrQoL, even if the Cronbach's alpha for the overall scale was satisfying (0.89). Therefore, the Department of Clinical Social Medicine, Heidelberg is currently developing a hand-eczema-specific instrument for measuring HrQoL, which is comprised by items relevant to hand eczema patients and which has a psychometric well evaluated structure measuring the following dimensions: impact of symptoms, functional impairment, emotions, and impairment due to the treatment and prevention. We suggest that impairment due to the treatment and prevention is an important aspect in hand eczema patients, since it is a chronic and recurrent disease, the mean duration of the disease in our sample was 7.9 years (SD 9.3).

Methods: First, a literature search on generic and dermatology HrQoL-questionnaires was performed. After reviewing the literature, a preliminary version of a hand eczema-specific HrQoL-questionnaire was developed by expert groups. This version is currently translated into English using a forward-backward procedure. Next, a second group of international experts will get involved in the development, in order to obtain a culturally equivalent version of the instrument which can be used for international comparisons. Subsequently, the instrument will be reconsidered in focus groups by patients for comprehensibility and content validity. The psychometric evaluation of the instrument will be done in a three time-point survey and statistical tests will be performed according to classical test theory, using reliability analysis, a sensitivity analysis, hypotheses based validity analysis, and a confirmatory factor analysis to verify the dimensional structure of the instrument.

#### P067

##### The BRAFV600E kinase inhibitor vemurafenib induces apoptosis through a process involving induction of endoplasmic reticulum stress in melanoma cells

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In a previous study, we observed that the pan-RAF inhibitor sorafenib induces upregulation of endoplasmic reticulum (ER) stress-related genes and apoptosis in melanoma cells *in vitro*. In this study, we investigated whether vemurafenib, which selectively inhibits the BRAFV600E kinase and demonstrates potent antitumor activity in melanoma patients with the BRAFV600E mutation, induces ER stress-mediated apoptosis.

The BRAFV600E kinase inhibitor vemurafenib inhibited growth, induced caspase-dependent apoptosis and upregulated the ER stress-related genes p8, CHOP, ATF4, ATF3 and TRB3 mRNA levels exclusively in BRAFV600E mutated melanoma cell lines. Apoptosis was correlated with the induction of the proapoptotic BH3-only protein Bim-particularly Bim short, which is linked to ER stress-mediated apoptosis. Western blot analysis showed that vemurafenib increases the protein levels of the ER stress marker CHOP in BRAF mutated but not in NRAS mutated melanoma cells. Treatment with vemurafenib resulted in a rapid increase in cytosolic calcium levels which is believed to reflect ER stress which can promote induction of the unfolded protein response. Furthermore, electron microscopy showed typical morphological signs of ER stress, in particular significant swelling of the endoplasmic reticulum lumen of BRAFV600E mutated melanoma cells treated with vemurafenib. siRNA inhibition of p8 diminished melanoma cell apoptosis induced by vemurafenib, overexpression of p8 significantly enforced melanoma cell apoptosis induced by vemurafenib. Furthermore, classical ER stress inducers such as thapsigargin and tunicamycin potently inhibited growth and induced apoptosis. Moreover, both thapsigargin and tunicamycin upregulated p8 and CHOP and induced apoptosis in vemurafenib-resistant melanoma cells.

These data suggest that the BRAFV600E kinase inhibitor vemurafenib induces apoptosis in BRAFV600E mutated melanoma cells through upregulation of ER stress-related genes, and that melanoma cells which acquired resistance to vemurafenib may be sensitive to agents which induce ER stress-mediated apoptosis through a different mechanism.

#### P068

##### Potency of new duplex drugs linking 3'-C-ethynylcytidine and 5-fluoro-2'-deoxyuridine against human melanoma *in vitro* and *in vivo*

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Background: Melanoma is increasingly common and currently no cure exists for patients with stage IV disease. There is an urgent need for novel drugs in order to effectively treat this potentially fatal malignancy. 2'-Deoxy-5-fluorouridylyl-(3'-5')-3'-C-ethynylcytidine [5-FdU(3'-5')ECyD] and 3'-C-ethynylcytidinyl-(5'->1-O)-2'-O-octadecyl-sn-glycerol-(3-O->5')-2'-deoxy-5-fluorouridine [ECyD-lipid-5-FdU] are so called duplex drugs that chemically link two antimetabolites into one molecule. They act as cytostatics by metabolisation into a mixture of active antimetabolites with different properties. This concept could use the advantage of a combination therapy avoiding its inconveniences.

Methods: The duplex drugs were evaluated along the National Cancer Institute (NCI) Developmental Therapeutic Program up to xenograft models. We assessed the cytotoxicity and mechanism of these heteronucleoside phosphate analogues and their corresponding monomers ECyD and 5-FdU on six metastatic melanoma cell lines and six ex-vivo patient-derived melanoma cells in comparison to current standard cytostatic agents and combinations thereof. Further, *in vitro* (real-time)-proliferation assays were performed. The embryotoxicity was also evaluated in the chick embryo model.

Results: Cell proliferation assays demonstrated that 5-FdU(3'-5')ECyD and ECyD-lipid-5-FdU had a high cytotoxic efficacy causing 75% melanoma cell death at concentrations ranging between nano- and micromolar. Cytotoxicity was conducted by induction of DNA cleavage/apoptosis, and via senescence in cells not undergoing apoptosis. Embryotoxicity demonstrated that the duplex drugs were less toxic than their parental monomers. *In vivo* results of the NCI showed that the duplex drug 5-FdU(3'-5')ECyD was efficacious in the LOX IMVI solid tumor xenograft model when 11.2 mg/kg/injection were administered every fourth day.

Conclusions: Both duplex drugs represent potential new chemotherapeutic drug combinations for malignant melanoma which are more potent than available standard monocytostatics and mixture thereof and thus further development is justified and desirable.

#### P069

##### The diagnostic yield of wells score and D-dimer testing in dermatological patients with symptoms of deep vein thrombosis

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Background: D-Dimer analysis in conjunction with a clinical probability score (Wells-score) has been shown to have a high sensitivity and a high negative predictive value for the exclusion of deep vein thrombosis (DVT). In patients with a low clinical probability and negative D-Dimer results, DVT can be excluded without ultrasound examination. More recently the diagnostic value of D-Dimer levels has been questioned in patients aged over 60 years, in patients with underlying malignancy, infections, or in outpatients with suspected distal DVT.

Objective: To identify the diagnostic performance of D-Dimer testing and Wells-score in hospitalized patients with dermatological conditions.

Methods: This study included 110 patient episodes of suspected DVT in 102 hospitalized patients with dermatological conditions (63 female, 39 male, median age 72 years, range 20-94 years). All data were retrospectively retrieved from standard operation procedure (SOP) files that were routinely used in the documentation of patients investigated to rule out DVT. Examinations were performed by Wells-score, Tina-quant® D-Dimer testing, and whole-leg duplex ultrasonography to rule out DVT.

Results: The Wells-score signalled a probability of DVT (score  $\geq 2$ ) in 30 out of 110 (27.3%) examinations. D-Dimer testing revealed pathological results in 91 out of 110 (82.7%) cases.

DVT was detected by whole-leg duplex ultrasonography in 14 patients (seven female, seven male, median age 73 years).

All 14 DVT patients showed elevated D-Dimers (100% sensitivity). Among the 16 patients that showed D-Dimers within normal limits, none was diagnosed with DVT (100% negative predictive value). A high rate of false-positive D-Dimer results ( $n = 79$ , 71.8%) led to a low specificity (16.8%) and a low positive predictive value (14.1%). In a multivariate statistical analysis D-Dimer levels were significantly associated with the dermatological main diagnosis ( $P = 0.0153$ ) and with the presence or absence of DVT ( $P = 0.0050$ ).

The two most frequent dermatological conditions in patients with suspected DVT were erysipelas of the lower leg ( $n = 34$ ) and chronic venous leg ulcer ( $n = 25$ ) making up approximately 53% of the examined patients.

The highest D-Dimer values were found in patients with metastasized/systemic malignancy (median 2.56 mg/l), inflammatory skin condition (e.g. generalized psoriasis, median 2.22 mg/l), and stasis eczema ( $n = 13$ , median 1.24 mg/l). The lowest D-Dimer values were measured in patients with venous leg ulcers (median 0.88 mg/l). DVT patients showed an intermediate high D-Dimer level (median 1.36 mg/l). The Wells-score allowed no discrimination of DVT and non-DVT patients (Sensitivity 27.3%, specificity 69.7%, negative predictive value 88.6%).

Conclusions: In this trial with hospitalized patients affected by severe skin diseases a negative D-Dimer test could safely rule out DVT. However, the reduction of whole-leg duplex ultrasonography examinations was low because only 16 out of 110 examined cases (14.5%) showed negative D-Dimer tests. Unspecific clinical criteria included in the Wells-score (e.g. pitting edema, calf swelling, localized tenderness) were also present in many skin conditions and clearly limited its diagnostic value.

In the light of high costs for D-Dimer testing we recommend to directly investigate all patients with suspected DVT and underlying inflammatory/malignant skin diseases by whole-leg duplex ultrasonography.

## P070

**Dermal open-flow microperfusion as a novel technique for *in vivo* measurement of dermal interstitium**

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**Background:** Dermal open-flow microperfusion (dOFM) is a novel minimal invasive sampling technique that provides direct access to dermal interstitium and therefore the possibilities of *in vivo* measurement of the cutis with respect to pathophysiological and pharmacological processes. The aim of this study was to describe the method of dOFM, and examine the tolerability and accuracy of insertion of this future technique in a prospective feasibility study.

**Materials and Methods:** We investigated the tolerability of dOFM in an open-labeled, uncontrolled single center trial. In 17 patients (12 otherwise healthy individuals, five psoriatic patients) up to nine catheters by means of two different needle sizes in diameter (0.5 mm and 0.7 mm) have been inserted intradermal and perfused with a sterile fluid at a constant flow rate. After application of dOFM, an equilibrium between the perfusate and the interstitium occurred, which allowed direct *in vivo* sampling of interstitial fluid for more than 24 h. A Visual Analogue Scale (VAS) and possible signs of local infection provided information regarding the tolerability of dOFM in our patients. Accuracy of insertion of dOFM catheters with respect to depth (<1 mm as reference value) was confirmed by ultrasonography in case of both needle diameters.

**Results:** Sampling was possible in all catheters for 24 h. All patients subjectively tolerated the application of dOFM technique very well. Analysis of VAS revealed a mean value of 2.75 in healthy patients versus 3.09 in psoriatic patients. The insertion of the 0.5 mm needle was more painful (mean VAS of 3.15) compared to the 0.7 mm needle (mean VAS 2.5). No signs of local infection could be observed in any patient. The 0.7 mm needle was easier to handle but provoked more capillary bleeding. The mean catheter depth was 0.81 mm in all patients, with 0.71 mm in healthy and 0.96 in psoriatic patients.

**Conclusion:** Dermal open flow microperfusion (dOFM) as a minimal invasive, constant *in vivo* sampling method is well tolerated with respect to pain after insertion and local reaction of the dermis by our patients. This technique is unique in its ability providing direct access to the biomarkers in interstitium and allowing sampling of molecules without limitations due to molecular size, protein binding or lipophilicity in an *in vivo* setting in humans.

## P071

**Sensitive and specific detection of collagen VII-specific autoantibodies by novel immunoassay using chimeric recombinant antigen**

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An autoimmune response to anchoring fibril component collagen VII is typically associated with the skin blistering disease epidermolysis bullosa acquisita (EBA), but also occurs occasionally in patients with systemic lupus erythematosus (SLE) or inflammatory bowel disease (IBD). Epitope mapping studies revealed that the major epitopes recognized by EBA autoantibodies reside within the non-collagenous (NC1) domain of the native collagen VII. In addition to very few cases showing reactivity to the triple helical domain of collagen VII, further important epitopes of EBA autoantibodies have been more recently mapped to the NC2 domain. Sensitive and specific immunoassays for the detection of collagen VII-specific autoantibodies are required for diagnosis and monitoring in EBA and for the study of autoimmunity in other conditions. Therefore, the aim of our present study was to develop an ELISA for the detection of autoantibodies against collagen VII. For this purpose, we have initially analyzed in silico both linear and conformational antigenic sites of collagen VII. Subsequently, based on these in silico and previous wetlab epitope mapping data, we designed a chimeric collagen VII construct containing the NC1, the hinge region of the triple helical and the NC2 domains that was expressed in stable transfected HEK-293. Serum IgG antibodies from EBA patient recognized the chimeric protein by immunoblotting. After optimization of working conditions by chessboard titration, a receiver operating characteristics analysis was performed yielding an area under the curve of 0.98 (95% CI: 0.9638–1.005), which allowed to set the cut-off at 0.32 OD at a calculated specificity of 97.5% and a sensitivity of 94%. Running the optimized test showed that serum IgG autoantibodies of 47 EBA (94%; 95% CI: 87.41–100%; n = 50), 2 Crohn disease (4%; 95% CI: 0–9.43%; n = 50), 8 ulcerative colitis (16%; 95% CI: 5.8–26%; n = 50), 2 bullous pemphigoid (4%; 95% CI: 0–9.44%; n = 50), and 4 pemphigus vulgaris (18.18%; 95% CI: 2–34%; n = 22) patients as well as from 4 (2.5%; 95% CI: 0–5%; n = 160) healthy donors reacted with the chimeric protein. Further analysis revealed that in 34%, 37%, 16% and 100% of sera autoantibodies of IgG1, IgG2, IgG3, and IgG4 isotype, respectively, have recognized the recombinant autoantigen. In conclusion, we developed a new ELISA assessing virtually all epitopes on collagen VII for the specific and sensitive detection of serum autoantibodies. This immunoassay should prove an useful tool for clinical and translational research and should essentially improve the routine diagnosis and disease monitoring in EBA and other conditions associated with collagen VII-specific autoimmunity such as IBD.

## P072

**Thermoablation (EUR? steam vein sclerosisEUroe) der Hautstammvenen**

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Die Thermoablation der Varikose mittels Wasserdruckkatheter (steam vein sclerosis) unter Ultraschallkontrolle stellt ein minimalinvasives Verfahren zur chirurgischen Behandlung der Varikose dar. Es ersetzt in vielen Fällen die Krossektomie mit Exhairese der V. saphena magna oder V. saphena parva. Die medizinische Indikation besteht in Stammvarikose der Vena saphena magna, Stammvarikose der Vena saphena parva, Seitenastvarikose und Perforansveneninsuffizienz. Diese hämodynamischen Störungen verursachen das Krankheitsbild der Chronischen Venösen Insuffizienz. Insbesondere bei bereits vorhandenen trophischen Hautstörungen inklusive Ulcus cruris venosum ist diese Therapieform ohne Schnittführung eine Alternative zur klassischen Stripping-Operation.

Ziel der Therapie: Ausschaltung/Zerstörung der klappeninsuffizienten Venenabschnitte zur Verbesserung des venösen Abstroms.

Methode: Unter Ultraschallkontrolle wird die zu behandelnde Vene (V. saphena magna, V. saphena parva, Seitenastvarize, Perforansvene) punktiert und der Wasserdruckkatheter positioniert. Nach dem korrekten Einbringen des Katheters werden die perivaskulären Gewebsschichten in unmittelbarer Umgebung der zu behandelnden Varize unter Ultraschallkontrolle mit einer subkutanen Infiltrationslösung infiltriert. Danach wird der Wasserdampf in den Katheter eingeleitet, an der Katheterspitze tritt der 110°C heiße Dampf aus und führt zum Shrinking (Verkleinerung des Lumens) der Varize, zum Verschluss. Der Katheter wird während des Dampfaustritts langsam aus der Varize gezogen, dabei kommt es zum langstreckigen Verschluss.

Patienten: n = 49; behandelte Venen: V. saphena magna: n = 36; V. saphena parva: n = 13

Nachkontrollen: nach 6 Wochen und 12 Monaten erfolgt die Anamnese im Hinblick auf die Stauungs-symptomatik, klinische Untersuchung sowie Duplexsonographie der extrafaszialen Venen und der Leit-venen und Venenfunktionsmessung mittels Photoplethysmographie.

Bewertung: Geringes Trauma, u.a. erkennbar an geringer Schwellung, Haematom, Schmerzhaftigkeit. Keine Schnittdurchtrennung der Haut und anderer Strukturen. Es resultiert eine geringe postoperative Morbidität, die Patientin / der Patient ist rasch wieder arbeitsfähig.

## P073

**Ustekinumab improves quality of life outcomes in psoriasis patients transitioned from methotrexate regardless of transition strategy: Week 16 results from the TRANSIT study**

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**Introduction and Objectives:** The TRANSIT study compared the safety and efficacy of two commonly used methods of transitioning psoriasis patients with inadequate response to methotrexate on to ustekinumab.

**Materials and Methods:** In this 52 week open-label, parallel group trial, 489 patients on methotrexate were randomised 1:1 into two arms: initiation of ustekinumab with either immediate cessation of methotrexate (arm-1) or 4 weeks overlap with methotrexate (arm-2). The study assessed impact of ustekinumab on health-related quality of life (HRQoL) using the Dermatology Life Quality Index (DLQI), Hospital Anxiety and Depression Scale (HADS), sub-divided into HADS-Anxiety and HADS-Depression scales, and EuroQOL-5D Visual Analogue Scale (EQ-5D VAS).

**Results:** Two hundred and forty-four patients entered arm-1, 245 entered arm-2. Mean baseline DLQI scores in arms 1 and 2 were 9.8 (±7.4) and 10.0 (±7.3) respectively, falling to 3.4 (±5.4) and 2.8 (±4.2) by week 16. The majority of patients in both arms achieved a clinically meaningful DLQI reduction of ≥5 points (57.0% arm-1, 59.7% arm-2), and a score of 0 or 1, indicating no negative effect on HRQoL (56.8% arm-1, 57.3% arm-2). Mean HADS Anxiety and Depression scores improved in both arms. Mean EQ-5D VAS improved from 63.5 (±25.5) and 63.7 (±26.2) to 73.1 (±25.9) and 74.5 (±25.4) at week 16 in arms 1 and 2, respectively.

**Conclusions:** Ustekinumab improves HRQoL outcomes in patients with moderate to severe plaque psoriasis transitioned to ustekinumab following inadequate response to methotrexate, with no difference observed between the two transition strategies used in this study.

## P074

**Ustekinumab is well-tolerated & effective in patients with psoriasis inadequately responsive to methotrexate: Week 12 results from the TRANSIT study**

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**Introduction and Objectives:** There are limited data on how to transition patients with psoriasis from conventional systemic agents to biologics. This study compared the safety and efficacy of two methods of transitioning patients from methotrexate to ustekinumab, following inadequate response to methotrexate.

**Materials and Methods:** In this 52-week open-label trial, 489 patients on methotrexate were randomised 1:1 into two arms: initiation of ustekinumab with either immediate cessation of methotrexate (arm-1) or 4 weeks overlap with methotrexate (arm-2). Inadequate response to methotrexate was defined as a PASI score ≥10 despite ≥8 consecutive weeks of methotrexate (10–25 mg weekly). Patients received 45 mg (≤100 kg) or 90 mg (>100 kg) ustekinumab. Primary endpoint was the proportion of patients experiencing ≥1 treatment-emergent adverse events (AEs) through week 12. Efficacy outcomes were secondary endpoints.

**Results:** Two hundred and forty-four patients were randomised to arm 1 and 245 to arm 2. Four subjects in each arm discontinued treatment through week 12. The proportion of patients experiencing ≥1 AEs was similar in both arms. AEs occurring in ≥5% of patients were headache, nasopharyngitis and arthralgia. Serious AEs were infrequent (2.9% arm-1, 2.0% arm-2), with 1 serious infection (arm 1, acute hepatitis B, week 2) and no cases of tuberculosis, malignancy or major adverse cardiovascular events. By week 12, baseline PASI scores fell from 17.44 (±6.97 SD) and 16.93 (±6.54) in arms 1 and 2 respectively, to 4.42 (±5.04) and 4.22 (±4.31). PGA of cleared/minimal: 65.3% (arm-1) versus 69.5% (arm-2).

**Conclusions:** Ustekinumab is well-tolerated and effective in patients inadequately responsive to methotrexate. Both transition strategies resulted in similar outcomes.

## P075

**Cutaneous effects of α-MSH-analogue afamelanotide in treatment of erythropoietic protoporphyria**

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The synthetic analogue of alpha-melanocyte-stimulating hormone [Nle4, D-Phe7]-α-MSH (afamelanotide) binds to the melanocortin-1 receptor (MC1-R) after sun exposure, thereby stimulating melanocytes to produce melanin. The resulting increase in skin pigmentation, along with putative anti-inflammatory properties of afamelanotide, offers the possibility of therapeutic benefit for some photodermatoses. The purpose of this report is to describe cutaneous effects of afamelanotide in a recent clinical trial for the hereditary photosensitivity disorder erythropoietic protoporphyria (EPP). Three dermatology clinical research centres recruited 41 patients with EPP for treatment with afamelanotide (Scenesse<sup>®</sup>) in two prospective, double-blinded, placebo-controlled, multicentre phase III trials and during a subsequent compassionate use period (supported by Clinovel Pharmaceuticals Ltd, Melbourne, Vic., Australia). All subjects received either the active drug or the placebo every 2 months by

means of a slow-releasing subcutaneous implant. The general cutaneous effects observed under this treatment comprised an increased skin pigmentation that was particularly striking in melanoctytic naevi and lentiginos located in sun-exposed body areas. In single cases, we observed pronounced perioral and localized labial pigmentation, arachnoid-shaped hyperpigmentations at the implantation site and linear postinflammatory hyperpigmentation. Additionally, some patients reported facial flushing shortly after implantation. Afamelanotide appeared to be effective in amelioration of the acute, burning and painful photosensitivity commonly experienced by EPP patients, although formal data analysis has not yet been reported. No major side effects were observed. In conclusion, this preliminary data has revealed significant cutaneous responses to this novel drug, which were generally acceptable to patients and would be of sufficient magnitude to explain a therapeutic response in EPP. It remains to be seen if a wider benefit for this drug emerges in other photosensitivity disorders.

**P076**  
**Anti-TNF treatment with etanercept results in early changes of functional gene cluster expression in psoriatic skin lesions**

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**Background:** Different subsets of T-helper (Th) cells are crucially involved in the pathogenesis of Psoriasis and other inflammatory skin disorders. There is accumulating evidence that cytokines secreted early in the affected skin regulate Th-cell differentiation. Using an animal model of Th-differentiation (experimental leishmaniasis) we previously identified cytokines produced by keratinocytes at the first day of infection which control Th-differentiation. Surprisingly we identified IL-4, known as a major Th-2 cytokine, as an important endogenous inducer of Th-1 immunity which highlights the importance of an unbiased experimental approach.

In humans the early phase of disease can not be analysed, but the effect of effective treatment strategies on functional gene clusters could reveal important molecular pathways in the affected skin. We therefore analysed gene expression patterns in psoriatic skin lesions early after etanercept treatment.

**Methods:** Skin biopsies were taken before and 1 week after treatment initiation from lesional and non-lesional skin in patients treated with etanercept. Skin RNA from responders was isolated using standard procedures and subsequently processed for RNA microarray and bioinformatic analysis. Results were confirmed using Real-Time PCR.

**Results:** We identified four gene clusters affected early during etanercept treatment in psoriatic skin lesions: (i) Genes induced in psoriatic skin compared to non-lesional skin and down-regulated by etanercept, (ii) genes down-regulated in psoriatic skin lesions and further down-regulated by etanercept, (iii) genes not regulated in psoriatic skin lesions and down-regulated by etanercept and (iv) genes not regulated in psoriatic skin lesions and up-regulated by etanercept.

Functional clustering revealed a striking overrepresentation of Interferon (IFN)- $\alpha$  responsive genes in cluster (i) and a significant overrepresentation of genes involved in keratinocyte differentiation in cluster (ii).

Interestingly only CCL18 was induced in psoriatic skin (three-fold) and further up-regulated by etanercept resulting in 10-fold induced expression levels compared to non-lesional skin.

**Conclusions:** Functional clustering indicates an effect of etanercept on keratinocytes. Moreover, CCL18 was recently described to induce tolerogenic DCs while overrepresentation of IFN- $\alpha$  regulated genes among genes down-regulated by etanercept could indicate an effect on plasmacytoid DCs. It is tempting to speculate, that etanercept treatment rapidly acts on the local status of proinflammatory and tolerogenic DC activity in psoriatic skin lesions which could subsequently influence Th-cell differentiation.

**P077**  
**Development and application of an autologous full-thickness skin equivalent for non-healing or slowly healing wounds**

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The surgical treatment regime of non-healing or slowly healing wounds is constantly improved. Mesh graft transplants, keratinocyte suspension and acellular dermis are common treatment regimens. For initial coverage of the affected areas skin-like grafts are also used. Although epidermal equivalents and autologous split skin are successfully used, but sometimes they do not give cosmetically satisfying results or it is not possible to achieve it. Aim of this study was development of an autologous full-thickness skin equivalent to acquire a transplant that more closely correlates to undisturbed healthy skin, and is less invasive than other surgical techniques.

Autologous epidermal and dermal cells were isolated, ex vivo expanded and seeded on a collagen-elastin scaffold. The developed full-thickness skin equivalent was morphologically and immunohistochemically characterized and subsequently transplanted onto a non-healing 9 x 6 cm wound located at the right forehead of a 71 year old female patient. The anamnesis showed a severe rheumatoid arthritis, its long-term medication with systemic corticosteroids caused an extensive skin atrophy with high vulnerability. Further efforts to close the wound by conservative and surgical treatment did not succeed. Therefore transplantation of a stratified and differentiated autologous full-thickness skin equivalent seemed to be a worthwhile attempt to cover the ulceration and to induce the healing process.

Characteristic epidermal strata and differentiation markers e.g. involucrin, Ki-67 and collagen IV were identified in the skin equivalent and proved its comparability to healthy human skin. Furthermore characteristics of a functional basal lamina could be verified by the expression of laminin in the area of the dermal-epidermal junction. Transplantation of the skin equivalent showed that within 10 weeks a marked improvement of the wound could be observed.

The present study demonstrates the comparability of the developed full-thickness skin equivalent to healthy human skin and the versatility for treatment of older patients with distinctive skin atrophy, excessive wounds and patients with chronic diseases.

**P078**  
**A prospective evaluation of quality of life, dosing and efficacy in patients with psoriasis and treatment with fumaric acid esters**

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Fumaric acid esters (FAE) has been approved in Germany for systemic treatment of severe psoriasis vulgaris since 1994. More recently, FAE have been recommended for the treatment of moderate and severe psoriasis vulgaris by the German S3 guideline for the therapy of psoriasis. This is the first study evaluating the improvement in quality of life under treatment with FAE. In addition, FAE dosing and efficacy were investigated. In this prospective study patients with different severity of psoriasis vulgaris treated with FAE were included. Main efficacy endpoints were Dermatology Life Quality Index (DLQI), EuroQoL 5 dimension (EQ-5D), Physician Global Assessment (PGA) and Psoriasis Area and Severity Index (PASI), before and at 3, 6 and 12 months of treatment.

A total of 249 patients (44% females, 56% males) were included at 84 dermatological centers. Mean age was 49.7 years, mean age at diagnosis was 33.3 years, mean PASI score was 16.8 and mean DLQI score was 10.5. At the beginning of the study 4.8% of the patients suffered from mild, 34.1% from moderate, 44.1% from moderate to severe, 16.2% from severe and 0.8% from very severe psoriasis by meaning of the investigator. The mean dosage of FAE (Fumaderm<sup>®</sup>) was 2.8 tablets per day. While

more than 70% of patients received one to three tablets per day, <30% received high dose FAE therapy (four to six tablets per day). The mean PASI score decreased from 16.8 to 5.6 after 12 months of investigation (overall PASI reduction of 67%). The overall mean DLQI score decreased from 10.5 to 3.3, whereby the quality of life correlated with the severity of the psoriasis. PGA and EQ-5D scores also showed an improvement, but less dominant than the improvement of PASI and DLQI. Treatment with FAE was generally well tolerated and the typical drug-related adverse events were documented. In conclusion, this study shows for the first time the significant improvement in quality of life in patients with psoriasis under FAE treatment. Furthermore, the data show that clinical efficacy and quality of life improvement can be achieved with a mean FAE dosage of only 2.8 tablets per day.

**P079**  
**Plasmacytoid dendritic cells in pemphigus vulgaris**

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**Background:** Pemphigus vulgaris (PV) is a severe autoimmune blistering skin disease in which autoantibodies (auto-ab) are developed mainly against the desmosomal cadherin desmoglein-3 (Dsg3). The contribution of innate immune cells to the initiation and perpetuation of this organ-specific autoimmune disease is largely unknown.

**Objectives:** Aim of this study was to investigate plasmacytoid dendritic cells (pDCs) and the expression of related chemokine-chemokine receptors systems by antigen presenting cells (APC) in PV patients. **Methods:** Blood from the PV patients and healthy donor were collected after an informed consent. PBMCs were isolated by Percoll gradient centrifugation. Cells were processed further for FACS analysis and also cultured in RPMI medium containing L-glutamine, Pen/Step and 10% FCS for *in vitro* TLR stimulation assays. In sera, we performed ELISA analysis for the chemokine ligands MIP-3 $\alpha$  (CCL20) and MIP-3 $\beta$  (CCL19), which are specific for the receptors CCR6 and CCR7 respectively.

**Results:** Based on flow cytometric analysis, our preliminary data showed a profound increase in APCs especially CD14+ monocytes, CD68+ macrophages, CD11c+BDCA-2+ dendritic cells and CD66b+ granulocytes in pemphigus patients compared to the healthy controls. The surface expression of HLA-DR was also increased on APC from the PV patients. Furthermore we analyzed the expression levels of major chemokine receptors expressed by the APCs such as CCR2, CCR4, CCR6 and CCR7. ELISA results showed a decrease in the average MIP-3 $\alpha$  levels in PV patients (7.65 pg/ml) having higher autoantibody titers than healthy controls (14.55 pg/ml). Apart from DCs, it has been previously shown that the chemokine receptor CCR6 is also expressed by regulatory T-cells (Tregs). Consistent with serum MIP-3 $\alpha$  levels, we previously observed a decrease in circulating Tregs in PV patients. Functional *in vitro* analysis of PBMC by culturing and stimulating with various TLR ligands such as LPS, R848 and RNA40, showed a significant increase in IL-6 and IFN- $\alpha$  secretion by PBMC from pemphigus patients compared to healthy controls. In order to rule out the possibility of increased global cytokine levels in the pemphigus patients due to an increased APC compartment, further investigations are underway to characterize these APCs at the cellular level for their cytokine secretion capabilities. In summary, these preliminary results suggest that both quantitative and qualitative differences in the APC compartment, especially in pDC, might contribute to the pathogenesis of PV. Our findings warrant further investigations of the role of innate immune stimuli in pemphigus.

**P080**  
**The TNF $\alpha$  blocker etanercept ameliorates psoriasis by downregulating proinflammatory slan (6-sulfo LacNAc) dendritic cells**

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Slan (6-sulfo LacNAc) dendritic cells (slanDCs) are potent proinflammatory TNF $\alpha$ , IL-6, IL-1 $\beta$ , IL-23 and IL-12 producing DCs. Because of this cytokine profile slanDCs have the capacity to induce Th1/Th17 directed immune responses. The numbers of slanDCs were found to be highly increased in lesional psoriatic skin.

Therefore, blocking the function of slanDCs could be an effective therapeutic intervention in psoriasis. Blockade of TNF $\alpha$  by biologic agents is one of the most powerful therapies for psoriasis. However, it is not known whether and how these biologics influence proinflammatory slanDCs. We therefore analysed slanDCs in skin and blood during a 24 week treatment period of 10 patients suffering from psoriasis vulgaris with etanercept.

In skin of responding patients (70% PASI response) the number of slanDCs was significantly reduced already after 4 weeks of treatment and reached values of healthy skin after 24 weeks. The decline of slanDCs was accompanied by a reduction of CD11c+ cells in psoriatic skin. Before treatment 50% of slanDCs were stained positive for CD11c in psoriasis. After 24 weeks of treatment CD11c could only be detected on 25% of slanDCs indicating their reduced activation status. The reduction of slanDCs was, in contrary to CD11c+ cells, not induced by caspase dependent apoptosis, because staining for slanDCs and active caspase3 did not show double positive cells after 4 weeks of treatment. It might be mediated by reduced recruitment of slanDCs in the skin because lesional expression of the chemokine CX3CL1 was downregulated by etanercept.

In blood of patients the percentage of slanDCs among mononuclear cells increased two times with a maximum at week 4. Other mononuclear cells did not show this increase. In parallel, the mean fluorescence intensity of HLA-DR, but not CD86, expressed on slanDCs declined by 30% after 4 weeks of treatment. This was not changed during the following 20 weeks and indicates a reduction of their proinflammatory capacity.

Furthermore, therapeutic concentrations of 10 g/ml etanercept significantly reduced the secretion of IL-1 $\beta$ , IL-6, IL-23, IL-12 by slanDCs after LPS stimulation *in vitro* by 25, 13%, 50% and 40%, respectively. This indicates an inhibitory effect of etanercept on maturation and function of slanDCs.

In summary our data suggest, that the decline of the number of slanDCs in skin and reduction of their proinflammatory potential is one new mechanism of TNF blockade by etanercept in psoriasis. This finding further supports the importance of slanDCs as proinflammatory DCs in the pathogenesis of psoriasis.

**P081**  
**Persistence of bacteria like *Pseudomonas aeruginosa* in non-healing venous ulcerations**

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**Background:** There is evidence, that chronic inflammation due to bacterial wound colonization especially by pathogens with biofilm-producing capabilities delays wound healing. Only few data are available if or how the microbiological spectrum changes over a long time in a chronic wound in an individual patient. Consequently, the aim of this clinical investigation was to analyze possible changes in the spectrum of microbial colonization in patients with non-healing venous leg ulcers in a specialized dermatological outpatient wound clinic comparing.

**Objective:** In this study, in a cohort of chronic wound patients in our out-patient clinic the microbiological spectrum between the year 2005 versus 2010 was analyzed as well as in out-clinic patients with continuous ulceration sequentially over a decade from 2001 to 2011.

**Methods:** We retrospectively analysed 126 patients with single or multiple chronic leg ulcers with chronic venous insufficiency as the only or a major contributing cause. Microbiological tests were car-

ried out with the so-called Levine technology after removal of wound dressings, before cleaning the wound, with light pressure on a central area of the wound surface. Following 2009, the 'Essener Kreis' was instituted, a centripetal technique in which the bacterial smear is taken moving from the outer edge of the wound toward the center by circular and rotating motion in order to gain a representative sample of material from all areas of the wound.

Results: In a cross-sectional analysis, in both 2005 and 2010 *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Proteus mirabilis* and *Enterococcus faecalis* respectively were the most common pathogens colonizing chronic leg ulcers. In a longitudinal analysis from 2001 to 2010 with patients with non-healing leg ulcerations during this time, strains like *P. aeruginosa* or *S. aureus* with biofilm producing capacities seem to persist over the years in individual patients despite antiseptic treatment. In 20/35 patients of those patients the same bacteria species could be identified in more than three consecutive years. If we refer to our results of the most frequent bacteria like *P. aeruginosa* or *S. aureus* with about 20% occurrence in our population, the statistical probability according to the probability law to detect the same bacteria three times in a row is only 0.8%.

Limitations: This is a retrospective study. Therefore, swab procedure and time point of sampling are different in some ulcerations. Unfortunately, there might also be differences in culture techniques of detecting all species of bacteria.

Conclusion: This 'domestic' persistent colonisation and subsequent chronic subclinical inflammation might result in delayed or absent wound healing of the colonized ulcerations. Persistence of pathogens might not only be due to the patient's wound environment but due to the capability of chronic residency of those bacteria. There are data confirming that the use of serial debridement to continually remove biofilms, followed by biofilm wound management strategies while the bioburden is still immature and more susceptible is an effective therapeutic regime. Consequently, strict hygienic management of chronic wounds in combination with more aggressive physical or antiseptic treatments to eradicate pathogenic bacteria, especially *P. aeruginosa* is needed.

## P082

### Prospective, randomized, investigator-blind, controlled therapy study on treatment of haemangioma of infancy: pulsed dye laser versus cryotherapy versus observation

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Treatment of superficial haemangioma of infancy is still a matter of debate because the majority of lesions shows spontaneous involution. However, unpredictable proliferation and complications such as scarred appearance of the lesional skin after involution, ulceration, cosmetic problems, and psychologic strain on both parents and infants are strong arguments for treatment. We assessed the benefit/risk ratio of two minimal invasive treatment options [pulsed dye laser therapy (PDL), wavelength = 585 nm; cryotherapy (CRYO) using Peltier effect, T = 32°C] which can be done in an outpatient setting versus the presently practised "wait-and-see" strategy.

We included 182 infants (55 m/127 f) with one (n = 124) or several (n = 58) superficial haemangioma in the early progressive or the indifferent phase with a maximum diameter of 30 mm and randomly assigned them to three groups: PDL (n = 70), CRYO (n = 54), and observation (OBS; n = 58). In cases with two or more haemangioma we assigned one haemangioma indicator lesion, handled all lesions in the same way, but evaluated only the indicator lesion. Treatment and assessment were done separately by two teams. The parents were obliged not to inform the investigator team which group their child had been assigned to. The lesions were treated up to three times in monthly intervals. Follow-up was done at 1, 2, 3, 4, 6 and 12 months after inclusion. We defined the following evaluation criteria: complete remission (CR), partial remission (PR), stop of growth (SG), progression (PRO); blistering, crust, scar, hypopy hyper pigmentation. Furthermore, there was a grading of the cosmetic appearance of the vascular tumour by parents: 1 (cosmetically acceptable) to 4 (cosmetically not acceptable). We took standardized clinical photos by using a colour and a metric scale.

At the start the size of the lesions was as follows: area (0.75–27.0 mm; mean = 7.6 mm); height (0.0–5.9 mm; mean = 0.89 mm); parental cosmetic assessment (value 1 = 36.7%; value 2 = 20.1%; value 3 = 18.3%; value 4 = 24.9%). The distribution of area and height was rather equal in all groups except height in CRYO where we found significantly higher haemangioma. At the 12-months follow-up the overall assessment was: PDL (CR 37.1%/PR 28.6%/SG 4.3%/PRO 30.0%), CRYO (CR 49.1%/PR 17.0%/SG 1.9%/PRO 32.1%), OBS (CR 8.8%/PR 26.3%/SG 10.5%/PRO 54.4%). The frequency of adverse effects was higher in the therapy groups (PDL 34.7%; CRYO 37.0%) when compared to the observation group (10.7%). Furthermore, we looked at the change of area and height separately by calculating the ratio of the mean diameter/height at 12 months and the mean diameter/height at the beginning. Treated haemangioma showed a significantly faster decrease both in area (PDL and CRYO  $P < 0.05$ ) and in height (PDL  $P < 0.05$ ; CRYO  $P = 0.06$ ) with time. 64.6% of parents in PDL stated cosmetic improvement, respectively 49.0% in CRYO, and 43.4% in OBS.

Both, PDL and CRYO showed a significantly faster and more pronounced involution of infantile haemangioma when compared to the natural course. The efficacy is the higher the smaller the lesion is at the start of treatment. However, the risk of complications appears to be higher in treated haemangioma. Our study clearly shows that both treatment strategies are rather safe and effective in infantile haemangioma in the early progressive phase.

## P083

### Age and skin site related differences in steroid metabolism in male skin point to a key role of sebocytes in cutaneous hormone metabolism

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Hormone concentrations decline with advancing age, promoting the worsening of skin structure and the visible signs of skin aging. Up to now it was not clear, whether age-dependent differences of steroid hormone concentrations in blood samples are also present in cutaneous suction blister fluid, and whether skin from different anatomical sites shows different steroid hormone concentrations.

Analysis of suction blister fluids and paired blood samples from young and old male subjects by UPLC-MS/MS showed inverse patterns of DHEA and androstenedione concentrations. The concentration of DHEA in blood samples was age-dependently significantly reduced from 46.8 nM (young cohort, mean age 27.8 years) to 21.6 nM (old cohort, mean age 62.6 years). On the contrary, the concentration of DHEA in suction blister fluids was age-dependently increased from 9.0 to 22.6 nM. The androstenedione concentration in blood samples of young subjects was lower (1.2 nM) than of old subjects (2.5 nM), with an inverse pattern measurable in suction blister fluids (1.2 nM vs 0.6 nM). Testosterone decreased age-dependently from 12.9 to 10.8 nM in blood samples and from 4.9 to 3.2 nM in suction blister fluids. Regarding skin sites, DHEA was lower in samples from upper back (9.0 nM) compared to samples from the forearm (18.1 nM). In contrast, the concentrations of androstenedione (1.2 nM vs 0.9 nM) and testosterone (significant 4.9 nM vs 2.8 nM) were higher in samples from upper back. *In vitro* analyses showed that only SZ95 sebocytes, but neither primary fibroblasts nor keratinocytes, were able to use DHEA as precursor for testosterone. Additionally, expression of 3-beta-hydroxysteroid dehydrogenase could only be observed in sebocytes, by Western blotting of SZ95 sebocytes lysates and immunostaining of sebaceous glands in facial biopsy samples.

In conclusion, our experiments show an inverse pattern of DHEA and androstenedione concentrations in blood versus suction blister fluids, highlighting age-dependent changes of dermal testosterone bio-

synthesis, and a stronger testosterone metabolism in young skin. Furthermore, sebocytes play a central role in cutaneous androgen metabolism.

## P084

### Evidence for epigenetic modulation of vitamin D signaling in melanoma cell lines

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We previously showed that some melanoma cell lines are resistant to the antiproliferative effects of biologically active vitamin D metabolite 1,25(OH)<sub>2</sub>D<sub>3</sub>. We also reported that 1,25(OH)<sub>2</sub>D<sub>3</sub>-sensitivity can at least in part be restored by co-treatment with histone deacetylase inhibitor (HDACI) Trichostatin A (TSA) or with DNA methyltransferase inhibitor (DNMTI) 5-azacytidine (5-Aza). Treatment of 1,25(OH)<sub>2</sub>D<sub>3</sub>-responsive and -resistant melanoma cell lines and normal human melanocytes NHM with 1,25(OH)<sub>2</sub>D<sub>3</sub> and/or epigenetic drugs (5-Aza, TSA) modulated VDR mRNA expression in 1,25(OH)<sub>2</sub>D<sub>3</sub>-responsive and -resistant melanoma cell lines and NHM. Treatment with 5-Aza, but not with TSA, reduced expression of the putative vitamin D receptor (VDR)-regulating microRNA miR-125b in 1,25(OH)<sub>2</sub>D<sub>3</sub>-responsive and -resistant melanoma cell lines and in NHM. Treatment with 1,25(OH)<sub>2</sub>D<sub>3</sub> and/or epigenetic drugs (5-Aza, TSA) reduced miR-27b expression in three out of four melanoma cell lines. Moreover, we found no difference in VDR protein expression in 1,25(OH)<sub>2</sub>D<sub>3</sub>-responsive as compared to 1,25(OH)<sub>2</sub>D<sub>3</sub>-resistant melanoma cell lines. Transfection with miR-125b antisense did not affect VDR mRNA/protein expression in IGR cells. We speculate that responsiveness to the antiproliferative effects of 1,25(OH)<sub>2</sub>D<sub>3</sub> corresponds to the expression level of VDR in target cells, which in turn might be regulated by VDR microRNAs or epigenetic modulating drugs. In summary, we report new insights into the molecular mechanisms that underlie the responsiveness of malignant melanoma to 1,25(OH)<sub>2</sub>D<sub>3</sub>.

## P085

### Association of genetic variants of the vitamin D receptor (VDR) with cutaneous squamous cell carcinomas (SCC) and basal cell carcinomas (BCC): a pilot study in a German population

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Background: Vitamin D receptor (VDR) polymorphisms have important implications for vitamin D signalling and are associated with various malignancies.

Patients and Methods: In a German population the frequency of several VDR polymorphisms (ApaI, TaqI, BglI) in basal cell carcinomas (BCCs, n = 90) and cutaneous squamous cell carcinomas (SCCs, n = 100) as compared to healthy controls (n = 51), was analyzed.

Results: Impressive variations in the frequency of some VDR genotypes were found when comparing skin of cancer patients and controls. An association of the genotype AaTtBb with BCC risk was found (BCC: 45.7%, SCC: 39.8% and controls: 38.0%). The genotype aaTTBB was exclusively found in the control group (20%), which suggested that this genotype may be protective against skin carcinogenesis. Moreover, the aaTtBb genotype was associated with skin cancer risk, being found at a much higher frequency in BCCs (21%) and SCCs (17%) as compared to controls (8.0%). Comparison of the frequencies of the VDR genotypes in sunlight-exposed versus not sunlight-exposed skin areas revealed BB 30.1% versus 7.1% respectively in BCCs and BB 28.1% versus 0.0% respectively in SCCs, indicating that vitamin D signalling may be of importance for photocarcinogenesis of the skin. Associations also indicated that the ApaI and TaqI genotypes may be of importance for photocarcinogenesis of BCCs, but not for SCCs. Comparison of the VDR genotype frequencies by age (younger than 60 years versus 60 years or older) revealed no evidence of age-dependent variations in patients with BCCs or SCCs. Conclusion: VDR polymorphisms are of importance for the development of BCCs and cutaneous SCCs, but further explorations of these findings and their implications are required.

## P086 (V02)

### Low serum 25-hydroxyvitamin D concentrations are associated with increased risk for melanoma and unfavourable prognosis

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Background: Low serum vitamin D (25(OH)D) concentrations are associated with an increased incidence and an unfavourable outcome of various types of cancer. However, there are limited data on influence of serum 25(OH)D on risk and prognosis of malignant melanoma (MM).

Patients and Methods: Basal serum 25(OH)D levels were analyzed in MM patients (n = 324) and healthy controls (n = 141). The retrospective study started in 2000, patients were observed until death or December 2004, whichever came first.

Results: Patients with low serum 25(OH)D concentrations (<10 ng/ml, n = 77) had a significantly (P = 0.006) greater thickness (median: 1.9 mm) of their primary tumors as compared to patients with higher levels (>20 ng/ml; n = 108; median: 1.00 mm). Patients with 25(OH)D serum levels in the lowest quartile had a shorter survival (median: 80 months) comparing with the highest quartile (median: 195 months; P = 0.0049). Patients of younger ages (<51 years) had statistically significant (P = 0.0049) thinner primary tumors than MM patients of older ages (>52) as well as higher median serum 25(OH)D levels (P = 0.003). Conclusions:

Our data strongly support the hypothesis that serum 25(OH)D levels are associated with risk and prognosis of MM. Further studies are urgently needed to investigate the role of the vitamin D endocrine system in MM.

## P087

### Development of a non radioactive aromatase assay with primary human skin fibroblasts

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Aromatase is a member of the P450 enzyme family. It is expressed in a variety of tissues and cell species e.g. in liver, skin and adipose tissue. The aromatase is located in the membrane of the smooth endoplasmic reticulum and catalyzes two reactions in the process of estrogen biosynthesis. Principally the aromatase catalyzes the transformation of androgens to estrogens, specifically androstenedione is transformed to estrone and testosterone is converted to estradiol.

In our experimental setup primary human skin fibroblasts were incubated for 1–14 days with different concentrations of testosterone (0.1, 1, 5, 10 and 20 M) as substrate for the aromatase. As analytical parameter of aromatase activity the estradiol concentration was measured in the cell free culture medium using the Roche Elecsys 2010 system. Additionally, the cell viability was investigated by AlamarBlue.

To determine a suitable aromatase inhibitor without side effects the skin fibroblasts were treated for 3 days with 20 M testosterone and different concentrations (1, 5, 10, 20 and 50 M) of apigenin, 7-OH-flavone and chrysin, respectively. In addition to the estradiol measurement the cell viability was determined by AlamarBlue, the cell proliferation by BrdU incorporation and the cytotoxicity by LDH assay. To study the stimulating effect on the aromatase activity the dermal cells were treated for 3 days with 10 M testosterone and different concentrations of the glucocorticoid dexamethasone (0.01, 0.1 and 1 M).

It could be shown that the measured estradiol concentration increases in primary human skin fibroblasts depending on the substrate concentration. Furthermore, our data reveal that 7-OH-flavone inhibits aromatase without side effects while dexamethasone stimulates aromatase activity.

From these results we conclude that the described non radioactive assay is suitable to monitor aromatase activity in primary human skin fibroblasts.

## P089

### No association of vitamin D signaling-related polymorphisms and melanoma risk as well as melanoma prognosis: a case–control study

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The vitamin D endocrine system (VDES) contributes to the pathogenesis and prognosis of malignancies including cutaneous melanoma. An expression of the vitamin D receptor (VDR) and an anti-proliferative effect of vitamin D in melanocytes and melanoma cells have been shown *in vitro*. More recently, an association of several VDR polymorphisms with melanoma risk and prognosis has been reported. However, other genes are also crucial for the functional integrity of the VDES. Studies examining associations of polymorphisms in genes coding for vitamin D metabolism-related proteins (1-hydroxylase [CYP27B1], 1,25(OH)2D-24hydroxylase [CYP24A1], the vitamin D-binding protein [VDBP]) and cancer risk are scarce, especially with respect to melanoma. In our hospital based case-control study including 305 melanoma patients and 370 healthy controls single nucleotide polymorphisms in the genes CYP27B1 (rs4646536), CYP24A1 (rs927650, rs2762939), VDBP (rs1155563, rs7041), and VDR (rs757343, rs731236, rs2107301, rs7975232) were analyzed for their association with melanoma risk and prognosis. Except rs2762939, rs731236, and rs2107301 the other five polymorphisms have not been analyzed regarding melanoma before. After the identification of age, skin type, and number of nevi as independent demographic and phenotypical melanoma risk factors in our study group (Schoof et al., 2009; Blankenburg et al., 2005a) we analyzed every single SNP for its association with melanoma risk in a dominant as well as an additive logistic regression model integrating these risk factors. As the primary tumor thickness was documented we also analyzed the association of every single SNP with melanoma prognosis in a dominant and an additive linear regression model predicting the Breslow tumor thickness. In summary, none of the analyzed SNPs showed an association with melanoma risk or melanoma prognosis neither in the dominant logistic regression model nor in the additive logistic regression model in our representative study group. Further studies with larger study populations and an optimized age match of controls, drawbacks in our setting, may be indicated.

## P090

### Anti-inflammatory and anti-pruritic activity of topical kappa-opioid receptor agonists in a murine model of oxazolone-induced delayed type hypersensitivity

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Small molecule kappa-opioid receptor (KOR) agonists have been widely investigated as potential analgesics and for the treatment of addiction. However, effects in the central nervous system are accompanied by side effects such as sedation, dysphoria or diuresis. In 2009 nalurfaline hydrochloride (Remitch<sup>®</sup>) was launched in Japan as treatment for uremic pruritus. The compound is a potent KOR agonist with low selectivity versus mu-opioid receptors (MOR) and a high selectivity against delta-opioid receptors (DOR). Most importantly, nalurfaline is not restricted to the periphery and is described to exhibit its anti-pruritic action by activating central KORs. Nalurfaline has been successfully tested in various murine models of itch induced by histamine, substance P, deoxycholic acid, morphine and compound 48/80. Furthermore, it has been evaluated in a spontaneous atopic dermatitis model in mice. The compound was applied orally, and in some instances subcutaneous dosing was used. Anti-inflammatory effects of nalurfaline hydrochloride *in vivo* have not been described, yet.

In this study, the efficacy of topical nalurfaline or of a proprietary, potent and selective KOR agonist (WO20090450-007) was assessed in a mouse model of oxazolone-induced delayed type hypersensitivity regarding amelioration of itching and inflammation. Female BALB/c mice were sensitized with oxazolone (1% in 100 I acetone) on day 0 and challenged on days 7, 9, and 11. Mice were treated daily beginning from day 11 (1 h before challenge with oxazolone) until day 18 with DMSO (50 I, topically), nalurfaline (1% and 0.2% in 50 I DMSO, topically) and WO20090450-007 (1% and 0.2% in 50 I DMSO, topically) or betamethasone dipropionate (0.05% in 50 I DMSO, topically) as positive control. Interestingly, on day 11 both KOR agonists as well as the positive control did reduce scratching as measured by an electronic scratch movement recording significantly not only in the early phase after the challenge with oxazolone but over a period of 22 h. Similar results were obtained on day 18. Ear thickness increased from day 11 to day 14 and 15, resp., in contrast to the betamethasone dipropionate group. However, ear swelling decreased in mice treated with KOR agonists significantly faster as compared to vehicle control. Histological analysis (H&E) of treated ears showed dose dependent effects on ear thickness, epidermal thickness and dermal infiltrate.

The presented results show that topical application of KOR agonists reduces not only scratching in a contact dermatitis model but also swelling and inflammation.

## P091

### Where do human Merkel cells come from? Immunohistochemical analyses of putative progenitor cell markers and potential regulators

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The origin of Merkel cells, neuroendocrine cells of skin, has been a controversial issue for several decades. Two alternative lineages were discussed, an epidermal and a neural lineage. Recently, three studies using transgenic mice demonstrated that in the mouse, Merkel cells arise from epidermal stem cells

during ontogenetic development. Moreover, it was shown that epidermal stem cells are responsible for Merkel cell homeostasis in adult mice. Although these findings may imply an epidermal origin of human Merkel cells, it has to be considered that Merkel cell distributions in human and mouse skin are significantly different, suggesting functional differences. We therefore aim at identifying progenitor cells responsible for Merkel cell homeostasis in human skin. During differentiation from stem cells to Merkel cells, cells are likely to pass through a transition state characterized by the parallel expression of stem cell marker proteins and Merkel cell marker proteins. To identify such transitional cells, we investigate human epidermis by double immunofluorescence analyses of epidermal stem cell markers and Merkel cell markers. We do not observe an overlap between expression of CK14 or CK15, expressed by Merkel cell progenitors in the mouse, on the one hand and CK20, a cytokeratin specific for Merkel cells, on the other hand. However, even though striking, this does not exclude a differentiation from epidermal stem cells to Merkel cells, as the postulated transition state might be characterized by the expression of further proteins, e.g., the epidermal stem cell markers Lrig1 and CD200 and the Merkel cell-specific cytokeratins CK8/18, but an absence of CK20. In ongoing experiments, we therefore analyze the potential co-expression of additional proteins typically present either in epidermal stem cells or in Merkel cells. To investigate a potential neural origin of human Merkel cells, proteins typically found in neural stem cells of skin, e.g. p75<sup>NTR</sup>, are also included. Moreover, we study putative regulators of stem cell differentiation to Merkel cells, including the transcription factors Atoh1, NFI-A, and the transcriptional cofactor PC3/Btg2. NFI-A and PC3 were immunohistochemically detected in human epidermis for the first time.

In conclusion, our data suggest that different mechanisms might underlie Merkel cell homeostasis in mouse and man.

## P092

### The pregnane X receptor (PXR) controls Langerhans cell migration via CCR7

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The pregnane X receptor (PXR) is a ligand-activated transcription factor regulating genes central to drug and hormone metabolism in the liver. We here show that PXR is highly expressed in different subsets of mouse and human immature DC and is down-regulated in mature DC including Langerhans cells (LC). PXR activation down-regulates CCR7 expression at the cell surface of mouse LC without affecting expression of other maturation markers such as CD40, CD86 and CXCR4 *in vitro*. Similarly, transgenic overexpression of the huPXR also called SXR (Tg-SXR) in LC decreases expression of CCR7, mimicking effects of pharmacological activation of PXR. Interestingly, transgenic co-expression of CYP3A4 (Tg-SXR/CYP), a well-known PXR downstream gene, further decreased CCR7 expression by LC, suggesting an involvement of CYP3A4 in this regulation. In contrast, treatment of cells with A-792611, a novel potent and specific antagonist of PXR, up-regulates CCR7 expression at the cell surface of mouse and human LC. *In vivo*, PXR deficiency increases percentages of LC while transgenic expression of huPXR decreases percentages of LC in skin draining lymph nodes of mice after skin sensitization with a contact allergen. Transgenic overexpression of both SXR and CYP3A4 in LC further increases the percentages of LC in skin draining lymph nodes of sensitized mice. Furthermore, langerin+ cells loose PXR while migrating into skin tumors and PXR expression is lowered in intra-tumoral CCR7+ cells in mice. All together these results demonstrate that PXR controls LC migration via CCR7 with relevance to tumor context.

## P093

### Differential effects of high glucose on human keratinocytes and fibroblasts and the impact of KdPT on glucotoxicity

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Foot ulcers are a major complication in diabetes leading to significant morbidity and mortality among patients. Here, we investigated the effects of high glucose on normal human keratinocytes (NHK) and human dermal fibroblasts (HDF), two effector cell types in wound healing. High glucose (10–30 mM) time- and dose-dependently reduced metabolic activity and viability of NHK. This correlated with decreased cell proliferation, but in contrast to other cell types, with no significant induction of apoptosis. Hyperglycemic conditions led to increased vacuolisation of the cytoplasm which was however not correlated with an increased expression of autophagic markers. Moreover, high glucose significantly reduced migration of NHK as shown by scratch- and transwell-migration assays. Mechanistically, high glucose increased intracellular oxidative stress and induced the expression of glucose-regulated-protein-78, a marker of endoplasmic reticulum stress. Next, we investigated the effects of antioxidants and KdPT, the latter a truncated tripeptide of alpha-MSH with cytoprotective properties, on glucotoxicity. Vitamin C and KdPT significantly reduced high glucose-induced intracellular oxidative stress. However, only KdPT, but not vitamin C, significantly increased metabolic activity and viability of NHK, indicating that mechanisms other than oxidative stress are involved in the above mentioned effects of high glucose on NHK. In marked contrast to NHK, high glucose did neither affect cell viability nor increased oxidative stress in HDF. This could be attributed to an altered metabolic rate of HDF or differential expression of glucose transporters. In summary, our data create a basis for better understanding the mechanisms of impaired wound healing in diabetes and possibly point towards novel therapies for diabetic foot ulcers.

## P094

### Skin quality and stress reaction of young and old skin can be influenced by skin care products

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Introduction: Skin alterations throughout life mirrors body aging. Skin aging is associated with dryness, roughness and changes of surface texture. It is also associated with changes in the composition of sebum as well as a disturbed response to stress. To evaluate the latter process the effect of skin aging on its reaction to mechanical stress was studied in different age groups. In addition, the effectiveness of skin care products in normalizing the signs of skin stress in different ages was studied. Patients and Methods: Skin quality parameters were measured (hydration level of stratum corneum by Corneometer, transepidermal water loss by Tewameter, skin pH and erythema level by Mexameter) and skin mechanical stress was evaluated (stripping with D-squame tapes 18x) at the forearms of two groups of healthy individuals younger than 37 years and older than 65 years. Measurements were performed at baseline and 1, 6, 24 h and 7 days after administration of mechanical stress. Three different 4 cm<sup>2</sup> areas of the stressed skin were treated daily with three different skin care products (two creams supplied by LVMH and a standard DAC base cream). A forth area was left untreated.

Results: The aged subjects' skin exhibited higher pH level, stronger erythema and higher hydration of the stratum corneum in comparison to young subjects' skin. Young and aged subjects reacted differently to stress. The aged subjects showed an immediate elevation of TEWL and hydration levels but a



delayed erythema reaction, in comparison with the young skin. The application of topical products – but not all – were able to diminish the skin reaction to stress in young but not in old individuals and only some products can slow down the normal repair process.

**Conclusion:** Young and aged skin differ in pH level, erythema and hydration. They respond to stress in a different manner, whereas young skin reacts immediately with erythema as early as the first hour and old skin shows a delayed and weaker response. Certain skin care products – but not all – can normalize the skin reaction to stress and improve the skin quality measurements.

#### P095

### Tropisetron, a serotonin antagonist, modulates the inflammatory cell response of human epidermal melanocytes and keratinocytes after exposure of UVB light or TNF-alpha

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Ultraviolet (UV) light has a key role in skin carcinogenesis. Proinflammatory cytokines such as tumor necrosis factor (TNF)-alpha mediate some of the inflammatory responses of epidermal cells after UVB treatment and sustained activation of their signaling pathways are implicated in photocarcinogenesis. Interestingly, there is increasing evidence for a modulatory role of serotonin (5-hydroxytryptamine, 5-HT)-mediated pathways in the control of inflammatory responses in various organs of the human body. However, the role of anti-serotonergic strategies in the inflammatory UVB response remains largely unexplored. Using tropisetron, a 5-HT-receptor (5-HT-R) modulating agent approved as an antiemetic, we investigated the effect of this drug on UVB- and TNF-alpha-mediated induction of proinflammatory mediators such as interleukin (IL)-6, IL-8 and cyclooxygenase-2 (COX-2) in human epidermal keratinocytes (NHK) and melanocytes (NHM). Tropisetron at doses from 10 to 10 µg/ml attenuated UVB- and TNF-alpha-induced IL-6, IL-8 and COX-2 mRNA expression in both cell types as shown by real-time RT-PCR. This suppressive effect of tropisetron on UVB- and TNF-alpha-mediated increase of IL-6 and IL-8 expression was confirmed at protein level in NHK. The kinetics of the attenuating effect of tropisetron differed among NHK and NHM. Accordingly, NHK were in general more sensitive than NHM towards the drug. Mechanistically, tropisetron reduced TNF-alpha-mediated nuclear translocation of p65/NF-KappaB in NHK but neither affected p38-signaling nor IKKalpha/Beta-degradation. Importantly, this effect of tropisetron was independent of endogenously produced 5-HT. In support of a 5-HT-receptor-independent action of the drug, the putative tropisetron receptors 5-HT3-R and 5-HT4-R were undetectable in both cell types at RNA and protein level. However, the expression of the closely related alpha7 nicotinic acetylcholine receptor (alpha7 nAChR) was detected in NHK but not in NHM suggesting that this receptor could be the mediator of the anti-inflammatory effect of tropisetron in NHK. In summary, our results highlight an anti-inflammatory potential of tropisetron in epidermal cells and point towards future strategies in the treatment of inflammatory skin diseases via targeting the alpha7 nAChR.

#### P096

### Macrophage-activating lipopeptide two regulates the stearyl-CoA desaturase/fatty acid desaturase two proinflammatory signalling in human SZ95 sebocytes

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**Background:** Previous own studies have shown that stearyl-CoA desaturase (SCD) is not only involved in lipogenesis as previously suggested, but also in the proinflammatory sebaceous gland signalling in animals and human SZ95 sebocytes *in vitro*. According our data, SCD is a key enzyme in bactericidal fatty acid biosynthesis together with fatty acid desaturase-2 (FADS2), an enzyme catalyzing the conversion of the essential fatty acid linoleic acid to the proinflammatory arachidonic acid. On the other hand, we have reported that bacterial antigens, and not P. acnes itself, may be involved in the development of inflammatory acne lesions. Indeed, lipopolysaccharides but mostly macrophage-activating lipopeptide 2 (MALP-2), a ligand of the Toll-like receptor-2 (TLR-2), induces the expression of SCD and FADS2 and stimulates the secretion of interleukin (IL)-6 and IL-8 in SZ95 sebocytes.

**Objectives:** To further elucidate the inflammatory signalling interaction and biological significance of MALP-2, SCD and FADS2 in SZ95 sebocytes.

**Methods:** SZ95 sebocytes were treated with MALP-2 (10 and 50 ng/ml), and the combination of MALP-2 and the SCD inhibitor FPCA ( $10^{-8}$  and  $10^{-6}$  M). Phorbol myristate acetate and dexamethasone were used as controls. Cytotoxicity was determined by the lactate dehydrogenase assay. SCD, FADS2 and TLR-2 mRNA levels were assessed by semi-quantitative RT-PCR and protein expression by western blot analysis. Determination of IL-6 and IL-8 cell release was evaluated by ELISA.

**Results:** A single MALP-2 treatment induced an increase of TLR-2 mRNA levels (up to 128%) at 12–24 h, SCD mRNA (up to 25%) and protein (up to 63%) levels at 1.5–12 h, and FADS2 mRNA (up to 49%) and protein (up to 42%) levels at 6–24 h in SZ95 sebocytes. A clear proinflammatory pattern was registered: MALP-2 strongly upregulated IL-6 (up to 110%) and IL-8 (up to 335%) SZ95 sebocyte secretion. Concomitant treatment with MALP-2 and the SCD inhibitor FPCA reduced the MALP-2-enhanced SCD and FADS2 mRNA and IL-8, but not IL-6, protein levels.

**Conclusions:** MALP-2 induces the expression of its receptor TLR-2 and stimulates the SCD/FADS2 pathway in human sebocytes. While the MALP-2-enhanced proinflammatory IL-8 secretion is – at least partially – SCD/FADS2-dependent, the sebaceous gland characteristic IL-6 increased secretion is independent.

#### P097

### Microarray-based analysis to identify new genes relevant for aging in human female hair follicles

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Hair aging as a so far quite unaddressed topic in dermatological research. Since diffuse hair loss, often occurring in women of mid or higher age, is only a symptom-based diagnosis, but has not been explained by a causative mechanism, hair aging is a process, which has been investigated in the present study.

Scalp skin biopsies from 24 women were taken to obtain single hair follicles to be subjected to microarray-based genome-wide analysis. Volunteers were allocated to three groups, (i) 20–25 years of age, no hair loss, pre-menopausal, (ii) 40–45 years of age, diffuse hair loss, pre-menopausal, (iii) 60–65 years of age, diffuse hair loss, post-menopausal. A fourth group was a parallel group to the group of 20–25 years old young women, but with smoking habits to compare the influence of smoke-induced oxidative stress as a reference to age-induced oxidative stress. Groups (i) to (iii) were all non-smokers. Single-gene analysis, pathway-analysis and confirmation of target genes by PCR as well as *in situ* protein detection by immunofluorescence microscopy (IF) were performed.

Single-gene analysis and pathway-analysis identified genes and key-pathways of hair follicle biology that were significantly regulated dependent on increasing age. Microarray-based single-gene analysis of 41 000 genes revealed genes which belong to four main biological processes: neuronal stress, inflammation, innate immune response and endocrinology. Additional software-based high-throughput pathway analysis identified four additional main areas of biological processes: oxidative stress, collagen, transporter channels and cell cycle.

Analysing the different age groups, the comparison between 20 and 25 years versus 60–65 years old women revealed the highest number of regulated genes (2424 genes), followed by 20–25 years versus 40–45 years (1365 genes) and 40–45 years versus 60–65 years (819 genes). After applying selection criteria (i) two-fold regulation and (ii) significant regulation  $P < 0.05$ , genes were further selected, and 163 known genes were found to be regulated in the group 20–25 years versus 60–65 years and 68 genes were regulated in 20–25 years versus 40–45 years. In the first comparison, 83 genes were down- and 80 genes up-regulated, whereas in the latter, 42 genes were down and 44 genes up-regulated.

Comparing the smoker age group 20–25 years versus the different non-smoker age groups, most regulated genes were found in the comparison with the same age group 20–25 years (1511 genes), followed by 60–65 years (1115 genes) and 40–45 years (997 genes).

To refine the search for biologically relevant genes in the non-smoker groups, a high information yield online search machine iHOP was used, and in combination with PubMed-reference search, 111 genes of interest in the context of 'skin', 'hair', 'aging' and connected biological processes such as 'apoptosis', 'cell cycle', 'stress', 'inflammation' and 'autoimmunity' were found. From these 111 genes, a refined re-analysis of the gene-relevance for hair aging related biological processes with the use of iHOP and PubMed was performed and a list of the 'Top 20'-genes created. Age-related regulation of several genes was confirmed by PCR, and *in situ* detection and localization of their respective proteins was performed by IF.

These data strongly suggest that hair aging is a profiled process which is characterized by specifically regulated genes and pathways that are involved in essential processes of hair biology, suggesting that their age-related involvement may be a causative factor for hair loss in women after the age of 40. This study represents a promising search strategy to identify new targets for the development of hair loss treatment in women at higher age.

#### P098

### Identification of novel caffeine target genes in human hair follicles

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The xanthine derivative, caffeine, promotes human hair growth *in vitro* by inhibiting catagen induction, stimulating hair shaft elongation and enhancing hair matrix keratinocyte proliferation. Besides its classical inhibitory effects on phospho-diesterase, very little is known about additional bioregulatory effects of caffeine in human skin and its appendages. Therefore, a large-scale genome-wide gene analysis (41 000 genes) was performed to identify previously unknown target genes and pathways regulated by caffeine in human hair follicles (HFs).

HFs from six men and six women with androgenic alopecia were acquired by elective scalp skin biopsies and cultivated for 24 or 120 h *in vitro* with caffeine (male: 0.001%; female: 0.0001%) or vehicle, and HF RNA extracts were subjected to microarray analysis (Agilent system). Genes found to be differentially regulated into the same direction (up-or-down,  $P < 0.05$ ) in at least three patients, were validated by PCR, and their *in situ* protein expression and localization within the HF was analysed by immunofluorescence microscopy (IF).

After 120 h cultivation, 2756 genes were regulated by caffeine, and 2- or more-fold, significant equidirectional regulation was observed in 19 genes in caffeine-treated male, and 22 genes in female HFs. Most of these genes had never before been implicated in human hair growth. Software-based pathway analysis additionally revealed involvement of TGFβ-, Wnt-, Notch-, insulin-, hormone-signaling pathways in male and female HFs. Gender-dependent differences in the HF response to caffeine were evident from the fact that female HFs showed involvement of a far higher number of additional pathways than male HFs, e.g. chemokine and death receptor signaling as well as stronger involvement of hormones and hormone receptors. Gene categories up-regulated by caffeine were further identified and associated with ribosomal processes, protein-biosynthesis and -modification in both male and female HFs, therefore representing enhancement of metabolism and proliferation. Down-regulated genes were mostly associated with keratin and keratin-associated proteins (KAP), namely in female HFs. Novel caffeine-regulated genes that were most profoundly regulated in the majority of investigated HF samples, were dermcidin (DCD), 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3), lactotransferrin (LTF) and L-kynurenine hydrolase (KYNU). In addition, female HFs showed significant regulation of chorionic gonadotropin and COX18 after 24 h incubation with caffeine. RT-PCR confirmed strong up-regulation of DCD in all male and female HFs, whereas LTF was only confirmed in male HFs. PFKFB3 and COX18 were confirmed by qPCR in female HFs. Finally, IF showed *in situ* protein expression of DCD, LTF, PFKFB3 and COX18 at specific anatomic sites of the HF, and up- or down-regulation was mostly corresponding to the respective gene regulation.

In conclusion, this gene profiling study identifies novel (direct or indirect) caffeine target genes in human HFs and characterizes relevant protein expression *in situ* as an important basis for the development of new hair growth-modulatory agents.

#### P099

### Analysis of the putative association of genetic variants of key components of the vitamin D endocrine system (vitamin D receptor, vitamin D binding protein, CYP27B1, CYP24A1) with melanoma risk and prognosis

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Gene polymorphisms (SNPs) of vitamin D receptor (VDR), vitamin D binding protein (DBP), CYP27B1, and CYP24A1 have important implications for vitamin D signalling and are at least in part associated with various malignancies. Using TaqMan SNP genotyping assays, we have analyzed in a German population the frequency of SNPs of VDR ( $n = 6$ ), DBP ( $n = 2$ ), CYP27B1 ( $n = 1$ ), and CYP24A1 ( $n = 1$ ) in malignant melanomas (MM,  $n = 360$ ) as compared to healthy controls ( $n = 376$ ). At present, we are analyzing statistically the putative association of these SNPs with melanoma risk and prognosis (Breslow thickness of primary MM at time of diagnosis, disease-free and overall survival). Results will be presented at this meeting.

#### P100

### Galanin and its receptors as modulators of eccrine sweat gland physiology

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Galanin, a member of the galanin family of neuropeptides, exerts a variety of biological functions in skin physiology. It has been shown that galanin peptide and galanin binding sites are present in human sweat glands, suggesting a possible function in sweat gland physiology and maintenance of the skin. The human sweat gland cell line NCL-SG3 was used to determine the expression of galanin receptor subtypes and to analyze possible intracellular signaling pathways involved upon galanin-receptor activation. We found that NCL-SG3 cells express mRNA of GalR2 and GalR3 but not GalR1. Similar results were obtained using primary cultures derived from human sweat glands.

Western blot analysis of whole cell lysates of NCL-SG3 cells and immunohistochemistry performed on paraffin-embedded cultured NCL-SG3 cells confirmed the presence of GalR2 and GalR3 in these cells. Furthermore, eccrine sweat glands stained also positive for GalR2 and GalR3, but not for GalR1 in human skin biopsies.

Results from Ussing chamber experiments showed that galanin can mediate chloride ion secretion in the NCL-SG3 cell line, which could be abolished by the application of SNAP 37889, a nonpeptidergic selective antagonist of GalR3. Additionally, intracellular calcium levels were not altered by galanin application in NCL-SG3 cells and indicate that galanin effects are more likely mediated via GalR3 at least in this cell line.

By radioimmunoassay we found that galanin is produced and actively secreted by the NCL-SG3 cells. Moreover, galanin was detected in human sweat samples derived from volunteers exercising on a bicycle ergometer in concentrations up to 8.9 fmol/ml.

Detailed understanding of the modulatory mechanisms of galanin in eccrine sweat gland physiology will be the basis for further investigations to elucidate its role under healthy and pathologic conditions.

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## P101

### Galanin is a vital activation agent for polymorphonuclear neutrophils

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Primary afferent nerve fibers control cutaneous blood flow and vascular permeability by releasing neuropeptides, such as galanin, that in turn can evoke inflammation. These vascular reactions and the additional recruitment and activation of leukocytes are the key features of neurogenic inflammation. Recently, we were able to show that accumulation of polymorphonuclear neutrophils (PMNs), upon induction of different inflammatory stimuli in the skin, is disrupted in galanin knockout mice (Gal-KO). Consequently, we aimed to elucidate if galanin is directly influencing PMN function *in vivo* and *in vitro*.

When galanin was co-injected along with the inflammatory stimulant carrageenin in the skin of Gal-KO, comparable quantities of PMN accretion to that of the corresponding wild-type mice could be observed. Additionally, local administration of galanin to inflamed knee joints caused a dose-dependent increase in leukocyte rolling velocity and adhesion within the synovial microvasculature in wild-type mice.

In human PMNs, isolated from healthy individuals, galanin exposure enhances the expression of the beta-2-integrin CD11b and L-selectin 62L, together with expression of CD66b and CD63, markers of secondary and azurophilic granules, respectively, that are activated during cellular activation and adhesion. In accordance with these findings, we were able to demonstrate that treatment of galanin at concentration of 10<sup>-7</sup> Molar results in significant changes in neutrophil adherence *in vitro*.

These effects are mediated possibly through the galanin receptor subtypes GalR2 and/or GalR3, which are expressed in human PMNs.

Here, we have identified and characterized galanin as another crucial regulator for PMN recruitment, rolling and adhesion, establishing this peptide as another important partaker in inflammatory processes.

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## P102

### SZ95 sebocytes induce melanocyte dendricity and proliferation *In Vitro*.

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Background: Although the regulatory effects of epidermal keratinocytes and Langerhans cells on epidermal melanocytes (HMe) are well known, no similar information regarding other cutaneous epithelial cells, such as sebocytes is available.

Objectives: In this study, we evaluated the effects of SZ95 sebocytes in both low Ca<sup>2+</sup> (0.05 mM) and high Ca<sup>2+</sup> (1.5 mM) culture conditions on human epidermal melanocytes (HMe) morphology, proliferation and melanin synthesis.

Methods: Pure serum-free HMe were incubated with SZ95 sebocytes in direct contact and SZ95 sebocytes in inserts and evaluation of morphological changes were done on day 0 (24 h after SZ95 sebocytes addition) and every 3 days thereafter for 12 days. Assessment of HMe proliferation was done at the same time points after incubation with SZ95 sebocytes conditioned media (SZ95-CM). Likewise, estimation of HMe melanin contents was performed but while SZ95 sebocytes were in inserts.

Results: SZ95 sebocytes co-cultured with HMe, whether in direct SZ95 sebocyte-HMe contact or with SZ95 sebocytes in cell culture inserts, resulted in HMe flattening with increase of cell surface area and multiple small dendrites formation. However, only in high Ca<sup>2+</sup> level and direct cell contact co-culture, the HMe dendrites were specially long and preferentially targeted and attached to SZ95 sebocyte surface membrane; features likely mediated by E-cadherin being strongly expressed by SZ95 sebocytes in high Ca<sup>2+</sup> level cultures. Likewise, only high Ca<sup>2+</sup> SZ95-CM stimulated HMe proliferation in a time-dependant manner reaching significant levels at day 9 (the percentage increase was 142.9%, *P* < 0.01) and at day 12 (the percentage increase was 179.2%, *P* < 0.001) of incubation and when compared to day 0. However on day 12 of incubation, the percentage increase was 1078% higher than its corresponding low Ca<sup>2+</sup> SZ95-CM (>5-folds, *P* < 0.001). In contrast, melanin contents of HMe significantly decreased on incubation with high Ca<sup>2+</sup> SZ95 sebocytes and in comparison with low Ca<sup>2+</sup> SZ95 sebocytes at day 6 (4.5 g + 0.2 vs 15.4 g + 0.8, *P* < 0.01) and at day 9 (6.9 g + 0.4 vs 10.9 g + 1.6, *P* < 0.05) of incubation.

Conclusions: Our results revealed, for the first time, that sebocytes also modulate HMe functions in a Ca<sup>2+</sup>-dependent manner and may contribute to constitutive and facultative skin color in sebaceous glands rich body regions.

## P103

### Functional influence of vitamin D and its receptor on adult human scalp skin epithelial progenitor biology *in situ*

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Calcitriol, the biologically active form of vitamin-D (vitD3), and its receptor were shown to contribute to cutaneous homeostasis. Recent murine studies demonstrated that calcitriol inhibits keratinocyte proliferation and stimulates differentiation in a dose-dependent manner and that targeted-mutation of the vitamin D receptor (VDR) induces alopecia. Additionally, calcitriol was also recognized as a potent immunomodulatory substance in the murine skin.

In isolated adult human epithelial hair follicle progenitors (eHFPs), we recently demonstrated first evidence that calcitriol significantly up regulated protein expression of CK15 and the immunomodulatory CD200, both recognized as human eHFP markers. Parallel, we detected also an up regulation of the VDR expression. In line with these findings, calcitriol slightly enhanced eHFP proliferation *in vitro* and

colony forming efficiency (CFE), whereas higher doses strongly induced apoptosis and impaired CFE as expected from the literature.

To get further ideas of the functional relevance of calcitriol and VDR and to directly recognize effects of blocked VDR on epithelial progenitors *in situ*, we treated organ cultured human scalp skin with calcitriol and/or a VDR antagonist and evaluated CK6 (basal keratinocyte proliferation), CK10 (differentiation) and Ki67 (proliferation), as well as the expression of the activated immune response markers MHC-I and -II.

Focusing on epithelial progenitor rich regions (epidermal basal layer and hair follicle bulge) differences in the expression of the investigated proteins were quantified by immunofluorescence reactivity.

By that, we discovered a significant reduction of CK6 expression in 0.1 nM calcitriol treated organ cultured scalp skin after 1 and 3 days and a significant increase of CK10 expression within the epidermal basal layers. After 3 days there is still an ongoing increase of CK10 expression. The combination of calcitriol and the VDR antagonist shows the expected inhibitory effect to CK10 expression but has no effect on CK6 expression.

Furthermore there is a significant increase of MHC-I and MHC-II expression in the 0.1 nM calcitriol treated scalp skin after 3 days within the epidermal basal layer.

In summary, we conclude that epithelial progenitors of human scalp skin and its appendages underlie prominent vitD3 and VDR controls. This encourages one, to systematically explore the molecular background of these controls and how these findings may be clinically exploited.

## P104 (V14)

### Inhibition of subtilisin-kexin isozyme-1, a member of the prohormone convertase family, induces apoptosis of human melanoma cells

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Prohormone convertases (PCs) are mostly known as enzymes processing prohormones. However, their role in pigment cells is poorly investigated. To elucidate the function of PCs in human melanocytes we focused here on subtilisin-kexin isozyme-1 (SKI-1). SKI-1 was constitutively expressed at mRNA and protein level in normal human melanocytes (NHM) and nine human melanoma cell lines as determined by quantitative real-time RT-PCR and Western immunoblotting. Via *in silico* promoter analysis we detected several putative transcription factor binding sites for transcription factors which are typically transactivated by melanocyte growth factors and extracellular or intracellular stressors. Among several mitogens and stimuli tested only the growth factor / tumor promoter phorbol-12-myristate-13-acetate as well as tunicamycin, an inducer of endoplasmic reticulum stress, regulated SKI-1 expression in NHM. Next, we assessed the biological function of SKI-1 in human pigment cells by employing the cell-permeable SKI-1 inhibitor decanoyl (dec)-RRL-choromethylketone (CMK) *in vitro*. Treatment of both normal and transformed melanoma cells led to a dose-dependent inhibition of metabolic activity and proliferation as assessed by XTT and crystal violet assay. Melanoma cells were more sensitive towards dec-RRL-CMK than NHM. Mechanistically, dec-RRL-CMK dose-dependently induced apoptosis of melanoma cells as shown by cell death detection assay, Annexin-V staining and processing of poly-adenosine diphosphate-ribose polymerase 1/2. This effect was associated with reduced expression of two prototypical SKI-1 target genes, caveolin-1 and glucose-regulated-protein 78, which are implicated in melanoma growth and progression. Indeed, treatment of melanoma cells with dec-RRL-CMK time-dependently reduced SKI-1 enzyme activity as determined by a newly established bioassay. In a first approach to assess the *in vivo* relevance of these findings, SKI-1 expression was examined in cryopreserved material of normal human skin and melanoma metastases. SKI-1 mRNA amounts as measured by real-time RT-PCR were significantly higher in tumor samples. Our findings suggest that SKI-1 could play a functional role during melanocyte transformation or metastasis by controlling apoptosis.

## P105

### UVB triggers the NALP1 inflammasome and S100 'alarmins' for IL-1 beta release in epidermal keratinocytes

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The skin is the first line of defense and protects against physical stress, such as environmental irradiation. UVB induces a cutaneous inflammation through IL-1 beta release with subsequent infiltration of inflammatory cells. In epidermal keratinocytes, UVB leads to activation of caspase-1 through activation of both NALP3 and AIM2 inflammasomes that are required for IL-1 beta secretion. In the present study, we confirm that UVB increases IL-1 beta expression which is further amplified by NALP3- and AIM2-inducing IFN gamma in human keratinocytes. Further, we observed a novel inflammasome that is additionally involved in UVB induced IL-1-beta production. The recently discovered NALP1 inflammasome activates caspase-5 in addition to caspase-1 for IL-1 beta release. We show that UVB induces caspase-5 dose- and time-dependently compared with caspase-1 in epidermal keratinocytes. In addition, UVB differently increases the antimicrobial peptides psoriasin (S100A7) and koebnerisin (S100A15) in keratinocytes that amplify the inflammatory response as chemoattractants and 'alarmins'. Previous studies showed that 'alarmins', e.g. cathelicidin (LL-37), interfere with inflammasome activation and subsequent IL-1 beta release. Here, we demonstrate that psoriasin is able to potentiate the UVB-triggered IL-1 beta activation in keratinocytes. We therefore suggest an UVB-mediated amplification loop through activation of inflammasomes, such as NALP1, and 'S100-alarmins' that synergize to promote skin inflammation.

## P106

### Examination of cellular processes in induced pluripotent stem cells from healthy and patient-derived human skin biopsies comparison with their corresponding parental cells

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Background: Application of somatic cell-derived induced pluripotent stem (iPS) cells with close features of human embryonic stem (hES) cells in regenerative medicine is of particular importance, but before this can be realized, there are some questions to clear up. We have previously performed cell infection with retroviruses expressing the four transcriptional factors OCT4, SOX2, KLF4 and C-MYC and we generated iPS cells from skin-derived fibroblasts obtained from a healthy neonate (BJ, HFF1, purchased from ATCC) and from an 84 year-old patient diagnosed to suffer from type II diabetes (NFH-2). However, little is known about the nature and sequence of molecular events accompanying nuclear reprogramming.

Objectives: The aim of the project is to produce systemic organ cells from iPS cells *in vitro* as a model to investigate the molecular pathogenesis of systemic diseases and molecular processes in iPS cells. Methods and Results: In order to address the molecular cell processes of the generated iPS cells, we analysed among others the parental somatic cells and their corresponding iPS cells with regard to chromosomal integrity and formation of reactive oxygen species (ROS) and we found chromosomal abnormality in iPS cells from old women in contrast to iPS cells from neonate fibroblasts. Both parental cells

showed chromosomal normality. Finally we differentiated the iPSC cells into neuronal cells despite chromosomal abnormality.

**Conclusions:** In summary, we succeeded to produce systemic organ cells from iPSC cells *in vitro*, despite the detected chromosomal abnormalities.

#### P107

##### Inactivation of integrin $\alpha 2\beta 1$ and $\alpha 11\beta 1$ results in severely diminished IGF-1 levels leading to dwarfism

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The extracellular matrix (ECM) is a key regulator of cellular functions controlling a multitude of cellular activities in different organs. Contact of cells with ECM is mediated by integrin receptors, which transmit signals from the outside environment into the cell and from inside-out. We have concentrated on the biological functions of collagen-binding integrins and have used a genetic approach to dissect the functions of the major collagen-binding integrins,  $\alpha 2\beta 1$  and  $\alpha 11\beta 1$ .

By gene targeting we generated mice with whole body ablation of either one or both integrin receptors. Strikingly, integrin null mice developed dwarfism, which was mildest in  $\alpha 2$  nulls, strong in  $\alpha 11$  nulls and very pronounced in double nulls lacking both receptors. Analysis of femora revealed that dwarfism does not result from growth plate abnormalities. Osteoblast differentiation and function also turned out to be normal, implicating that dwarfism cannot be attributed to osteoblast-autonomous dysfunction. Moreover, dwarfism was not restricted to the skeleton but affected all organs; thus, constitutive ablation of integrins  $\alpha 2\beta 1$  and/or  $\alpha 11\beta 1$  results in proportional dwarfism.

These findings suggested a systemic cause for the overall size reduction. In accordance with a critical role of insulin-like growth factor (IGF)-1 in growth control, circulating IGF-1 levels assessed by Elisa in the sera of double null mice were sharply reduced by 85% of normal levels. These results stimulated the analysis of the growth hormone/IGF-1 axis by micro-dissection of defined brain regions. This approach revealed that low IGF-1 levels resulted from reduced growth hormone releasing hormone (GHRH) expression in the hypothalamus and subsequently reduced growth hormone (GH) expression in pituitary glands of null mice, which is directly linked to IGF-1 production. These findings point to a novel and unexpected role of collagen-binding integrin receptors in the GH/IGF-1 axis and in growth regulation. Thus coupling of hormone secretion to ECM signaling via integrins may represent a novel concept in control of endocrine homeostasis.

#### P108

##### Cortisol and brain derived neurotrophic factor levels are altered by exam stress: consequences for tissue regeneration in healthy young women

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Tissue regeneration in peripheral organs at the self – environment border such as the skin is greatly affected by stress and results in disturbed wound healing and disregulated inflammation. In mice, activation of the third stress-axis – the neurotrophin/neuropeptide stress axis (NNA) – results in neurogenic inflammation and increased apoptosis in skin, followed by precocious termination of a physiologically occurring tissue regeneration process: hair growth. Human anagen hair follicle organ culture experiments with the neurotrophin brain derived neurotrophic factor (BDNF) confirmed these observations. Here we analysed stress perception, morning serum and plugged hair follicles in 20 healthy young women undergoing the final medical exam at the Humboldt-University in Berlin, Germany in comparison to 16 women participating in a regular semester. We found that basal morning serum levels of cortisol were decreased while BDNF was increased 12 weeks prior to the exam and significantly at the exam. These results corresponded well with the subjective perception of demands and anxiety as measured by the perceived stress questionnaire (PSQ) and the STAI. These levels returned to control levels 12 weeks after the exam. In parallel, participants in the exam group displayed significantly less hair follicles in the growth phase of the hair cycle in their scalp skin. These results confirm that continuous stress alters stress axis activity not only of the classical hypothalamus pituitary adrenal axis (HPA) but also of the NNA with consequences for tissue regeneration in healthy individuals and presents a simple and reproducible clinical approach to objectify stress and stress effects.

#### P109

##### Chronic stress upregulates Langerhans cell-nerve fibre contacts and counter-regulates allergic inflammation

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Stress is usually considered to be detrimental to allergic diseases. Some published data however suggest a training effect of certain stress-paradigms that attenuate inflammation depending on intensity and frequency of stress encounter. To address this issue, we here compared the effect of acute versus chronic stress on atopic dermatitis-like allergic dermatitis (AID) in C57BL/6 mice i.e. sensitized and i.d. challenged with chicken egg ovalbumin. A singular 2 h noise exposure together with restraint was considered to be acute stress while 2 h of noise and restraint stress applied daily over 5 days was considered to be chronic stress. SP expression and mast cell degranulation was increased both after acute and chronic stress exposure. However, only after chronic stress exposure the number of PGP 9.5- and GAP43-immunoreactive nerve fibres increased in skin together with an increasing number of their contacts with epidermal antigen presenting cells (Langerhans cells). At the same time, chronic stress exposure was associated with a down-regulation of eosinophilic infiltration of the skin. Moreover, cytokine production and TH2 bias were shifted towards control levels after chronic stress exposure. These findings indicate a training effect of this chronic stress paradigm promoting downregulation of detrimental inflammatory effects. Further investigations have to reveal whether these effects can be used to treat AID.

#### P110

##### Effects of the combination of vitamin D and calcium in SZ95 sebocyte regulation

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role in calcium homeostasis, has also been investigated in SZ95 sebocytes. Expression of vitamin D receptor (VDR), as well as the hydroxylases vitamin D-25-hydroxylase (25 OHase) and 25-hydroxyvitamin D-1 $\alpha$ -hydroxylase (1  $\alpha$ OHase), and 1,25-dihydroxyvitamin D-24-hydroxylase (24 OHase) was detected in SZ95 sebocytes *in vitro* using real time quantitative polymerase chain reaction (qRT-PCR). Sebocytes cultured under serum-free conditions containing 0.4 mM calcium treated with high doses of vitamin D ( $10^{-7}$  M) show a statistically significant increase in numbers and decrease of lipid accumulation. On the other hand, pharmacological doses of vitamin D induce an antiproliferative effect in SZ95 sebocytes in the presence of fetal calf serum in medium.

In our study we elucidate the role of vitamin D at  $10^{-7}$  and  $10^{-9}$  M in SZ95 sebocytes adapted to four different calcium concentrations (0.05, 0.15, 0.4, 1.4 mM). We managed to show that induction of sebocyte proliferation and suppression of lipogenesis through vitamin D ( $10^{-7}$  M) is significantly higher for high calcium concentrations (0.4, 1.4 mM).

#### P111

##### Human keratinocytes express AIM2 and respond to dsDNA with IL-18 secretion

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It is currently well accepted that keratinocytes, beside their barrier function, participate actively in the skin immune system and are able to sense harmful pathogens by using receptors that recognise evolutionarily conserved microbial components. To date it seems to be verified that keratinocytes express all components which are necessary to form the NLRP3 inflammasome complex including the adapter protein ASC and caspase-1. In this study we investigated the presence and activity of the recently identified AIM2 inflammasome in human keratinocytes. On the basis of our findings, we conclude that keratinocytes express a functional AIM2 inflammasome which triggers a strong IL-1 release in response to cytosolic dsDNA. IL-1 production by keratinocytes plays a pivotal role in inflammatory processes in the skin. Therefore, activation of the AIM2 inflammasome in keratinocytes represents another potential trigger factor for the development and maintenance of inflammatory skin diseases.

#### P112

##### Deciphering the mechanism of pseudosyndactyly in recessive dystrophic epidermolysis bullosa

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In recessive dystrophic epidermolysis bullosa (RDEB), wound healing is severely impaired, since repeated friction and infections lead to persistent inflammation and consequently to chronic wounds and atrophic scars. Almost all RDEB patients develop pseudosyndactyly with contractures leading to the loss of finger function. The main goal of this study is to elucidate the mechanisms underlying scarring and pseudosyndactyly in order to contribute to improving those symptoms in RDEB.

Therefore, we studied the expression of inflammation and fibrosis markers in scarring and non scarring skin of EB patients and healthy donors. We found strong upregulation of the pro-inflammatory cytokines interleukin-1 (IL-1) and IL-6 in RDEB skin, especially in scar tissue, indicating that repeated wounding in RDEB patients leads to permanent local and maybe also systemic inflammation. Furthermore, expression of TGF- $\beta$  and the ECM protein tenascin-C, a fibrosis marker, were also enhanced in RDEB scar skin. However, in scarring and not scarring RDEB skin, we did not find upregulation of alpha smooth muscle actin, a marker for myofibroblasts and fibrosis, on the RNA as well as on the protein level. Therefore, the fibrotic changes leading to finger contractures in RDEB may not be caused by myofibroblasts, but by another, yet unknown mechanism. To further improve the understanding of wound healing, scarring and pseudosyndactyly in RDEB, we are currently performing a genome wide study of gene expression in scarring and non scarring skin of RDEB patients and healthy persons by cDNA microarrays.

#### P113

##### Prognostic factors in squamous cell carcinoma of the skin

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**Background:** Cutaneous squamous cell carcinoma (SCC) is one of the most common malignant neoplasms of the human skin. In many cases, SCCs tend to arise out of non-invasive precursor lesions, like actinic keratosis. Such lesions can persist (remain 'latent') for a long time, even many years, before further developing into invasive malignant squamous cell carcinoma of the skin. Although SCCs in most of the patients take a rather benign course due to low metastatic capacity, some patients develop metastatic disease and some patients eventually die of their cutaneous SCC. Tumor classification for prognosis, malignity and outcome is therefore important.

**Objectives:** Although some suggestions for the classification for cutaneous SCC exist, still no mandatory parameters for prognostic assessment are available so far. The aim of this study is to identify relevant factors for prognosis and diagnosis of cutaneous SCCs to achieve the best way for patient's individual treatment.

**Methods:** Our study analyzes patients with histologically verified SCC who were diagnosed for primary cutaneous SCC between 2006 and 2009 at the Dermato-Oncology clinic, Department of Dermatology, Medical University of Vienna, Austria. For this purpose, histology is reviewed and analyzed for defined criteria (including immunohistochemical stainings) and further compared with patient's clinical data. Histological findings were correlated with the patient's individual clinical record to identify potentially new prognostic factors. Multivariate logistic regression has been performed to identify/confirm clinical, histological and pathologic factors for an improved predictive classification for cutaneous SCC.

**Results and Discussion:** We present the results of this retrospective analysis and discuss the impact of the histological report in cases of cutaneous squamous cell carcinoma on further treatment procedures.

#### P114

##### Transformed cutaneous CD30 positive T-cell lymphoma of the lymph node mimicking classical Hodgkin lymphoma

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Classical Hodgkin lymphoma (cHL) following mycosis fungoides (MF) or lymphomatoid papulosis (LyP) in the same patient has been debated in the literature. There is considerable morphologic and immunophenotypic overlap between cHL and nodal involvement of CD30-positive T-cell lymphomas (TCL). Whether such cases represent TCL with Hodgkin-like cells or cHL is often difficult to resolve. Biopsies from patients with a prior history of cutaneous TCL or primary cutaneous CD30-positive T-cell lymphoproliferative disorder and lymph node biopsies reported as either CD30-positive TCL with Hodgkin-like cells or cHL were retrieved from the authors' institution. We analyze the clinical,

morphological, and immunophenotypic features of these cases and report T-cell receptor gene rearrangement studies (TRG). Laser capture microdissection was performed in one case. Of 11 cases identified, 10 were considered CD30-positive TCL with Hodgkin-like cells, while one was confirmed as cHL upon review. Four cases originally diagnosed as cHL were revised as CD30-positive TCL. The CD30-positive TCL showed a male predominance (M:F, 4:1) with a median age of 53 years (range 44–72 years). Nearly all patients (9/10) initially presented with skin lesions and later developed nodal involvement, although in some cases lack of knowledge of the cutaneous lesions led to a misdiagnosis of cHL. In 8/10 patients the draining lymph node was involved, whereas in two cases generalized skin disease was present. Tumor cells of these biopsies morphologically resembled Hodgkin/Reed-Sternberg (HRS) cells. These HRS-like cells were strongly positive for CD30 and negative for B-cell markers (i.e. PAX5, CD20) in all cases. Expression for CD15 was observed in the majority of cases (9/10, 90%). Also, 7/10 cases of CD30-positive TCL with Hodgkin-like cells had tumor cells that expressed at least one T-cell marker and all (9/9) cases studied revealed a clonal rearrangement by TRG. *In situ* hybridization studies for EBV were negative for all studied cases. In one case the diagnosis of cHL followed by LyP was confirmed, with HRS-cells expressing PAX5, CD30 and CD15. In some cases of transformed MF/LyP with nodal involvement, the distinction from cHL can be challenging, but combined morphologic, immunophenotypic, and molecular studies together with careful clinical correlations help to differentiate these lesions. Misdiagnosis as cHL remains a diagnostic pitfall.

#### P115 (V03)

##### Role of IL-24 in psoriasis pathogenesis

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Like its relatives IL-22 and IL-20, IL-24 is over-expressed in the diseased skin of psoriasis patients. However, the role of IL-24 in psoriasis pathogenesis is largely unknown. To understand the significance of this cytokine in psoriasis, this study aimed to characterize the producers, target cells and effects of IL-24. Among resident tissue cells of the skin, keratinocytes were able to produce IL-24, and this was increased by TNF- $\alpha$ , IL-17A, and IL-22, but not by IL-19, IFN- $\gamma$ , or IL-21. With levels similar to stimulated keratinocytes, IL-24 was expressed also by activated monocytes. However, these cells lost their ability to produce IL-24 upon differentiation into either macrophages or dendritic cells. Interestingly, activated T-cells, e.g. Th17 cells, were also able to express IL-24. Actually, IL-24 expression levels in Th17 cells were even approximately 10 times higher than in activated monocytes or keratinocytes. The development of IL-24-producing Th17 cells was critically dependent on the action of TGF- $\beta$  during the generation of these cells but was not altered by the presence of IL-10. The relevance of Th17 cells as important source of IL-24 in psoriasis lesions was substantiated by significant positive correlation between IL-24 and IL-17F levels in samples of diseased skin from psoriasis patients. We previously excluded immune cells from being targets of IL-24. Among tissue cells of the skin, only keratinocytes and to a minor extent dermal fibroblasts but not dermal endothelial cells, melanocytes or subcutaneous adipocytes expressed relevant amounts of the IL-24 receptor R1-components. IL-24 enhanced the expression of antibacterial proteins and inhibited the terminal differentiation of keratinocytes. The latter was associated with psoriasis-like morphological changes of reconstituted human epidermis. Our data suggest that in the psoriatic skin T-cells are main producers of IL-24, and that this cytokine contributes to the epidermal alterations.

#### P116

##### Specific and overlapping functions of T-cell mediators in psoriasis pathogenesis

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Psoriasis is a common chronic immune-mediated skin disease with significant negative impact on the quality of life of the affected people. The enhanced proliferation of keratinocytes, their disturbed terminal differentiation, a massive cutaneous infiltration of immune cells, and the increased innate immunity are characteristic microscopic and molecular alterations in psoriatic lesions. It is commonly assumed that immune cells by means of their mediators alter the keratinocyte biology and induce the psoriasis lesions. However, the concrete contributions of individual T-cell cytokines to these alterations are largely unknown. The aim of our work was to shed light on the role of the individual T-cell cytokines in psoriasis pathogenesis. In the first study part, the expression levels of seven Th1- (IL-2, IFN- $\gamma$ ), Th17- (IL-17A, IL-17F, IL-21, IL-26), and Th22-cell (IL-22) cytokines and of markers of each above-mentioned psoriasis characteristic skin alterations was determined in lesions from 12 patients. The analyses of positive correlations between levels of individual T-cell cytokines and psoriasis marker suggested that some alterations are mediated by several interleukins, whereas other alterations are induced by specific mediators. *In vitro* application of selected individual T-cell cytokines to three-dimensional human epidermis models confirmed these assumptions. Furthermore, the application of a cytokine mix that should reflect the inflammatory situation in psoriatic lesions and a mix without individual cytokines to three-dimensional human epidermis models confirmed that a joint action of IL-17 and IL-22 was necessary for the strongest induction of antimicrobial peptides and IL-17 had a dominant role in CCL20 induction. Importantly, Th17- and Th22-cell cytokines induced secondary mediators in keratinocytes (e.g. IL-17 induced IL-19, IL-22 induced IL-20) with similar effects to their own, thereby enhancing and extending their own effects. Finally, mouse experiments with transgenic over-expression (IL-22) or cutaneous application (IFN- $\gamma$ ) of cytokines confirmed the key *in vitro* results. Taken together, our study suggests that psoriasis is a multi-step and multi-cellular process in which some alterations are mediated by several cytokines and other by individual mediators. These data do not only improve our knowledge about the pathogenic cascade of psoriasis but may also lead to the development of specific therapies in future.

#### P117 (V15)

##### A Humanized HLA-class II transgenic mouse model for the analysis of desmocollin 3-specific T – and B cell responses in pemphigus

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Desmosomal cadherins particularly desmoglein (Dsg) 3 and 1, are targeted by IgG autoantibodies (auto-ab) in autoimmune blistering disorders such as pemphigus. Several *in vitro* and *in vivo* studies have supported the concept that IgG Dsg3- and I-specific auto-ab are necessary for blister formation. Recently, we and others have identified pathogenic IgG auto-ab against an additional desmosomal protein, desmocollin (Dsc) 3, in atypical pemphigus patients. Furthermore, the relevance of Dsc3 in stratified epithelia has been fully observed in a conditional epidermal Dsc3-null mice. Thus, the animals demonstrate a severe cutaneous phenotype with suprabasal blister formation resembling pemphigus vulgaris (PV).

HLA-DR0402-DQ8-human CD4-transgenic-murine MHC class II-deficient mice were established in our laboratory as an *in vivo* model to study the humoral and cellular immune response to human Dsg3 protein. The aim of this study was to investigate whether the HLA-class II-transgenic mice could also provide a promising *in vivo* system to characterize the CD4+ T cell and antibody response to human Dsc3-protein. Immunization of the transgenic mouse with recombinant Dsc3 led to the production of antibodies recognizing the native Dsc3-protein as demonstrated by ELISA and immunofluorescence using human keratinocytes. Applying the dispase-based keratinocyte dissociation assay we were able to show that Dsc3-specific IgG from immunized animals induced loss of keratinocyte adhesion. The cellular immune response to human Dsc3 was measured *ex vivo* by CFSE dilution assays of splenocytes isolated from Dsc3-immunized mice. We could identify proliferative responses of CFSE-diluted CD4+ T cells by flow cytometry after *ex vivo* restimulation with the human extracellular Dsc3 domains. Moreover, there was a relation between antibody titers and T cell responses against Dsc3. Further investigations aimed at characterizing the CD4+ T cells and IgG responses to human Dsc3 protein in more detail, such as looking at epitope specificity of Dsc3-reactive IgG and subtypes of Dsc3-specific CD4+ T cells, are being carried out.

In principle, these findings support the use of the present mouse model for understanding early activation steps of the autoimmune cascade leading finally to the secretion of IgG auto-ab responses against Dsc3 which may largely contribute to the immune pathogenesis of pemphigus.

#### P118

##### Anxiety and depression are less frequent in patients with autoreactive as compared to non autoreactive chronic spontaneous urticaria

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Background: Around 50% of patients with chronic spontaneous urticaria (csU) exhibit at least one psychiatric comorbidity such as anxiety and depression. Autoreactive urticaria is a common subgroup of csU characterised by circulating histamine-releasing serum factors that is readily identified by the autologous serum skin test (ASST). As of yet, it is not clear whether the quality of life impairment as well as the frequency of psychiatric comorbidities varies in different subforms of csU, particularly between ASST positive and negative patients. Methods:

Data of 209 csU patients was collected in the tertiary referral centers in Berlin and Athens. One hundred and sixty-four were subjected to the ASST and all patients were asked to complete the Urticaria Activity Score for four consecutive days (UAS4), the Dermatology Life Quality Index (DLQI) and the Hospital Anxiety and Depression Scale (HADS).

Results: The csU populations in Berlin ( $n = 137$ ) and Athens ( $n = 72$ ) were found to be not different in terms of age, gender distribution, urticaria activity, quality of life impairment and rate of ASST positive patients (36.4% vs 34.3%). Accordingly, both populations were pooled for further comparisons of autoreactive and non autoreactive csU patients. In both groups, age and gender distribution was not different, whereas the mean UAS4 (9.8 vs 8.6) and DLQI total scores (7.7 vs 6.6) showed a non-significant trend towards higher values in ASST positive patients. In contrast, the proportion of patients with anxiety and depression as assessed by use of the HADS was significantly higher in ASST negative patients (20.0% vs 36.9%,  $P < 0.05$  and 7.3% vs 20.4%,  $P < 0.05$ ).

Conclusions: Patients with autoreactive csU suffer less frequently from anxiety and depression although they tend to show a higher disease activity and quality of life impairment. These results strongly suggest that autoreactive csU is truly different from other subtypes of csU and that psychiatric comorbidity plays less of a role in these patients.

#### P119

##### Epidemiology and care of childhood urticaria – results of an expert survey

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Background: While there is increasing information about the pathogenesis and treatment of chronic urticaria in adults, there are only little published data for children. Consequently, most of the current recommendations for the management of childhood urticaria are based on extrapolation of data obtained in adults.

Methods: In this nationwide, cross sectional survey study on chronic urticaria in children, 49 German dermatologic and pediatric hospital-based physicians answered questions on their patient numbers, the natural course of disease and their diagnostic and therapeutic approach with a special focus on chronic spontaneous urticaria (csU).

Results: On average, the participating hospitals care for 19.8, 3.4 children with urticaria every month. Most of these patients present with acute spontaneous urticaria (15.1, 2.8). In contrast to what has been reported for adults, the gender ratio was estimated to be 1:1 for csU. Almost all participating hospitals offer programs to search for underlying causes in their csU children, which are successful in 30%, 5% of attempts. Infections and autoreactivity were indicated to be the most common identified causes in csU (45% and 27%). In their symptomatic therapeutic approach most hospitals (84%) follow the current EAACI/GA2LEN/EDF/WAO-guidelines by using non sedating antihistamines (nsAHs) in standard doses as first line therapy, followed by up dosing of nsAHs as the preferred second line approach (62%). Importantly, the tolerability of high dosed nsAHs was rated to be equally good as that of standard dosed nsAHs by the majority of respondents (84%).

Conclusions: Our study identified and characterised important differences in children and adults suffering from csU. Further research is needed to better understand the causes, course and response to treatment of csU patients and to enable the development of better diagnostic and therapeutic approaches for childhood urticaria.

#### P120

##### Association of utility-based and psychometric measures of quality of life of patients with psoriasis vulgaris

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Background: Clinical trials and health-economic evaluations apply different approaches to measure health-related quality of life (HrQoL). In clinical trials, health status instruments are applied to measure the influence of disease on the patient's physical, emotional and social functioning by means of a score such as for example the DLQI. Health-economic evaluations, however, require preference-based descriptors of disease severity (e.g. health utility scores) on a scale from 0 (minimum HrQoL) to 1 (perfect health). It is unclear, how well these different approaches of HRQL-assessment are inter-related.

Objectives: To compare the two different approaches to measure HRQL in patients with psoriasis vulgaris.

Methods: Standardized assessment of HrQoL, assessed by means of the DLQI and by means of health utilities (time-tradeoff method) in 58 patients with psoriasis vulgaris at two different points in time (baseline, after 6 months). Investigation of the strength of correlation of DLQI and health utilities and correlation of changes in DLQI and changes in health utility scores by means of Spearman correlation coefficients.

Results: Fifty-eight patients (38% female, mean age 50 years) with different disease control levels (mean PASI: 6.4; range 0–26.1) were included. At baseline, the mean (range) DLQI and health utility scores were 7.4 (0–23.0) and 0.81 (0.19–1.00), respectively. The correlation of the DLQI and health utilities was significant and of moderate strength ( $r = 0.49$ ;  $P < 0.001$ ). The correlation between changes in DLQI and changes in health utility scores between baseline and 6-month follow-up was likewise significant and of moderate strength ( $r = 0.51$ ;  $P < 0.001$ ).

Conclusions: Both absolute (static) scores of HRQL assessed by the DLQI and health utility scores assessed by means of the TTO-method and changes in these scores are significantly correlated in patients with psoriasis vulgaris. This finding is a prerequisite to investigate appropriate models aiming to estimate health utilities to be used in health economic evaluations from DLQI scores collected in clinical trials.

#### P121

##### Translation and cross-cultural adaption of the Nordic Occupational Skin Questionnaire (NOSQ-2002) to German

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Background: Occupational skin diseases are among the most frequent work-related diseases. The Nordic Occupational Skin Questionnaire (NOSQ-2002) is a validated instrument that allows a standardized assessment of occupational and non-occupational exposures/risk factors of skin diseases as well as the prevalence and course of skin diseases with relevance for occupational medicine. However, a validated German version of the NOSQ is missing limiting the use of the NOSQ in Germany.

Objectives: Translation and cross-cultural adaption of the NOSQ-2002 to German with particular attention to the clarity, comprehension and appropriateness of the translated version.

Methods: The adaptation of the German NOSQ-2002 followed the principles of the International Society for Pharmacoeconomics and Outcomes Research (ISPOR). It comprised a forward and backward translation by native speakers as well as a qualitative evaluation of the target version of the German NOSQ-2002 in cognitive debriefing interviews. Eighteen people were interviewed, representing different age-groups, both genders, people with and without skin disease as well as persons with and without occupational risk factors for skin diseases.

Results: Overall, the translated German NOSQ-2002 was well understood by all 18 participants of the qualitative assessment. The process of translation and modification resulted in some minor transformations of the German version of the NOSQ-2002 in comparison to the initial version (e.g. addition of examples and definitions, redraft of instructions, formal rearrangement).

Conclusions: The linguistic validation of the German NOSQ-2002 is a prerequisite for assessment of occupational skin diseases in accordance with international standards in future studies. The next step will be the examination of the psychometric measurement properties in a larger sample of persons at risk for occupational skin disease. After final adaptation the German version of the NOSQ-2002 will enable the standardized survey of skin diseases in the occupational setting in Germany.

#### P122

##### Predictive value of food sensitization and filaggrin mutations in children with eczema

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Background: It was reported that in infants with eczema and food sensitization the presence of a filaggrin (FLG) null mutation predicts future asthma with a specificity and a positive predictive value (PPV) of 100%.

Objectives: To evaluate the predictive value of food sensitization and food allergy, FLG haploinsufficiency, and their combination in infants with early-onset eczema for persistent eczema and childhood asthma

Methods: The two birth cohorts GINI and LISA as well as a collection of 65 early-onset eczema cases with and without food allergy were investigated.

Results: The risk for asthma was significantly increased by food sensitization (positive likelihood ratio PLR = 1.9 (95% CI 1.1–3.4) in GINI, PLR = 5.5 (2.8–10.8) in LISA) and presence of a FLG mutation (PLR = 2.9 (1.2–6.6) in GINI, PLR = 2.8 (1.0–7.9) in LISA) with a rather high specificity (79.1% and 92.9% in GINI, 89.0% and 91.7% in LISA), but low sensitivity (40.0% and 39.3% in GINI, 31.6% and 23.5% in LISA). Likewise, the risk for persistent eczema was increased. In the clinical cases neither food allergy nor FLG mutations had a significant effect. The combination of both parameters did not improve prediction and reached PPVs of 52.3% (GINI), 66.9% (LISA) and 30.6% (clinical cases) assuming an asthma prevalence in children with early eczema of 30%.

Conclusion: Early food sensitization and presence of a FLG mutation in infants with early eczema increase the risk for later asthma, but the combination of the two factors does not represent a clinically useful approach to reliably identify children at risk.

#### P123

##### Prevalence analysis of frequent dermatoses in probands of a population-based study in Pomerania (SHIP-1)

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Objective: Although in many dermatologic disorders prevalence data are given, there are only a few recent data available from population – based studies. The aim of this prevalence study was to define the distribution of different common dermatoses in a northeast german study population.

Methods: The SHIP Study (Study of Health in Pomerania) began with a sample size of 4310 (68.8% of invited 7008 people). In SHIP-1, a follow-up examination of the first cohort 5 years later with 3818 participants, dermatologic investigations were implemented as a 'module'. Data from 2040 participants who underwent both a standardized dermatologic questionnaire and a whole body investigation were collected to determine the prevalence of common dermatologic diseases in the study population. As cumulative UV exposition is the most important risk factor for non-melanoma skin cancer, its clinical assessment was performed in addition to the assessment of dermatoses. Criteria for the assessment of UV-induced skin damage were cutis rhomboidalis nuchae, erythrosis interfollicularis and elastosis. Other important variables were common nevi (presence and semiquantitative assessment of the num-

ber), the presence and count of clinically atypical nevi, cutaneous precancerous lesions subclassified in different entities, skin tumours, (basal cell carcinoma, squamous cell carcinoma, malignant melanoma, other skin tumours), psoriasis, atopic eczema, mycoses (differentiated).

Results: Data are still in a review process above all in terms of weighting. Comparisons will be shown with other investigations both population – based and non – population based. The analysis of the primary unweighted data seem to be in a pre-described range for some common dermatoses as psoriasis and atopic dermatitis but to be elevated in signs of UV-related skin aging (elastosis, cutis rhomboidalis nuchae).

#### P124

##### Prevalence and health care situation of juvenile psoriasis in Germany

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Introduction: Population-based data on epidemiologic parameters and health care of psoriasis in children are rare. It was the aim of the study to analyse the prevalence, comorbidity and health care situation of children and young adults with psoriasis using nationwide health insurance data.

Methods: A secondary data analysis was performed using a database of about 1.7 million non-selected individuals from a German health insurance organization which covers all geographic regions. The study population consisted of all in 2009 continuously insured persons <18 years with at least one confirmed diagnosis of psoriasis. Furthermore data were obtained for two control-groups: (i) children without psoriasis and (ii) children with a confirmed diagnosis of atopic dermatitis (AD).

Results: In total, 1313 patients <18 years out of 293.181 were identified with psoriasis; the overall 1-year prevalence was 0.45% (girls 0.48%, boys 0.42%). AD was identified in 30.354 children (prevalence 9.66%). Regional differences were observed ranging from 0.35% to 0.63%. Comorbidity of diagnoses known to be related to psoriasis in adults was also found in children: e.g. prevalence of obesity was 7.08% in children with psoriasis, 3.61% without this diagnose and 4.11% in children with AD. Other comorbidities with increased rates in psoriatic children included diabetes, depression, arthritis, hypertension, hyperlipidemia, iridozyklitis, keratitis and nail disease. Patient care was provided by almost equal numbers of dermatologists, ( $n = 900$ ), general practitioners (GP) ( $n = 886$ ) and paediatricians ( $n = 971$ ).

Psoriasis treatment differed markedly by specialisation: GP and paediatricians prescribed systemic steroids more often than dermatologists and made less use of distinct topical drugs.

Conclusion: Juvenile psoriasis is frequent and associated with substantial comorbidity. Therapeutic care for psoriasis shows considerable differences between care givers. The study results underline the need for the development and use of an interdisciplinary treatment-guideline in juvenile psoriasis.

#### P125

##### One-year mortality rate in bullous pemphigoid – significant correlation of autoantibody levels against BP180 at time of diagnosis

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Introduction: Bullous pemphigoid (BP) is characterised by circulating pathogenetic autoantibodies against bullous pemphigoid antigens I (BP230) and II (BP180). The individual course of disease is unpredictable. Factors like old age and multiple comorbidities probably lead to an increased 1-year mortality rate and must be considered before therapy is initiated.

Objective: In this retrospective study we investigated the possible correlation of various epidemiological, clinical and immunopathological characteristics with the 1-year and overall mortality rate in a cohort of 41 patients with BP.

Methods: Using a data collection sheet we collected epidemiological, clinical and immunopathological data of 41 patients diagnosed with BP at our department between October 2001 and June 2009. Clinical severity at the time of diagnosis was assessed through evaluation (ABIS) of clinical photographs ( $n = 26$ ). Missing immunopathological data (ELISA, indirect immunofluorescence and/or split skin studies) were completed through analysing stored serum samples. All patients were followed up for at least 1 year.

Results: The 1-year mortality was 17.5%; this is a 2.5-fold higher probability of death compared with an age- and gender-matched control group. Levels of autoantibodies against BP180 at the time of diagnosis correlated significantly (i) with disease activity ( $n = 26$ ,  $P = 0.03$ ) and (ii) with the 1-year mortality ( $n = 37$ ,  $P = 0.008$ ). Moreover, 1-year mortality correlated significantly with the initial dosage of systemic steroids ( $n = 41$ ,  $P = 0.034$ ). The number of comorbidities only correlated with the mortality over the whole observational period of 25 months on the average ( $n = 41$ ,  $P = 0.027$ ).

Conclusions: Our data show a significant correlation of autoantibody levels against BP180 at the time of diagnosis with the 1 year mortality rate, implying that disease-specific factors contribute to a higher mortality rate in patients with BP compared to a gender and age matched control group. Therefore, antibody levels measured by ELISA could provide a prognostic factor which should be taken into account in the management of BP patients. Initial steroid dosage should also be considered as a potential risk factor for the increased 1-year mortality. Correlation of the number of comorbidities with the overall mortality underscores the importance of close collaborations with other specialities in the management of BP patients.

#### P126 (V04)

##### Remission of eczema in children and influencing factors: a prospective population-based Swedish study

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Introduction: Atopic dermatitis (AD) is the most frequent inflammatory childhood disease with high impact on the wellbeing of affected children and of society. It has been reported that about half the cases heal before adolescence. However, there is a lack of knowledge regarding factors associated with remission of AD.

Aim: To determine predictors of importance for remission of AD in childhood. Setting and participants: All children aged 1–3 years in 2000 living in Värmland, Sweden were eligible to participate in the Dampness in Building and Health study, and 4779 (64%; 4779/7509) children participated in both the baseline and follow up survey of the study. The study population consisted of 894 children participating in both surveys with 'eczema during last 12 months' at baseline.

Methods: AD remission was defined as no 'eczema during last 12 months' at follow-up in 2005. The association between remission of eczema, and a set of background, lifestyle and environmental variables was estimated by means of univariable analysis, and multivariable logistic regression modelling based on a hierarchical model.

Results: Of all children with eczema at baseline 54.1% had no eczema 5 years later. In adjusted analysis, the milder the eczema (aOR 1.43; 1.16–1.77), and the later the onset (aOR 1.40, 1.08–1.80), the higher the odds of remission. Further, in multivariable analysis, rural living (adjusted odds ratio (aOR) 1.48; 1.07–2.04), non-flexural eczema (aOR 2.57; 1.62–4.09), and absence of food allergy (aOR 1.51; 1.11–2.05) were independent factors associated with remission of eczema 5 years later. In crude

analysis, absence of family history of allergic disease (aOR 1.47, 0.99–2.17), or only one parent with history of allergic disease (1.74; 1.18–2.56) compared to two parents with history of allergic disease, absence of doctor-diagnosed rhinitis (aOR 3.50; 1.49–8.31), and doctor-diagnosed asthma (1.65; 1.06–2.56) increased the odds of remission of eczema; and year of building the house between 1960–1984 compared to construction before 1960 (0.67; 0.49–0.91) decreased the odds of remission. The factors birth order, gender, parental smoking, breast feeding, antibiotic consumption, bedrooms with PVC flooring material, and day care attendance were not found to be associated with remission of eczema. In separate analysis, current pet ownership was associated with remission of eczema, although the strength of the association attenuated in analysis adjusted for avoidance behaviour and concomitant allergic diseases (aOR 1.28; 0.90–1.82). Conclusions: Features of eczema are the most important predictors of remission. The milder the eczema, and the later the onset, the higher the chance of remission. Early identification and treatment is of great value. Rural living may be beneficial for remission of eczema.

## P127

### Germline mutations in BAP1 predispose to melanocytic tumors

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Common acquired melanocytic nevi are benign neoplasms that are composed of small, uniform melanocytes and are typically present as flat or slightly elevated pigmented lesions on the skin. We describe two families with a new autosomal dominant syndrome characterized by multiple, skin-colored, elevated melanocytic tumors. In contrast to common acquired nevi, the melanocytic neoplasms in affected family members ranged histopathologically from epithelioid nevi to atypical melanocytic proliferations that showed overlapping features with melanoma. Some affected individuals developed uveal or cutaneous melanomas. Segregating with this phenotype, we found inactivating germline mutations of BAP1, which encodes a ubiquitin carboxy-terminal hydrolase. The majority of melanocytic neoplasms lost the remaining wild-type allele of BAP1 by various somatic alterations. In addition, we found BAP1 mutations in a subset of sporadic melanocytic neoplasms showing histological similarities to the familial tumors. These findings suggest that loss of BAP1 is associated with a clinically and morphologically distinct type of melanocytic neoplasm.

## P128

### FGFR3, PIK3CA and RAS mutations in lichenoid keratosis

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Background: Lichenoid keratosis (LK) are benign solitary skin lesions which have been proposed to represent a regressive form of pre-existent epidermal tumors such as solar lentigo or seborrheic keratosis. However, the genetic basis of LK is unknown.

Objectives: FGFR3, PIK3CA and RAS mutations have been shown to be involved in the pathogenesis of seborrheic keratosis and solar lentigo. We thus investigated whether these mutations are also present in LK.

Methods: After manual microdissection and DNA isolation, 52 LK were screened for FGFR3, PIK3CA and RAS hotspot mutations using SNaPshot<sup>®</sup> multiplex assays.

Results: We identified 6/52 (12%) FGFR3 mutations, 10/52 (19%) PIK3CA mutations, 6/52 (12%) HRAS mutations and 2/52 (4%) KRAS mutations. FGFR3 and RAS mutations were mutually exclusive. One LK showed a simultaneous PIK3CA and HRAS mutation. In nine LK with a mutation, non-lesional control tissues from the epidermal margin and the dermal lymphocytic infiltrate were wild type, indicating that these mutations are somatic. To demonstrate that these findings are specific, 10 samples of lichen ruber planus were analyzed without evidence for FGFR3, PIK3CA or RAS mutations.

Conclusion: Our results indicate that FGFR3, PIK3CA and RAS mutations are present in approximately 50% of LK. These findings support the concept on the molecular genetic level that at least a proportion of LK represent regressive variants resulting from former benign epidermal tumours such as seborrheic keratosis and solar lentigo.

## P129

### Subcutaneous panniculitis-like T-cell lymphomas do not show genetic aberrations with array CGH

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Background: Subcutaneous panniculitis-like T-cell lymphoma (SPTCL) is currently defined as a T-cell lymphoma characterized by a cytotoxic alpha/beta T-cell phenotype with typical immunohistochemical findings and a good prognosis. In contrast to SPTCL, cutaneous gamma/delta T-cell lymphoma, that may present with prominent subcutaneous involvement, shows an aggressive behavior with poor prognosis. In 2008, Hahtola et al. using single-cell comparative genomic hybridization (CGH) reported in nine cases of SPTCL a large number of genetic aberrations including losses of chromosomes 1, 2, 10, 11, 12, 16, 19, 20, 22 and gains of chromosomes 2 and 4. However, no confirmation of these genetic data by other groups has been presented yet.

Methods: We performed array CGH in skin biopsies of three patients with diagnosed SPTCL. To ensure high purity of tumor DNA, areas with over 75% tumor cell infiltration were laser microdissected before DNA isolation. In addition one case of gamma/delta T-cell lymphoma with prominent involvement of the subcutaneous fat has been added.

Results: All three cases of SPTCL did not show any genomic aberrations with array CGH. The case of gamma/delta T-cell lymphoma showed a large number of copy number changes with losses on chromosomes 1, 6, 10 and 13 as well as gains on chromosome 2. On follow up this patient presented with a rapidly progressive course typical of this aggressive lymphoma.

Conclusion: In contrast to Hahtola et al. we were not able to detect copy number alterations in SPTCL. The lack of genetic aberrations observed by CGH may be explained by two mechanisms. Either, neoplastic lymphocytes of SPTCL do not show major gains and losses, consistent with the low-grade malignancy of these tumors, or that even after accurate laser microdissection, neoplastic cells are too few to be evaluable by CGH. However, the prominent chromosomal aberrations in the gamma/delta T-cell lymphoma with subcutaneous involvement (morphologically comparable to the SPTCL) suggests that the genomic characterization of few tumor cells with array CGH is valid.

## P130

### Characterization of novel XP-G patients: prognostic assessment on the basis of mutational analysis

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Individuals suffering from xeroderma pigmentosum (XP) have a defect in the nucleotide excision repair pathway (NER). Consequences are premature skin ageing and a 1000-fold increased risk for the development of UV-induced skin cancers. To date, 7 XP genes (XPA to XPG) have been identified, which have a role in repair of UV-lesions and are part of NER. There is considerable phenotypic heterogeneity between the different XP complementation groups. Even within a single XP complementation group a high variability regarding skin cancer risk as well as the severity of neurological abnormalities is observed. We have access to the largest collection of patients suffering from NER-defective syndromes in Germany including clinical data as well as primary fibroblast cells. We perform clinical and molecular diagnostics of these patients in order to provide prognostic assessments of patients with a defect in NER in the future.

We have established a cell and RNA/DNA bank for further molecular characterization of the patients. For phenotypic characterization we have measured UV-sensitivity and UV-lesion-repair-capacity. Furthermore, we determined their complementation group and have localized the mutation in the gene by sequencing. Three patients could be assigned to the rare XPG complementation group, where only approximately 14 XP-G patients have been described worldwide so far. All three patients have novel missense and nonsense mutations never described before. Two patients are compound heterozygous, whereas the other patient is homozygous. The functionality of the mutated XPG proteins has been tested and showed some residual repair activity depending on the mutation. We conclude that the phenotypic heterogeneity depends on the type and location of the disease causing mutation in the XPG gene.

## P131

### Association of gene polymorphisms with cutaneous melanoma

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Cutaneous melanoma is the most aggressive type of skin cancer. The risk of melanoma increases with phenotypic risk factors such as family history of melanoma, dysplastic nevi, number of nevi or skin type. However, these phenotypic risk factors are not very specific in predicting the individual melanoma risk. Therefore, identification of molecular melanoma risk factors is very desirable for improved patient stratification. The development of skin cancer is a multi-step process in which the accumulation of genomic mutations finally leads to tumour formation. Gene polymorphisms may lead to a 'mutator-phenotype' that facilitates the development of such mutations. The DNA damage binding protein 2 (DDB2/XPE) is involved in the nucleotide excision repair pathway. Mutations in the gene can cause the DNA repair-deficiency syndrome xeroderma pigmentosum. The polymorphism nt18100C>G in intron 3 of the DDB2 – gene is associated with a 1.3-fold increased lung cancer risk. The PTCH – gene is part of the Sonic – Hedgehog signal (SHH) pathway. Inactivating mutations in PTCH lead to activation of the pathway which is an early event in basal cell carcinoma (BCC) development. It is reported that the polymorphism C3944T (Pro1315Leu) in exon 3 of the PTCH gene is associated with an increased BCC and breast cancer risk.

To assess an association between the described polymorphisms and melanoma we collected DNA from 286 Caucasian melanoma patients from Germany and from 342 healthy control individuals from the same area matched by gender.

The allele frequencies (cases: controls) were for DDB2 nt18100C 46.1%: 53.9% and for PTCH exon 3 3944C 45.6%: 54.4%. In summary we found no association of the DDB2 intron 3 nt18100GG and the PTCH exon 3 3944TT genotypes with increased risk of melanoma development.

## P132 (V05)

### Host genetics play a dual role in controlling both skin bacterial species and autoimmune blistering skin disease

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Establishing the relationship between the host-genome and skin microbiota is key to understanding the overall genetic basis for skin autoimmune diseases. To study this, a cohort of 261 mice from the G4 population of an advanced intercross line originating from four strains partially susceptible to epidermolysis bullosa acquisita (EBA) was used. Mice were genotyped using a 1400-SNP Illumina microarray and their EBA scores were determined. Pyrosequencing was then used to quantitatively measure the skin microbiota. The sequences were classified to species level Operational Taxonomic Units (OTUs) (using a 97% threshold). A Measurable Microbiota (MM) was defined as those species level OTUs that quantitatively varied across the animal population. Through MM, the total number of species OTUs was reduced to 134. When MM was tested for co-segregation against the genotype, 335 overlapping host Quantitative Trait Loci (QTLs) (LOD scores  $\geq 3.9$ ) were identified for 97 OTUs. Using the unweighted UniFrac metric, highly significant species differences between healthy and EBA mice were observed (Monte Carlo permutation test,  $n = 1000$  permutations,  $P = 0.001$ ). This further led to identifying common QTLs for EBA disease (from previously published data) and species level OTUs, resulting in 19 QTLs from 18 species OTUs. Out of these 18 species OTUs, clearly 11 species OTUs were significantly higher in healthy mice compared to EBA mice. This data is currently confirmed using knockout mice of candidate quantitative trait genes mapping within the common microbial/EBA QTLs. Our findings show that the host QTL controlling EBA disease also controls certain skin bacterial species, indicating that host-genetics plays a dual role in controlling both microbiota and disease.

## P133

### No evidence of viral genomes in whole-transcriptome sequencings of melanomas

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Several viruses induce the development of skin tumors, such as human herpes virus 8 causing Kaposi's sarcoma and Merkel Cell Polyoma Virus (MCPyV) causing Merkel cell carcinoma. Similarly to these

types of tumors, melanoma incidence is increased in immuno-suppressed patients, raising the suspicion that viral infections might contribute to melanoma formation. We recently searched for viral sequences in 454 next generation whole-transcriptome sequencings of three melanoma metastases using digital transcriptome subtraction (DTS) analysis and found no evidence for viral transcripts. Considering the low sample number, we have now investigated 10 melanoma samples using sequencing-by-synthesis (SBS) technology, which offers a better coverage at a lower cost. We were able to detect viral sequences in a dataset derived from a HPV-positive cervical cancer, but did not find viral sequences in our melanoma samples, confirming our previous findings.

**P134**  
**Filaggrin deficiency in ichthyosis vulgaris (IV): increase of skin pH and evidence for imbalance of KLK activity in a 3D keratinocytic model for IV**

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Ichthyosis vulgaris (IV) is a common genetic skin disease characterized by dry skin and scaling. It is caused by nonsense mutations in the filaggrin gene (FLG) that are also associated with an increased risk of atopy. Recently, it was shown that the natural moisturizing factor (NMF) is decreased in IV, and a slight impairment of the epidermal barrier has been postulated.  
We address the question whether the pH is altered, thus leading to changes of the balance of epidermal kallikrein (KLK) activity. Here, we have clinically studied a cohort of 20 patients with IV. All patients were screened for mutations within the filaggrin gene. Electron microscopy confirmed reduction or absence of keratohyaline granules. We conducted a skin physiological study, i. e. transepidermal water loss (TEWL), pH and corneometry in all patients and in 17 controls. Moreover, we developed a 3D organotypic cell model for patients with complete FLG-deficiency. Cell culture experiments were performed for six patients, which have been analysed for activity of KLK5, 7 and caspase-1. The mean TEWL in patients with IV and healthy controls was 7.78 and 5.28 g/mhr, respectively. Hence, there is a low increase of TEWL in IV ( $P = 0.008$ ). The mean pH was 5.56 and 5.27, respectively. The minimal difference in pH was of low significance ( $P = 0.031$ ). The preliminary results of KLK5 and 7 activity of the primary keratinocytic cell culture showed an increase of KLK7 activity in patients with IV compared with controls, whereas only a low increase of KLK5 activity has also been detected. There was no shift of caspase-1 activity.  
We confirm a difference in pH and a slight change of the epidermal barrier function in filaggrin deficiency. These issues are under further investigation.

**P135**  
**2-photon fluorescence lifetime imaging microscopy (2P-FLIM) to assess pH in filaggrin-deficient skin equivalents**

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Recently, we reported an inverse relationship between amounts of FLG gene products and skin surface pH in Filaggrin (FLG)-deficient patients, with double-allele subjects displaying the greatest elevations in skin surface pH. In FLG deficient patients, reduced amount of FLG might lead to a decreased downstream production of acidic metabolites, known to be endogenous acidifiers of the stratum corneum. Thus, we hypothesized that decreased generation of FLG products could result in an increase in skin pH that deviates from the pH optima of lipid metabolizing enzymes and epidermal proteases. Prolonged pH alterations could precipitate downstream structural and functional alterations.  
Skin equivalents were stained with BCECF (2',7'-Bis-(carboxyethyl)-5(6)-carboxyfluoresceinacetoxymethyl), a widely used fluorescent pH indicator for near-neutral pH measurements. The fluorophore was added to medium of skin equivalents at a final concentration of 80 M and incubated at 37°C for approximately 7 h. For measurements of pH in skin equivalents we used 2-photon-fluorescence lifetime imaging microscopy (2P-FLIM) since lifetime of BCECF is pH dependent and increases with increasing pH. As detection unit, we used a 16-channel time-correlated single photon counter (TCSPC), which records single photon-events originating from an excitation laser pulse in single beam mode. The pH at each pixel was calculated from lifetime-resolved data.  
Skin equivalents of FLG-/- genotype showed a near-absent granular layer when compared to skin equivalents generated with FLG +/- cells which showed a normal, multilayered stratum granulosum. 2P-FLIM revealed an increased pH in skin equivalents of homozygous FLG genotype, compared to healthy control skin equivalents.  
These results support the hypothesis, that FLG deficiency leads to an increase in skin pH and suggest that skin equivalents from keratinocytes of homozygous FLG mutation carriers serve as a model for functional studies of FLG deficiency in ichthyosis vulgaris and atopic dermatitis.

**P136**  
**Late-onset erythropoietic protoporphyria caused by mosaicism after autologous blood stem cell transplantation and radiation therapy**

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Erythropoietic protoporphyria (EPP) is an autosomal semi-dominant disorder that is due to a marked deficiency of ferrochelatase (FECH), the eighth enzyme in heme biosynthesis. This enzymatic dysfunction results from inheritance of a germline FECH gene mutation on one parental allele in combination with a common hypomorphic intronic FECH variation, IVS3-48C, on the other parental allele. Usually, EPP manifests in early childhood with burning cutaneous photosensitivity. Here we present a 57-year-old Caucasian man who developed severe cutaneous photosensitivity 6 years after treatment of a large B-cell lymphoma with autologous blood stem cell transplantation and radiation therapy. Biochemical and enzymatic analyses confirmed the diagnosis of EPP. By leukocyte DNA sequencing we detected a nonsense mutation, p.R298X, in combination with the hypomorphic IVS3-48C variation in trans. Interestingly, the chromatographic intensity of the mutated T-allele at position 298 was reproducibly lower than that of the wild type C-allele, suggesting mosaicism. Most of the rare cases of late-onset EPP occurred in association with a myelodysplastic syndrome or myeloproliferative disorder due to a deletion on chromosome 18q, the region in which the FECH gene is located. Hence, we first excluded such a deletion by fluorescence *in situ* hybridization. Sequencing of DNA derived from cultured fibroblasts of the patient showed absence of p.R298X, confirming that this mutation arose as a

result of mosaicism. This is the first report on late-onset EPP due to blood cell mosaicism caused by a spontaneous FECH mutation following autologous blood stem cell transplantation and radiation therapy of a large B-cell lymphoma.

**P137 (withdrawn)**

**P138**  
**Pemphigus vulgaris in Egyptians is associated with the HLA Class II alleles DRB1\*04:02:01, \*14:56 and \*08:04:01.**

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Pemphigus vulgaris (PV) is a rare severe autoantibody mediated blistering disease that is strongly associated with human leukocyte antigen (HLA) II alleles. However, the HLA haplotypes vary with the ethnic background. The purpose of this study was to perform high resolution genotyping for the HLA-DRB1 locus in a German and an Egyptian cohort.  
The HLA-DRB1 locus of 46 German and 47 Egyptian PV patients versus 74 and 73 ethnically matched healthy blood donors were genotyped. Genotyping was performed in two steps. First, low resolution typing with the polymerase chain reaction sequence specific primer (SSP) method was used to reveal subtype associations of the DRB1 gene. The frequency of HLA-DRB1\*04 was 38% in German PV patients (controls 18%; corrected  $P$ -value:  $1.8 \times 10^{-7}$ ) and 45% in Egyptian PV patients from the Cairo area (controls 18%; corrected  $P$ -value:  $5.8 \times 10^{-7}$ ). HLA-DRB1\*14 was found in 22% of German PV patients (controls 2%; corrected  $P$ -value:  $1.42 \times 10^{-7}$ ) and in 14.89% of Egyptian patients (controls: 1.37%; corrected  $P$ -value:  $5.72 \times 10^{-7}$ ). In addition, a significant association of HLA-DRB1\*08 was seen in Egyptian patients (patients: 13.89%, controls 1.37%; corrected  $P$ -value:  $1.3 \times 10^{-7}$ ), but not in Germans.  
In a second step, we increased the resolution of the associated subtypes by a HLA sequencing-based typing (SBT) strategy (up to six digits). The high resolution analysis of \*04 revealed a strong association of \*04:02:01 in 83.33% of Egyptian and in 71.43% of German patients (33.33% and 0% in Egyptian and German controls, respectively). Egyptian patients and controls both exclusively carried HLA-DRB1\*08:04:01. HLA-DRB1\*14 was identified as \*14:56 in most cases without preference of the patient and control groups in both Germans and Egyptians.  
Our data confirms a strong association of HLA-DRB1\*04:02:01 and \*14:56 in PV patients in distinct ethnicities. In six-digit high resolution, associations to intronic gene variations were not found. In contrary to the German population, an additional association for HLA-DRB1\*08:04:01 was detected only in Egyptian PV patients.

**P139**  
**The Cockayne syndrome (CS) A and B proteins interact with the mitochondrial nucleoid involved in transcription of mitochondrial DNA: possible relevance for neurodegeneration of CS**

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Cockayne syndrome (CS) is a rare progeroid disorder, characterized by skin photosensitivity, loss of subcutaneous fat and neurodegeneration. It is caused by mutations in the genes encoding for the CSA and CSB proteins, which are known to be involved in repair and transcription of nuclear DNA. We showed previously, that in mitochondria CSA and CSB are involved in the repair of mitochondrial (mt)DNA and the protection from aging-associated loss of subcutaneous fat.  
Since in the nucleus CSA and CSB are also involved in transcription, we investigated interaction with proteins of the mitochondrial nucleoid, a large multiprotein complex known to be involved in mitochondrial transcription. We developed a new assay for screening direct interaction of CSA and CSB with nucleoid-proteins. After isolation of mitochondria and subsequent pull-down of nucleoid complexes these were separated and visualized by blue native gel electrophoresis with subsequent second dimension SDS gel separation, followed by western-blot detection of the complex-constituting proteins. We found CSA and CSB containing protein complexes also harbor proteins, essential for mitochondrial transcription: (i) mtRNA polymerase and mitochondrial transcription factor A (mtTFA), confirming potential involvement of CSA and CSB in transcription. This interaction was absent in CSA/B deficient controls. Unexpectedly, with this technique, we also identified interaction with a protein known to be involved in the pathogenesis of ParkinsonEUR(TM)s disease. These findings point to a role of CSA and CSB in mitochondrial transcription as well as a possible link to neurodegenerative symptoms previously unexplained in Cockayne Syndrome.

**P140**  
**How to measure quality in lymphedema care: development and application of a new quality of care index**

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Qualified and guideline-based treatment of chronic lip- and lymphedema (CLE) is challenging. It requires both long term patient compliance with time-consuming treatments and close cooperation between physicians, lymphtherapists, and medical supply store staff. Measurement of quality of care in CLE must include different facets of diagnostic, treatment and after-care. In spite of this complexity, a single quality index is needed for the assessment of measurement of change and comparison between patient groups.  
Indicators of quality of care of CLE were developed in a national Delphi expert consensus including three steps: (i) extraction of possible indicators from national and international literature, (ii) first questionnaire-based evaluation of indicators by 24 German lymphedema experts; reduction of the pool of indicators by national expert discussion ( $n = 12$ ), and (iii) second questionnaire-based evaluation by 28 lymphedema experts and finalisation of a non-redundant indicator set which found broad support in the evaluation. Quality of lymphedema care was assessed in a cross-sectional study involving a large spectrum of care providers in the metropolitan area of Hamburg. The quality indicators were transformed to one unweighted index on individual patient level.  
A total of 12 quality indicators were identified, covering history, diagnostics, therapy, and prevention. These indicators were applied to 348 patients with CLE of any origin (90.8% female, mean age  $57 \pm 14$  years). On average, 58% of the quality indicators were met (quality index of 0.58). Quality of care was significantly higher in patients treated in the 'Lymphnetz Hamburg', a network of lymph specialists (quality index 0.63 vs 0.53).  
The quality indicators and the quality index for CLE are feasible for comparisons of patient groups, regions and health care systems. Furthermore, use of the quality index may lead to process optimization in lymphedema care.

P141

### Disease related stress in children with Neurofibromatosis type 1 in Germany from their own and their parents point of view

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**Introduction and Objectives:** Neurofibromatosis type 1 (NF1) is a rare disease characterized by benign tumors of the peripheral nerves and typical skin disfigurements. There is evidence that children with NF1 experience psychosocial problems (Noll 2007).

**Aim of this study:** Obtaining data about NF1 childrens stress from their own and their parents perspective.

**Materials and Methods:** In a cross sectional study, a total of 109 children with NF1 and one parent of each child separately filled in a questionnaire on the child's clinical status, quality of life (QoL), disease related stress, body image, support options and diverse health care parameters. The parents statements were compared to the childrens views by paired *t*-tests.

**Results:** The children were aged between 10 and 17 (44.3% female). 27.5% had at least one relative with NF1. The childrens stress was rated higher by parents than by children themselves (3.82,8 vs 3.12,8, *P* < 0.01, scale from 0 = no stress to 10 = extremely stressed). The parents evaluated their own stress considerably higher than their childrens (5.13,0 vs 3.12,8, *P* < 0.001).

Additionally those parents and children with other affected family members considered themselves more strained and the children rated their quality of life lower (67.920,2 vs 78.415,4, *P* < 0.05, scale from 0 = minimum to 100 = maximum QoL).

**Conclusions:** Children with NF1 seem to have a more optimistic view on their burden than their parents. The finding that parents rate their own stress levels even higher than their childrens indicates a broader family concernment. It seems that those with affected family members do not find it easier to cope with NF1. Therefore psychosocial support of the family should be an essential part of health care of children with NF1.

P142

### Quality of health care of atopic dermatitis in Germany: results of the national health care study atopic dermatitis 2010

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**Objectives:** To obtain reliable data on quality of care of atopic dermatitis in adult patients in Germany from the patient's and physician's perspectives. Because dermatologists are the main health care providers in this disease, the study was conducted with a random sample of dermatology practices and clinics.

**Patients and Methods:** One hundred and seventy-four dermatological centres participated in the study, 91 of these were active (including at least one patient). In this non-interventional, cross-sectional study socio-demographic data, clinical history, health-related quality of life (DLQI), current medication and treatment and patient-defined treatment benefits (PBI) were assessed by standardized questionnaires. **Results:** Data from 1678 patients (60.5% female) were suitable for analyses. The mean age was 38.415.9 years. 49.2% of patients had a positive family history for atopic diseases. On average, 10.1% of the patients' body surface area was covered with dermatitis lesions. Regarding the concomitant diseases, rhinoconjunctivitis was – with up to 54% – the comorbidity of highest prevalence.

The most frequently used treatments during the last 5 years were basic ointments (90.4%) followed by topical corticosteroids (85.5%). 75.8% of patients 'felt not' or only 'moderately impaired' by their treatment. The most commonly named reasons for itching or for worsening of dermatitis symptoms were 'emotional factors and stress' (named by 73.1%). The global score of disease specific quality of life in skin diseases (DLQI) showed a mean of 8.565. 32.1% had a DLQI of more than 10. The self-rated state of health (EQ-5d-VAS, 100 = best possible state of health) was 63.622,0, on average. 26.6% of patients reported to suffer 'often' or 'every night' from sleeplessness because of severe itching during the last 7 days. On average, the patients rated the health care of their atopic dermatitis during the last years as 2.60,9 (1 = very good, 5 = poor). Patient-defined therapeutic benefit (PBI) was 2.41,1, on average (4 = maximum benefit).

**Conclusions:** Although a large part of patients consider their treatment-related burden as relatively low, the daily burden of disease seems to be comparatively high: Sleeplessness and scraping in about one third of patients indicate a high disease-related burden and insufficient therapeutic regimes in these cases.

Furthermore, about one third of patients had a DLQI of more than 10, which means a significant lack of quality of life. It is outstanding that a large part is treated with glucocorticoids. The fact, that 73.1% of patients believe emotional factors and stress to be the main reason for exacerbation of their atopic dermatitis, underlines the importance of psychological factors, which require much more than only medical treatment in patients with atopic dermatitis. Special patient training seminars e.g. can give a platform to this topic.

P143

### Kaletra®/Truvada® as post exposure prophylaxis (PEP) to HIV – an effective and well tolerated regimen

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**Introduction:** PEP to HIV is a course of antiretroviral drugs administered within 72 h after events with high risk of exposure to HIV aiming to reduce the odds of established infection.

We evaluated the putative HIV exposed individuals referred to the MUV and indicated for PEP in years 2008–2010.

**Methodology and Results:** Our so far analyzed data of 180 individuals (research in progress) demonstrated that:

1 44.1% are females.

2 Indication type: unprotected homosexual contact [28.5%, from which 45% of source patients (SPs) were HIV positive], needlestick injuries (22.8%, 37.5% HIV positive SPs), unprotected heterosexual contact (21.4%, 20% HIV positive SPs), occupational exposure (12.8%, 100% HIV positive SPs), rape (11.4%) and needle exchange by IVUDs (2.8%) where HIV status of SPs were unknown,

3 PEP regimens were Kaletra®/Truvada® (79.4%) or Kaletra®/Combivir® (20.5%),

4 58.8% of individuals tolerated the PEP without any adverse events, 35.3% had minor adverse events (nausea, fatigue, diarrhea, abdominal discomfort or slight elevation of pancreatic enzymes) and in 5.8% PEP was modified or discontinued (severe adverse events: strong diarrhea, abdominal pain and vomiting or significant elevation of liver function parameters),

5 77.1% of patients missed at least one of their follow-up visits planned at 1, 3 and 6 months after PEP start, and

6 No case of seroconversion was observed.

**Conclusion:** Approximately equal numbers of sexes seek counseling service for PEP. Most prevalent types of exposure include high risk sexual contact and needlestick injuries. Kaletra®/Truvada® combination seems to be a well tolerated and effective therapy.

P144

### Characteristics and care of patients with chronic hand eczema: update from the carpe registry

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**Aims:** The aim of the carpe (German acronym: Chronisches Handekzem-Register zum Patienten-Langzeitmanagement) registry is to investigate sociodemographic and clinical characteristics, applied treatment options and adverse events in patients affected by chronic hand eczema (CHE) in Germany. **Methods:** Patients with chronic hand eczema (CHE) are prospectively assessed by means of a dermatological examination and a patient questionnaire and followed-up at three, six, nine, 18 and 24 months. Socio-economic and clinical data are repeatedly collected as well as data on diagnostics, current skin status, atopy, severity and treatment of CHE. Patient-reported outcomes include health-related quality of life (HrQoL) measured by the Dermatology Life Quality Index (DLQI). Analyses of quantitative and qualitative variables was done using SAS for Windows.

**Results:** A total of 1058 patients with chronic hand eczema were eligible for this interim-analysis, of whom 55.0% were female. Mean age was 47.0 years [standard deviation (SD): 13.8 years]. The average duration of CHE was 7.7 years (SD: 9.2 years). The most frequent occupations of those included were: nursing and health care (20.0%), metal and electrical industry (17%) and food industry and gastronomy (14%). 32.0% had already received in patient-care (hospital or tertiary prevention) because of CHE. 25.0% were currently unable to work due to CHE. 36.0% had been on sick leave due to CHE in the 12 months prior to investigation. 5.3% had changed or given up their job due to CHE.

Irritant contact dermatitis was the leading diagnosis (44.6%). 59.3% had an atopic skin diathesis. At inclusion, CHE was very severe in 23.7%, severe in 47.3% and moderate in 19.4% of patients, and clear or almost clear in 9.7%. 94.0% of patients had received topical corticosteroids prior to inclusion in the register, 26.2% systemic antihistamines, 28.7% topical calcineurin-inhibitors, 38.4% UV therapy, and 33.0% had received systemic treatment.

Mean DLQI score decreased from a mean of 9.5 (SD 6.3) at baseline to 4.9 (SD 4.7) after 18 months. The proportion of patients whose CHE was clear or almost clear increased from 9.7% at baseline to 34.5% after 18 months.

**Conclusions:** Both subjective and objective parameters confirm the severity and chronicity of the CHE in the patients included. At inclusion, a considerable proportion of patients may not receive adequate treatment. A clear improvement of health-related quality of life and disease severity over time was observed.

P145

### Health care situation of patients with chronic pain after surgery in Germany – a secondary data analysis project

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According to several studies post-operative pain is a widespread phenomenon. At least every third patient in Germany suffers from pain after surgery. Furthermore, a few studies have shown that 20–60% of the patients developed chronic pain after specific surgeries within 6 months. However, currently published data show a lack of information on the real health care situation of patients with pain after surgeries.

The aim of the study is to analyse the prevalence, cost of illness and health care situation of patients with chronic pain after surgeries using nationwide health insurance data.

The actual health care situation of patients with chronic pain after surgery will be analysed using secondary data from a statutory health insurance. The study population consists of all in 2009 and 2010 continuously insured persons ≥18 years of age with at least one stationary surgery. Furthermore a control group will be obtained.

Health insurance data of the nationwide health care organisation 'Barmer GEK' will be analysed. These secondary data will show the actual health care situation of patients with chronic pain after surgery.

The data will give knowledge about prevalence of chronic pain after surgery in population-groups relevant for the allocation of resources and health care planning. As substantial parameter for analyses cost of illness and the use of health services as well as prescriptions of practitioners have been identified. Moreover patient subgroups, the patient treatment and therapy process and regional variations have to be considered. On the basis of this analysis specific surgeries with a high risk of chronic pain can be identified.

Methods were shown to be of high feasibility in pre-tests. At the present data are analysed; results will be presented at the ADF-meeting.

Secondary data, based on data-collection from a large pool of patients over a long period of time, can represent the health care situation of patients with chronic pain and complement primary data which are. This way over-use, under-use, and mis-use of pain therapy in patients with chronic pain can be identified.

P146

### Routine skin cancer screening in Germany 2008–2011: performance and evaluation from the dermatological perspective

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**Background:** A systematic evaluation of the German routine skin cancer screening (SCS), as requested by the Federal Joint Committee, has not yet been carried out. However, since 2009 an annual accompanying evaluation has been performed by the German Centre for Health Services Research in Dermatology (CVderm).

**Study goal** was the longitudinal assessment of the features of SCS and its impact on dermatological health care provision.

**Methods:** Standardized surveys were conducted in 2000 German dermatology practices (listed and available at the database of the Professional Association of Dermatologists, BVDD) in the years 2009, 2010 and 2011. Physicians were asked to answer a short questionnaire on their performance of and opinion on SCS. Data were analyzed descriptively and compared by years.

**Results:** Response rates were 34.7%, 29.3% and 31.2% (in 2009, 2010 and 2011 respectively). In total, 1902 questionnaires were suitable for analysis. Mean number of SCS performed was 345/341/337 per quarter and praxis. Mean payment remained about 21 EUR. Satisfaction with SCS rose during the study period from 32.0% of physicians who were (very) satisfied with SCS in 2009 to 53.4% in 2011. A preference for a combined compensation of SCC by statutory health insurance (SHI) and patients' out of pocket payments (IGeL) was expressed by 41.8%/54.1%/56.7%, whereas coverage by SHI only was preferred by 29.0%/25.1%/29.2% in 2009/2010/2011.



Results from the current 2011 survey ( $n = 623$ ) are: Almost all dermatologists (96.6%) used dermatoscopes, 26.1% as patients' out of pocket expenses only. The number of surgical procedures and specific prescriptions has increased by 65.3% and 34.2% in comparison to the previous year. Cooperation with general practitioners (referral) was rated as (very) good by 22.9% and as (very) bad by 32.6% of the dermatologists. 83.8% regarded the quality of health care for skin-cancer-patients better since SCS had been introduced. This proportion was higher than in 2009 (69.4%) and in 2010 (79.8%). Conclusion: German dermatologists to a high degree approve SCS. Although a need for improvement is expressed for specific conditions, satisfaction with SCS increased continuously.

#### P147 Cutaneous over-expression of RANKL up-regulates MHC class I-mediated anti-viral immunity

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Viral infections of the skin are controlled by the immune system and since RANK-RANKL signalling is critical for dendritic cell – T cell communication as well as for the regulation of immune responses we investigated whether RANK-RANKL interactions might play a role in cutaneous anti-viral immunity. Therefore, transgenic mice over-expressing RANKL (CD254) in basal keratinocytes (K14-RANKL tg) were epicutaneously infected with Herpes simplex virus type 1 (HSV-1). Interestingly, K14-RANKL tg mice developed significantly smaller skin lesions compared to wildtype controls. Moreover, the number of virus particles as well as virus replication was reduced in tg versus wildtype skin as evidenced by immunofluorescence staining and quantitative real-time-PCR indicating increased primary anti-viral immune responses in K14-RANKL tg mice. Since CD8+ T cells are known to be essential for anti-viral immunity we analyzed the numbers and function of CD8+ T cells in regional lymph nodes and cutaneous lesions. Flow cytometry and immunohistochemistry revealed up-regulated levels of total CD8+ T cells as well as an enhanced expression of markers associated with cytotoxic T lymphocyte (CTL) function, like IFN- $\gamma$ , Fas ligand, and granzyme B, in CD8+ T cells from K14-RANKL tg compared to wildtype mice. To analyze the specific role of CD8+ T cells for anti-viral immune responses we isolated CD8+ effector T cells from HSV-1-infected CD45.2+ wildtype as well as CD45.2+ K14-RANKL tg donors, transferred them into naive congenic recipients and subsequently, infected recipient mice with HSV-1. The transfer of CD8+ T cells from HSV-1-infected wildtype donors resulted in a slight reduction of skin lesion size compared to recipients that received PBS instead of CD8+ T cells. However, mice that were injected with CD8+ T cells from HSV-1-infected K14-RANKL tg donors failed to develop inflammatory skin lesions upon HSV-1 challenge suggesting a strong MHC class I-restricted anti-viral immune response. Notably, the transferred CD45.2+CD8+ T cells from K14-RANKL tg mice migrated to regional lymph nodes and showed an up-regulated expression of activation as well as cytotoxic markers, like CD43, CD69, IFN- $\gamma$ , and granzyme B, compared to adoptively transferred wildtype T cells. To investigate whether CD8+ T cells are indeed essential for the increased anti-viral immunity in K14-RANKL tg mice we depleted CD8+ T cells in wildtype and tg mice prior to HSV-1 infection by using specific antibodies. Upon HSV-1 challenge CD8-depleted wildtype mice developed skin lesions similar to those observed in mice treated with an IgG control antibody. Strikingly, CD8-depletion in K14-RANKL tg mice led to a substantial increase in skin lesion size suggesting the RANK-RANKL-mediated induction/expansion of anti-viral MHC class I effector cells. Since during the induction of cutaneous anti-viral immune responses skin-derived antigen presenting cells, such as epidermal Langerhans cells (LC), migrate from the skin to regional lymph nodes and induce the differentiation of virus-specific effector T cells we investigated the effect of RANK-RANKL signalling on the phenotype and function of LC. Interestingly, LC from regional lymph nodes of HSV-1-infected K14-RANKL tg mice showed an up-regulated expression of activation/maturation markers, like CD80 or CD86, suggesting an increased T cell stimulatory capacity. Together, these data indicate that RANK-RANKL signalling is crucially involved in cutaneous MHC class I-mediated anti-viral immunity.

#### P148 (V06) TNF- $\alpha$ blockade ameliorates systemic autoimmunity by inducing functional regulatory T cells

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T cell-mediated autoimmune disorders play an important role in public health. However, the pathomechanisms underlying the loss of immunotolerance against self are largely unknown but it has been shown that autoreactive T cells can be detected in peripheral blood and cutaneous lesions from patients with autoimmune diseases. Usually the activation of autoreactive T cells is inhibited by functional CD4+CD25+Foxp3+ regulatory T cells (Treg) that actively suppress MHC class II-mediated immune responses. Interestingly, in rheumatoid arthritis systemically increased TNF- $\alpha$  levels have been suggested to impair the suppressive activity of Treg resulting in increased proliferation of pathogenic effector T cells. Since in mice with cutaneous overexpression of CD40 ligand (K14-CD40L tg) leading to the development of a severe systemic autoimmune disease, TNF- $\alpha$  levels are also substantially increased we investigated the role of TNF- $\alpha$  blockade on the suppressor function of Treg and disease progression in this mouse model. Thus, after onset of autoimmunity K14-CD40L tg mice were injected with an anti-TNF- $\alpha$  antibody or an IgG control. Strikingly, TNF- $\alpha$  blockade resulted in a significant amelioration of autoimmune dermatitis, which was paralleled by the up-regulation of Treg numbers in regional lymph nodes. Analysis of the Treg subpopulations before and after anti-TNF- $\alpha$  treatment revealed the peripheral induction of a novel CD62L- subset characterized by a decreased expression of markers associated with thymic-derived Treg like Helios or neuropilin-1 but up-regulated levels of IL-10 and CTLA-4. Notably, in contrast to thymic-derived Treg from autoimmune-prone K14-CD40L tg mice anti-TNF- $\alpha$  induced CD62L- Treg were functional as evidenced by *in vitro* suppression assays. Since systemically increased TNF- $\alpha$  levels have also been implicated in disease progression in mouse models of psoriasis and in psoriasis patients we hypothesized that this might be mediated by an impaired Treg function. Therefore, we analyzed whether in psoriasis-like skin inflammation of mice induced by topical imiquimod treatment TNF- $\alpha$  blockade resulted in an up-regulation of Treg numbers and suppressive activity. Indeed, similar to the observations made in CD40L-induced autoimmunity anti-TNF- $\alpha$  treatment led to the peripheral induction of a functional CD62L- Treg subset. These Treg efficiently inhibited the proliferation of Th-17 cells in skin-draining lymph nodes of psoriatic mice and the migration of pathogenic effector T cells to cutaneous lesions resulting in the amelioration of disease. Next, we investigated the effects of TNF- $\alpha$  on the suppressor function of Treg from psoriasis patients. While Treg isolated from peripheral blood of healthy donors were able to suppress the proliferation of effector T cells, Treg from psoriasis patients failed to inhibit effector T cell proliferation suggesting that the high systemic TNF- $\alpha$  levels in psoriasis patients might have impaired Treg function. As anti-TNF- $\alpha$  treatment is known to ameliorate psoriasis we speculated that TNF- $\alpha$  blockade could restore the suppressive activity of Treg. Hence, Treg were purified from the same psoriasis patients before as well as 16 weeks after start of anti-TNF- $\alpha$  treatment and their suppressive activity was assessed. Indeed, similar to the observations made in mouse models TNF- $\alpha$  blockade resulted in the peripheral induction of an immunosuppressive CD62L- Treg subset. Together, these data suggest that TNF- $\alpha$  seemed to act as a modulator of autoimmunity by inhibiting the suppressive activity of Treg and moreover, indicate that blocking TNF- $\alpha$  signaling might restore the function of Treg leading to a decreased proliferation of pathogenic effector cells.

#### P149 (V17)

##### Mast cells establish effective anti-tumor immune defense

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Tumor-specific T cells are frequently detectable in cancer patients. However, in most cases cancer immune defense fails to reject the tumors. In melanoma, T cell mediated regression of the tumor can be detected and we identified high numbers of mast cells (MC) recruited to cutaneous melanomas and especially in melanomas with regression. We hypothesized that tumors recruit MC to profit from MC derived growth and angiogenic factors. However, in response to, 'danger' stimuli, MC already present in and around the tumors may turn against the tumors and orchestrate cancer defense. One possible receptor activating MC by binding endogenous or microbial danger signals is Toll-like receptor 4 (TLR4). Indeed, TLR4 ligand LPS was identified as potent activator of human and mouse MC *in vitro*. For further analyses, we established a murine model of cutaneous melanoma by adoptively transferring OVA-expressing B16-melanoma cells into mouse skin. Within 1 week of tumor growth, MC were already recruited to the melanomas, but tumors further increased in size. Importantly, the transfer of tumor-specific CD4+ and CD8+ T cells at day 7 significantly reduced tumor growth in wild type (wt) but not in MC-deficient sash mice. Moreover, additional application of MC danger signal LPS enforced T cell recruitment to the melanomas and resulted in significantly enhanced tumor defense in wt mice only. Tumor defense in this model was indeed mediated by MC as reconstitution of TLR4-deficient mice with wt MC but not with TLR4-deficient MC induced this effective tumor-defense. Screening for underlying mechanisms we identified a marked upregulation and secretion of the chemokine IP-10 (CXCL10) following TLR4 mediated MC activation. Importantly, CXCR3, the receptor of IP-10, is expressed by tumor-specific CD4+ and CD8+ T cells and tumor-specific T cells migrated towards IP-10. Consequently, IP-10 deficient mice were analyzed and we found that in the absence of IP-10 MC-dependent tumor defense was lost. To further prove that MC-derived IP-10 is crucial for effective tumor defense, MC deficient sash mice were either reconstituted with wt MC or with IP-10 deficient MC. Only mice reconstituted with IP-10 secreting MC established effective anti-tumor defense again proving the crucial role of MC and MC derived IP-10 for anti-tumor immune responses. In summary our data demonstrate that activation of MC can orchestrate effective tumor defense with MC derived IP-10 as the main effector molecule recruiting T cells and initiating tumor rejection.

#### P150

##### The tumor suppressive microRNA-34a and microRNA-34c control the expression of the NKG2D ligand ULBP2

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NKG2D is a receptor of Natural Killer (NK) cells and different subsets of T lymphocytes, involved in the detection of stressed abnormal self. Ligands of NKG2D are surface molecules, structurally related to classical MHC class I molecules that in humans belong to either the MIC or the ULBP molecule family. A variety of human malignancies show surface expression of MIC and ULBP molecules, sensitizing them for NK cell- and T cell-mediated cytotoxicity. Malignant melanoma cells express the NKG2D ligand ULBP2. Recently we demonstrated that elevated levels of soluble ULBP2 in sera from melanoma patients are associated with poor prognosis. The molecular mechanisms that control ULBP2 expression are poorly understood. This prompted us to ask for the role of microRNAs (miRNAs) in ULBP2 regulation. Members of the miR-34 family, known for their tumor-suppressive activity, were predicted as potential binders for the 3'-untranslated region (UTR) of ULBP2. To analyse the impact of miR-34, different fragments of the 3'UTR of ULBP2 were fused downstream to a luciferase reporter. The resulting constructs were transfected into HEK293 cells together with miR-34 mimics. We observed a significant reduction in luciferase activity in the presence of miR-34a and miR-34c. To prove that miRNAs directly bind the 3'UTR of ULBP2, point mutations were inserted into the predicted miR-34 binding site. Indeed, these mutations caused an abrogation of the negative effect of miR-34 mimics on luciferase activity, emphasizing the importance of this binding site for ULBP2 regulation. Accordingly, transfection of melanoma cells with miR-34 mimics induced a clear downregulation of ULBP2, detectable at the level of total protein and surface protein. Importantly, miR-34 transfected melanoma cells were less sensitive to NK cell cytotoxicity. In contrast, inhibiting endogenous miR-34 by transfection of anti-miR-34 led to a significant induction of the ULBP2 protein. Finally, the analysis on several melanoma cell lines revealed a predominant expression of miR-34a that was negatively correlated to the surface expression levels of ULBP2. Taken together these data demonstrate that members of the tumor-suppressive miR-34 family control ULBP2 expression levels, strengthening the model of the NKG2D receptor ligand system as a barrier to tumor development.

#### P151

##### CB1 receptor deficiency in epidermal keratinocytes promotes contact allergic inflammation and delays epidermal barrier repair response

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We previously reported that the 'endocannabinoid system' (ECS) attenuates allergic contact hypersensitivity (CHS) responses in the skin. The G-protein coupled cannabinoid receptor CB1, which is abundantly expressed in the central nervous system and on peripheral nerve fibres, is part of the ECS. Here we further investigated how CB1 receptors regulate inflammatory immune responses in the skin. Using a conditional gene-targeting approach, we observed that mice lacking CB1 receptors specifically on keratinocytes (KC-Cnr1<sup>-/-</sup>) largely recapitulated the phenotype of complete Cnr1<sup>-/-</sup> mice, thus indicating that the essential CB1 receptor function resides in this cell type. Histological analyses revealed an increased reactive epidermal hyperplasia due to enhanced proliferation of basal keratinocytes associated with persistent induction of the inflammatory keratin 6 expression in Cnr1<sup>-/-</sup> and KC-Cnr1<sup>-/-</sup> mice. Cultured CB1 receptor-deficient keratinocytes secreted lower levels of IL1 $\alpha$ , a proinflammatory cytokine which supports the regeneration of injured epidermis. In contrast CCL8, an important chemoattractant protein, was elevated after stimulation with IFN $\gamma$  *in vitro*. As an indicator of deficient IL1 $\alpha$ -dependent defensive function *in vivo*, we discovered that both Cnr1<sup>-/-</sup> and KC-Cnr1<sup>-/-</sup> mice showed a delayed repair of the epidermal permeability barrier. Elevated CCL8 levels *in vivo* led to an increased recruitment of myeloid immune cells. Taken together, these results demonstrate a previously unrecognized pathophysiological role of CB1 receptors on keratinocytes, limiting contact allergic inflammation and promoting the homeostatic regeneration of epidermal integrity.

P152

### Modulation of inflammatory macrophage functions by artificial extracellular matrices composed of collagen and sulfated hyaluronan

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**Objective:** Macrophages are key players in the onset of chronic inflammation at implantation sites; however their activation is crucial for the initiation of healing processes. Depending on the microenvironment they encounter macrophages develop different phenotypes displaying either inflammatory, immunoregulatory or wound healing functions. Tissue injury typically drives the activation of inflammatory macrophages at implantation sites. Their suppression in favor of wound healing macrophages is thus critical for fast implant integration and healing. Extracellular matrix (ECM) components such as collagen, fibronectin or glycosaminoglycans (GAGs) have been widely used as coatings for biomaterials to improve implant integration and performance. Hyaluronan (HA) is a non sulfated ECM-derived GAG that resolves inflammatory processes and sustains homeostasis as high molecular weight molecule. Recent advances in biomaterial research highlight the positive effect on biomaterial integration and wound healing of HA that was modified by insertion of sulfate groups. The development of artificial ECMs (aECMs) composed of both ECM proteins and GAG has evolved a new promising approach in the design of biomaterial coatings. Since the ECM provides positional and environmental cues essential for immune cell function aECMs are suggested to have comprehensive immunomodulating capacity. In this study we test aECM composed of collagen and either none, low or high sulfated HA in respect of their capability to modulate the function of inflammatory macrophages.

**Methods:** Artificial extracellular matrices are generated utilizing the natural self-assembly potential of collagen type I in combination with either hyaluronic acid (HA) or single or high sulfated hyaluronan (ssHA or hsHA). Human blood-derived CD14<sup>+</sup> monocytes are isolated and seeded on the aECMs or collagen (control). GM-CSF is added to generate inflammatory macrophages. After 6 days of culture macrophage functions including LPS-induced cytokine release, MMP production and phagocytosis of pathogens are evaluated.

**Results:** Hallmark of inflammatory macrophages is their release of inflammatory cytokines. We find that compared to collagen all aECM reduce the secretion of TNF, IL-1 $\beta$ , IL-12p40, MCP-1 and IL-8 by the inflammatory macrophages as detected both at protein and RNA level. Noteworthy, with increasing level of HA sulfation in the aECM inflammatory cytokine release is more decreased. Interestingly, only in the presence of sulfated aECMs the macrophages secrete the immunosuppressive cytokine IL-10, which is more pronounced with increasing grade of HA sulfation. Gen expression of pro-inflammatory cytokines typically involves the activation and nuclear translocation of NF $\kappa$ B and is counter regulated by IL-10 promoted activation of STAT3. In accordance with the detected cytokine profile we find reduced level of NF $\kappa$ B activation in macrophages cultured in the presence of sulfated HA. However phosphorylation of STAT3 on these aECM is not induced. Macrophage phagocytosis of *E. coli* is also reduced by the aECM and displays the same dependency on HA sulfation. ROS production, however, is unaffected by all aECMs.

**Discussion:** Our data clearly show that the aECMs have immunomodulating potential. Furthermore, they indicate a functional switch of inflammatory macrophages towards a regulatory phenotype as a function of sulfation of HA. The mechanisms by which sulfation of HA elicits immunosuppressive effects are currently investigated.

P153

### Extracellular [64Cu]DOTA-antibody labeling of OVA-Th1 cells for *in vivo* positron emission tomography (PET) imaging yields less impact on OVA-specific T cell functioning and a higher contrast compared to intracellular [64Cu]PTSM labeling

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So far only few studies consider a role of IFN- $\gamma$  producing CD4<sup>+</sup> T cells (Th1) in ovalbumin (OVA)-induced airway hyperresponsiveness (AHR). High sensitive non-invasive imaging modalities, such as small animal positron emission tomography (PET) are capable to investigate basic migration properties of radiolabeled Th1 cells *in vivo*. We recently have shown that the main drawback of intracellular [64Cu]PTSM labeling is an impaired Th1 cell viability and functioning. Nevertheless we could follow [64Cu]PTSM-labeled Th1 cells in OVA-induced AHR for up to 24 h. Aim of our study was to improve murine T cell labeling. We labeled OVA-Th1 cells extracellular with a specific [64Cu]DOTA-linked monoclonal antibody (mAb) and compared this new approach with our established labeling method.

Freshly isolated OVA-T cell receptor transgenic CD4<sup>+</sup> cells were specifically cultured for 12–14 days to generate a Th1 phenotype. We labeled OVA-Th1 cells/ml with 0.7–2.1 MBq (approximately 15–45 g) of an OVA-TCR-specific mAb (KJ1-26) which was linked to 64Cu via the chelator DOTA. We analyzed OVA-Th1 cell viability by trypan blue staining and supernatants of OVA-peptide stimulated OVA-Th1 cells by IFNELISA 3, 24 and 48 h after labeling. We induced OVA-AHR for two intranasal OVA-challenges in BALB/c mice which were OVA-immunized 2 weeks earlier. Ten million viable [64Cu]DOTA-mAb-labeled OVA-Th1 cells were administered i.p. into diseased and healthy mice and monitored by PET and CT 3, 24 and 48 h after cell transfer. Finally we performed *ex vivo* biodistribution.

*In vivo* evaluation of [64Cu]DOTA-mAb-labeled OVA-Th1 cells revealed a similar impaired viability as observed for [64Cu]PTSM labeling. Accompanied to increasing activity of the radiolabel we observed an increase in unviable OVA-Th1 cells 24, 48 and 72 h after labeling. In sharp contrast to [64Cu]PTSM labeling we detected no impairment of specific T cell functioning due to [64Cu]DOTA-mAb until 24 h after labeling. Even after 48 h we detected only a slight reduction in specific IFN $\gamma$  production. *In vivo* using *in vivo* PET, we detected [64Cu]DOTA-mAb-OVA-Th1 cells already 3 h after i.p. transfer in the perithymic lymph nodes (LNs) of diseased and healthy mice and lung LNs of diseased mice. Compared to [64Cu]PTSM labeling we could follow specific OVA-Th1 migration for up to 48 h. Noticeable, we detected high-contrast signals within single LNs. Analysis of the PET scans confirmed these findings, as we detected higher organ to muscle ratios in the lung and the perithymic LNs. We could confirm PET data *ex vivo* by biodistribution. Accordingly, mAb-labeling minimizes the background activity as it deals with a much lower 64Cu efflux compared to [64Cu]PTSM labeling. Extracellular [64Cu]DOTA-mAb-labeling of T cells seems to be very stable and less harmful than intracellular [64Cu]PTSM labeling and provides a higher contrast and a longer observation time. Using this approach we could follow the dynamics of OVA-Th1 migration in perithymic and lung LNs of AHR-diseased mice for up to 48 h. It is a valuable method for *in vivo* cell trafficking studies in the realm of T cell immunology and can replace and refine invasive FACS analysis of organs.

P154

### Essential role of cathepsin B during contact hypersensitivity reactions

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Proteases, such as Cathepsin B (Cath B), have important functions in critical steps of inflammatory processes. Cath B, a cysteine protease, is involved in intracellular MHCII-processing and in remodeling the extracellular matrix. Our Aim was to study the role of Cath B in the effector phase of acute and chronic cutaneous delayed-type hypersensitivity reactions (DTHR) non-invasively *in vivo* using optical imaging (OI) and protease activatable probes.

Mice were sensitized at the abdomen with trinitrochlorobenzene (TNCB) and challenged at the right ear 7 days later to elicit acute contact hypersensitivity reactions (CHSR) and repetitively challenged every 2 days for up to five times to induce chronic CHSR. Ear swelling responses were measured 12–24 h after TNCB-challenge. We injected protease activatable probes 12 h after ear challenge and performed *in vivo* and *ex vivo* OI investigations 24 h later. We used ProSense 680, activatable by several proteases, such as Cath B, L, S and CatB 680, an almost exclusively Cath B activatable probe. Additionally we investigated ears and lymph nodes (LNs) *ex vivo* for active Cath B using an activity-based probe, which is an analog of the E-64 broad spectrum inhibitor of cysteine CatB. As the p38-MAP kinase pathway can induce Cath B we topically applied LN950, a selective p38-MAP kinase inhibitor, or sham-treatment every 24 h at the right ears starting 3 days prior the first challenge and performed CatB 680-OI measurements.

We first analyzed proteases activity in chronic DTHR. The OI probe ProSense 680 signal intensity after the 5th Challenge was 4.0-fold higher in the inflamed right ears compared to the untreated left ears. As control we injected the OI probe the ProSense 680 control, a non-activatable probe and detected almost no signal. To further focus specifically on Cath B we injected the OI probe CatB 680 into littermates 12 h after the first, third and 5th TNCB-challenge and detected signal intensity 24 h later. After the first TNCB-challenge CatB 680 signal intensity was 2.4-fold higher, 4.0-fold higher after the 3th, and 6.2-fold higher after the 5th TNCB-challenge compared to the untreated control ears. CatB 680 OI analysis displayed a strong signal intensity exclusively in TNCB-challenged ears, in their draining cervical LNs, and in some mice additionally in the axillary right LNs while we detected a slight signal in the thymus and no signal in the spleen. *Ex vivo* analysis of ears and cervical LN using activity-based probes confirmed an up regulation of active Cath B during acute and chronic DTHR. The selective p38 MAP kinase inhibitor LN950 significantly reduced ear swelling responses 24 h after the first TNCB-challenge. As a consequence of LN950 treatment we measured a reduced CatB 680 signal intensity in the draining cervical LN, only a slight decrease in TNCB-challenged ears and no differences in the thymus. Active-site-labelling confirmed a reduced Cath B expression in TNCB-challenged ears as well as in the draining cervical LN by LN950 treatment. Proteases, especially Cath B, are highly active in acute and chronic CHRS. Targeting p38-MAPkinase as well as Cath B might be a powerful tool to limit cutaneous DTHR to self antigens in auto immune diseases such as psoriasis.

P155

### Increasing numbers of circulating CD11b+ and CD33+ MDSC in melanoma patients lead to general reduction of T cell reactivity and correlate with melanoma progression.

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Myeloid-derived suppressor cells (MDSC) are a heterogeneous cell population functionally defined by immune suppressive activity not only in a variety of inflammatory settings but also in tumors. In experimental tumor models MDSC are supposed to be major contributors to tumor immune tolerance which facilitate tumor development and progression. In our study we used a panel of different MDSC associated markers including CD11b, CD33, HLA-DR, CD14, CD80, CD83 and IL-4R $\alpha$  to define and quantify MDSC frequencies in the peripheral blood of stage I-IV melanoma patients. We observed an accumulation of MDSC in the course of melanoma progression and in comparison to healthy volunteers. However, especially the surface marker CD11b and CD33 turned out to be marker with a significant correlation to melanoma progression. Notably, the presence of CD11b+ MDSC led to a general reduction of T cell reactivity to TCR stimulation and to recall antigens such as tetanus toxoid. These findings point out that tumor progression in melanoma patients results in increased and functional relevant systemic MDSC frequencies. The characterization of relevant markers to define MDSC frequency and function in melanoma patients allows us furthermore to analyze different immunotherapeutic settings regarding their modulatory effects on MDSC. Our findings may lead to an improved tumor immune monitoring and thus to the development of novel diagnostic and therapeutic reagents for cancer immunotherapy.

P156 (V18)

### Human CD8+ T cells need CD4 T-cell-help to escape deletion upon re-stimulation *in vitro*

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The importance of CD4<sup>+</sup> T-cell mediated help during secondary expansion of antigen-specific CD8<sup>+</sup> T cells rather than in priming was already examined in mice. Additionally, a great influence on the generation of a lasting and robust immune response was also corroborated in mice. Nevertheless, mice are no men, so the development of an experimental system by which the molecular and functional basis of the interaction between human dendritic cells (DC), CD4<sup>+</sup> and CD8<sup>+</sup> T cells can be revealed in more detail seems reasonable. Thus, we endowed *ex vivo* generated bulk CD4<sup>+</sup> T cells with a gp100/HLA-A2-specific TCR. RNA electroporation into the CD4<sup>+</sup> T cells resulted in approximately 90% transfection efficiency, and did not require prior activation of the cells. To elucidate the antigen-specific cross-talk of CD4<sup>+</sup> T cells and DC after co-incubation, DC were previously loaded with the corresponding gp100 peptide. After co-cultivation, a clear Th1 cytokine secretion, as well as the antigen-specific up-regulation of maturation markers (CD25, CD40, CD80, CD86, and CD70) on both immature (iDC) and mature (mDC) DC, and the up-regulation of activation markers (CD25, CD69) on CD4<sup>+</sup> T cells was identified in a time-dependent manner. Besides, the number of specific helper cells needed in our setting was determined by the analysis of the CD25 and CD70 expression on DC. Decreasing the number of helper cells resulted in a reduction of both antigen-specific maturation marker elevation and Th1 cytokine secretion. As CD40L is supposed to be eminent in CD4<sup>+</sup> T-cell help, we used a CD40L blocking antibody to dissect its role in our system. Concerning the analyzed surface markers no differences in the expression was detectable upon anti-CD40L antibody treatment. In contrast, the blocking completely inhibited TNF secretion in both iDC and mDC conditions and slightly reduced the release of IL-8 and IL-12p70. To explore the influence of this cross-talk between DC and CD4<sup>+</sup> T cells on the priming and re-stimulation of CD8<sup>+</sup> T cells, we mimicked both the sequential or simultaneous interaction of CD4<sup>+</sup> and CD8<sup>+</sup> T cells and DC. A sequential interaction of the DC with the CD4<sup>+</sup> T cells, which were then removed by flow cytometry sorting, and a subsequent encounter with the CD8<sup>+</sup> T cells did not significantly enhance the DC's capacity to prime and expand the CD8<sup>+</sup> T cells. Permitting the simultaneous interaction of all three cell types by not removing the CD4<sup>+</sup> cells, still did not lead to differences in the priming capacity, but upon a 2nd and 3rd stimulation, a clearly superior antigen-specific CD8<sup>+</sup> T-cell expansion was detected. This superior expansion was only measurable when the CD4<sup>+</sup> T-cell epitope and the CD8<sup>+</sup> T-cell epitope were present on the same DC, whereas in absence of the CD4 epitope the number of specific CD8<sup>+</sup> cells even decreased after repetitive stimulations. These findings corroborate the mice data that T-cell help plays a minor role during an initial immune response, but massively pro-

notes recall immune responses. A closer analysis of the transcriptional signatures of the DC and expanded CD8<sup>+</sup> T cells will bring new insights into the mechanisms of human T-cell memory formation that could improve the efficiency of the immunotherapy of cancer.

#### P157

##### Mutated tumor antigens as targets for cancer immunotherapy

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The identification of new candidate antigens as potential targets offers promising possibilities for the immunotherapy of tumors. Mutations in proteins like BRAF and NRAS, which frequently occur in melanoma, or in GNAQ and GNA11, which emerge in a majority of uveal melanomas, might generate such antigens. Interestingly, mutations in these antigens often occur in certain codons.

We analyzed 23 tumor cell-lines via PCR and subsequent sequencing for mutations in codon 209 of GNAQ and GNA11 (Q209P or Q209L). These mutations exclusively occurred in cell lines of uveal melanoma patients (1/2, and 1/2, respectively), but were not found in other melanoma cell lines (0/21). The cell lines were also tested for mutations in BRAF at codon 600 and NRAS at codon 61. In contrast, BRAF and NRAS mutations (V600E in BRAF and Q61R, Q61L, or Q61K in NRAS) were found in 11/22 and 4/18 cell lines, respectively, albeit these mutations only occurred in non-uveal melanoma cell lines.

To test the immunogenicity of the mutated antigens GNAQ and BRAF in comparison to their wild type versions, CD8<sup>+</sup> T cells were stimulated three times with autologous mature dendritic cells (mDCs), which had been equipped with the wild type (wt) or mutated (mut) antigens by RNA-electroporation. Successful transfection of the mDCs was confirmed by intracellular FACS analyses. The antigen-specific activity of the stimulated CD8<sup>+</sup> T cells was then determined by Multi Functional T Cell Assay (MFTC) with antigen-loaded DCs as target cells and by IFN $\gamma$ -Elispot with antigen-loaded peripheral blood mononuclear cells (PBMCs) as targets.

In the MFTC analyses of the GNAQ stimulation, an increase in CD8<sup>+</sup> T cells that produced TNF and IFN $\gamma$  was detectable, when CD8<sup>+</sup> T cells were stimulated in an antigen-specific manner by RNA-transfected mDCs. Elispot analyses revealed highest antigen-specific IFN $\gamma$ -production by T cells stimulated with PBMCs, which were electroporated with the GNAQ wild type antigen or the mutated variants Q209P and Q209L. However, CD8<sup>+</sup> T cells, which had been stimulated with mDCs, loaded with a pool of peptides that span the mutation, during long and short term stimulation produced much lower quantities of cytokines. In Elispot, co-cultivation with PBMCs loaded with a GNAQ-peptide-pool could not induce specific cytokine production by CD8<sup>+</sup> T cells.

The CD8<sup>+</sup> T cells, which had been stimulated over 3 weeks with mDCs expressing the BRAF wild type antigen or the mutated BRAF antigen showed an antigen-specific up-regulation of the IFN $\gamma$  production in the corresponding short time stimulation of the MFTC assay. A slightly higher amount of IFN $\gamma$  production by T cells was detectable, when the T cells were stimulated with the BRAF wild type antigen compared to T cells, which were stimulated with the mutated BRAF antigen. The Elispot analyses revealed IFN $\gamma$  secretion after stimulation of BRAF wt/BRAF mut pre-stimulated CD8<sup>+</sup> T cells with DCs which were electroporated with the BRAF wt- and BRAF mut-RNA. The T-cells showed no clear pattern of higher IFN $\gamma$  secretion after stimulation with the mutated or the wild type form of BRAF. In MFTC assays, T cells which were stimulated with DCs loaded with peptide, either wild type or mutated, showed no IFN $\gamma$  production. Also in Elispot, CD8<sup>+</sup> T cells which were stimulated with peptide-loaded target cells showed no IFN $\gamma$  secretion.

Additional stimulations will be analyzed to further investigate the immunogenicity of GNAQ and BRAF in their wild type and mutated forms. Other tumor antigens like GNA11 and NRAS, which also show mutated versions, will also be tested for their immunogenicity.

#### P158

##### Retargeting T cells with MHC-independent chimeric antigen receptors by RNA electroporation

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The transfer of tumor-specific T cells has become a promising approach in the immunotherapy of cancer. However, the clinical application of tumor-infiltrating lymphocytes (TIL) was hampered by problems in the generation of those cells or by their low anti-tumor activity *in vivo*. The efficiency of T cells that were redirected to the tumor by the transfer of wild-type T-cell receptors (TCR) suffered from the weak antigen-affinity of those receptors and the down-regulation of MHC-molecules on the tumor cell. Those issues can be overcome by the transfer of chimeric antigen receptors (CAR). In those receptors antibody-derived scFv molecules are fused to signaling domains of the TCR/CD3 complex, therefore allowing the generation of T cells with a high affinity to native surface-antigens of the tumor. To avoid the risk of inducing a permanent autoimmunity, which has been described as a possible side effect of the retroviral transduction of CAR-molecules, we introduced such CAR by mRNA electroporation to achieve a transient expression.

We efficiently transfected T cells with different CAR-molecules specific for the melanoma-associated chondroitin sulfate proteoglycan (MCSP). MCSP is expressed on most melanomas but has only a very limited expression pattern in healthy tissues. We transfected T cells with six different receptors to investigate the influence of the antigen-binding and the signaling domain on the expression and functionality of the CAR. The CAR molecules could be detected on the T cells for up to 9 days after electroporation. CAR-transfected T cells were able to recognize MCSP-positive tumor cells as shown by cytokine secretion and lysis of those cells. Our study also revealed that the antigen-binding domain does not only affect tumor-cell-recognition but also contributes to the expression of the CAR on the T-cell surface. We also could show that CAR-transfected T cells were able to prolong the survival of immunodeficient mice when co-injected with tumor cells into these animals. Furthermore, by using RNA-electroporation we provide a method that allows us to regulate the receptor density on the T-cell surface by adjusting the RNA concentrations used to transfect the T cells to investigate the influence of receptor density on antigen recognition and potentially avoid on-target off-tumor specificity. Taken together this study shows the selection of one MCSP-specific CAR that has in our opinion great potential for a clinical application. Furthermore, by using RNA-electroporation we provide a technology, that allows a faster and safer selection process of the transfected receptors.

#### P159

##### Improvement of the T-cell stimulatory capacity and IL-12p70 secretion of dendritic cells via constitutive activation of the NF $\kappa$ B-pathways

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Activation of the NF $\kappa$ B-pathways is a key process in the induction of full maturation of dendritic cells (DC) and is thus relevant for their T-cell stimulatory capacity. Given that tumor-antigen-loaded mature DC are a promising tool to induce significant numbers of high quality effector and memory T cells, we investigated the influence of the expression of constitutively active mutants of activators of the NF $\kappa$ B-pathways, with the main aim to improve the stimulatory capacity and their applicability in

cancer immunotherapy. Therefore, we transfected cocktail-matured DC with RNA coding for constitutively active mutants of activators of the classical (IKK $\beta$ E6A10) and alternative (IKK $\alpha$ E6A16) NF $\kappa$ B-pathway via electroporation. The expression of the constructs led to a higher expression of distinct favorable surface markers (CD25, CD40, CD70, CD83, CD86, and OX-40L) and to an increased secretion of several pro-inflammatory cytokines (IL-6, IL-8, TNF, and IL-12p70). Interestingly, those effects were clearly enhanced when mutant activators of both pathways were co-electroporated. Remarkably, the transfected DC kept their CCR7-mediated migratory capacity and secreted IL-12p70 in a high and long lasting manner, whereas IL-10 was secreted in very low quantities.

To investigate whether transfected DC are still able to respond to extracellular activation of the NF $\kappa$ B-pathway and secrete additional cytokines, especially IL-12p70, we co-cultured the transfected DC with the soluble CD40 ligand (sCD40L). We added sCD40L immediately or 24 h after electroporation to the DC-cultures or left the control conditions untreated. The addition of sCD40L did not increase the secretion of IL-12p70, but quite the contrary, the sCD40L-supplement even reduced the amount of secreted IL-12p70 when sCD40L was given directly after electroporation. No effects were detected, when sCD40L was supplemented 24 h after electroporation.

We also investigated the capacity of DC transfected with the NF $\kappa$ B-pathway mutant activators to prime and expand autologous T cells antigen-specifically, because low IL-10 and high IL-12p70 secretion favors the T-cell stimulatory efficiency of DC. Hence, autologous T cells were stimulated weekly with DC transfected with the NF $\kappa$ B-pathway activators. After the third stimulation, we achieved a ten-fold higher rate of antigen-specific CD8<sup>+</sup> T cells in comparison to the control DC transfected with the antigen mRNA only. Especially the T-cell condition which was stimulated three times with IKK-double-transfected DC showed a high IFN $\gamma$  induction after an overnight restimulation with Ag-loaded T2-A1 cells. The cytolytic capacity of the T cells was not affected by the stimulation with IKK-transfected DC.

The transfection of constitutively active activators of the NF $\kappa$ B-pathways appears to be an appropriate tool to generate potent DC because of the following features: (i) high IL-12p70 production up to 48 h post transfection, with low IL-10 secretion, (ii) sustained migratory capacity, and (iii) elevated capacity to expand autologous antigen-specific T cells.

#### P160 (V19)

##### Spleen tyrosine kinase (Syk) promotes granulocyte-mediated skin blistering induced by autoantibodies

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The passive transfer of collagen VII-specific antibodies into mice induces granulocyte-dependent skin blistering closely recapitulating the clinical and laboratory findings in patients with epidermolysis bullosa acquisita. The Fc $\gamma$ R-dependent signaling in granulocytes critically relies on immunoreceptor tyrosine-based activation motifs (ITAMs)-activation of the spleen tyrosine kinase (Syk). Therefore, in our present study, we addressed the relevance of Syk for the Fc $\gamma$ R-dependent granulocyte activation and skin blistering induced by collagen VII-specific autoantibodies. For this purpose, Syk (-/-) bone marrow chimeras carrying a Syk-deficient hematopoietic compartment were generated by transplanting Syk (-/-) fetal liver cells in lethally irradiated wild-type recipients. After complete repopulation of the hematopoietic compartment, skin blistering disease was induced by repeated injections of collagen VII-specific antibodies. Importantly, Syk deficiency in the hematopoietic compartment completely blocked the development of clinical and histological disease in mice ( $n = 15$ ), whereas Syk (+/+) chimeric animals ( $n = 15$ ) were fully susceptible to experimental antibody-induced blistering. Levels of circulating pathogenic IgG against collagen VII as well as the antibody and complement deposition were similar in both animal groups. Interestingly, granulocyte recruitment was low to absent in the Syk (-/-) chimeras. A small molecule inhibitor of Syk blocked the immune complex-induced activation of human and murine granulocytes in reactive oxygen species production assays. In addition, pharmacological inhibition of Syk abolished the dermal-epidermal separation induced by patient autoantibodies and granulocytes from healthy donors in frozen sections of human skin. In conclusion, we show that genetic and pharmacological inhibition of Syk protects from autoantibody-induced granulocyte-dependent skin damage. Our results strongly suggest that Syk-targeted approaches will offer significant therapeutic benefit in inflammatory and autoimmune diseases.

#### P161

##### Regulatory T cells induced by the AhR ligand nonylphenol express GARP

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Ultraviolet radiation (UVR) suppresses the immune system in an antigen-specific fashion via induction of regulatory T cells (Treg). The arylhydrocarbon receptor (AhR), a cytoplasmic receptor which is crucially involved in detoxification processes, has been recently observed to be activated by UVR in a ligand-independent fashion. Activation of the AhR appears to be involved in UVR-induced immunosuppression since the induction of Treg by UVR was prevented by AhR antagonists like 3-methoxy-4-nitroflavon and resveratrol. Hence, we postulated that in turn activation of the AhR by natural ligands should exert similar immunosuppressive effects like UVR. Injection of the AhR ligand nonylphenol (NP) into mice prevented the induction of sensitization against dinitrofluorobenzene (DNFB). In addition, Treg were induced in these mice as demonstrated by adoptive transfer experiments. NP-induced Treg act in an antigen-specific fashion since Treg obtained from DNFB- and NP-treated donors only blocked the sensitization against DNFB but not against oxazolone in the recipients. Recently, GARP (glycoprotein a repetitions predominant) was described as a protein specifically expressed in Treg upon activation of the T cell receptor. GARP appears to be functionally relevant since silencing of GARP in Foxp3-expressing Treg inhibits their suppressive activity. To determine whether NP-induced Treg express GARP, mice were injected intraperitoneally for 4 days with NP. Twenty-four hours after the last injection animals were sensitized against DNFB. Five days later lymph nodes and spleens were obtained for intravenous injection into naive mice. Before transfer, cells were depleted from GARP-expressing cells by magnetobead separation using a monoclonal anti-mouse-GARP antibody. Unfractionated cells served as controls. Twenty-four hours after injection animals were sensitized with DNFB and after 5 days ear challenge was performed. The contact hypersensitivity (CHS) reaction in recipient mice injected with unfractionated cells from NP-treated donors was remarkably suppressed. In contrast, transfer of GARP-depleted cells did not inhibit sensitization. In turn, injection of GARP-enriched cells resulted in a pronounced suppression of CHS. These results suggest that the AhR ligand NP induces Treg which express GARP. It is currently under investigation by which mechanisms GARP is involved in mediating the suppressive activity of Treg.

#### P162

##### Cutaneous innate immune sensing of TLR2 ligands induce myeloid-derived suppressor cells and potentially suppresses cutaneous immune responses to limit and terminate skin inflammation

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In order to investigate consequences of exposure to microbes on atopic dermatitis (AD) skin, Th2 dominated contact hypersensitivity (CHS) to the hapten FITC was investigated. Lipoproteins are

important components of Gram positive bacteria and function as TLR2 ligands. Therefore we established a model in which we applied FITC with or without TLR2/6 ligands Pam2Cys or FSL-1 and TLR2/1 ligand Pam3Cys to FITC sensitized mice to mimic the presence of Gram positive bacteria on AD skin lesions. FITC induced CHS ear skin inflammation was not changed in mice that previously received Pam3Cys. In sharp contrast, in mice previously exposed to Pam2Cys or FSL-1, ear swelling, FITC-specific antibodies, and FITC-specific T-cell proliferation were significantly reduced. Investigating underlying mechanisms, we identified Gr1+CD11b+ myeloid derived suppressor cells (MDSCs) to be massively increased after TLR2/6 ligand treatment only.

To now investigate the functional role of these MDSC in more detail, freshly isolated CD11b+ MDSC were adoptively transferred into FITC sensitized mice resulting in CHS suppression. Moreover, further characterization revealed that CD11b+Ly6C+ but not CD11b+Ly6G+ cells inhibit T cell proliferation. Interestingly, these T cells 'suppressed' by MDSC were able to proliferate following *in vitro* restimulations at later time points. In line with this, also FITC rechallenge of Pam2Cys treated mice at later time points completely abrogated previously detected immune suppression. As we could also show that MDSC induction by Pam2Cys was TLR2 dependent, this suggests that MDSC induced by innate immune signals are temporarily active to limit and terminate inflammation. We next wanted to investigate whether our findings are specific to the skin innate immune response. Therefore chimeric mice were generated by wildtype or TLR2 ko bone marrow transplantation to TLR2 ko or wildtype mice respectively. Analyses revealed that signals through TLR2 on skin cells but not hematopoietic cells are required and sufficient for MDSCs expansion. Surprisingly, TLR2 on MDSCs was not required for their induction, accumulation, activation, and suppressive function. To investigate whether TLR2 mediated cutaneous innate immune sensing leading to MDSC and immune suppression is a general mechanism and not limited to FITC CHS, we, in addition, analyzed cutaneous tumour defense of ovalbumin expressing B16 melanomas. To this end, mice were sensitized to ovalbumin and with or without exposure to Pam2Cys the consecutive immune responses to ovalbumin expressing B16 melanomas was analyzed. Again, MDSC induction and ameliorated tumor defense was detected as a consequence of cutaneous innate immune sensing of Pam2Cys.

Collectively, we show for the first time that the presence of certain lipoproteins on skin is sufficient to potentially inhibit cutaneous immune responses by TLR2 dependent induction of highly suppressive MDSC. Importantly, this potent MDSC mediated immune suppression is active within a narrow window following cutaneous innate immune sensing indicating a mechanism to limit and terminate skin inflammation.

### P163 Mapping of pathogenic epitopes in experimental epidermolysis bullosa acquisita

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Epidermolysis bullosa acquisita (EBA) is an autoimmune subepidermal blistering disease of mucous membranes and skin caused by autoantibodies against collagen VII. The passive transfer of collagen VII-specific antibodies into mice results in subepidermal blister formation. *In silico* and wetlab epitope mapping studies revealed numerous distinct epitopes recognized by EBA patients' autoantibodies within the noncollagenous (NC1) and NC2 domains of collagen VII. However, the distribution of pathogenic epitopes on collagen VII has not yet been described. In this study, we therefore performed an *in vivo* functional epitope mapping in the previously established mouse model of EBA. As a first step, five overlapping fragments (mCVII-1, aa 1–300; mCVII-2, aa 281–594; mCVII-3, aa 561–879; mCVII-4, aa 871–1125; mCVII-5, aa 1108–1323) spanning the entire NC1 domain and a fragment (mCVII-Z, aa 2795–2944) corresponding to the NC2 domain of murine collagen VII were used to produce specific antibodies in rabbits. Subsequently, the purified IgG fractions specific to different regions of collagen VII were injected into wild type BALB/c mice ( $n = 8/\text{group}$ ). The animals injected with antibodies against fragments of the NC1 domain developed to different extent experimental EBA. Antibodies against the NC2 domain similarly to the normal rabbit IgG used as control did not induce skin disease. Our results clearly demonstrate for the first time that antibodies targeting multiple, distinct epitopes distributed over the entire NC1 domain of collagen VII induce blistering skin disease *in vivo*. Our present findings have crucial implications for the development of antigen-specific B- and T cell-targeted therapies in EBA.

### P164 (V21) Treg exert anti-inflammatory activity by A2A adenosine receptor mediated, cAMP-driven downregulation of adhesion molecules on endothelial cells.

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In TNBC-induced contact hypersensitivity reactions in mice we have shown that adenosine produced by *i.v.* injected regulatory T cells (Treg) is able to block the expression of E- and P-selectin on inflamed endothelium. This leads to abrogation of the extravasation of T cells and prevents the ear swelling response. To determine which adenosine receptor is engaged, we set up an *in vitro* culture system, in which endothelial cells (EC) were cultured on glass slides. After reaching confluence, the EC were superfused with isolated, fluorescently labelled T cells and the adhesion of T cells to the EC was quantified using videomicroscopy. In this system we show that conventional T cells (Tcon) displayed a strong adherence to EC activated by TNF- $\alpha$ . In contrast, addition of Treg prior to activation of the EC blocked the adherence of Tcon significantly. This Treg-mediated blockage was abrogated by adding the A2A adenosine receptor specific antagonist SCH58261 to the EC, whereas antagonists against A1, A2B or A3 adenosine receptors did not affect Treg-mediated suppression. Further support for the role of the A2A receptor in mediating these effects became evident by experiments showing augmented cAMP levels and increased ERK1/2 phosphorylation in EC after Treg-EC cocultures. This signalling is typical for engagement of A2A receptors and has been reported to occur also in other cells after triggering the A2A receptor. As Treg generate adenosine by cleavage of ATP via CD39, we further reasoned about the source of ATP and found that endothelial cells release ATP after activation, which subsequently activates Treg and enables them to produce adenosine. Thus, activated EC may turn on anti-inflammatory processes *in vivo* by activating Treg to produce adenosine, which then engages endothelial A2A receptors and suppresses adherence of Tcon to EC. To underline the *in vivo* relevance of these findings, we further demonstrated expression of the A2A adenosine receptors in CD31+ EC isolated from murine ears. Moreover blocking of A2A receptors *in vivo* prior to injection of Treg abrogated the immunosuppressive effect of Treg in the CHS model. Therefore, the Treg mediated suppression via adenosine in the CHS model is triggered by the A2A adenosine receptor, leading to elevated cAMP levels and ERK1/2 activation in inflammatory stimulated endothelium.

### P165 Immunization with the antigen MOG fused to a novel single chain fragment variable (scFv) for murine DEC-205 suppresses development of experimental allergic encephalomyelitis (EAE) in mice

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The DEC-205 prototype receptor for antigen uptake is expressed specifically by Dendritic cells (DC) and greatly increases antigen presentation. We have shown that targeting of antigens to non-activated

DC *in vivo* induces immunosuppressive regulatory T-cells (Treg). Therefore our aim was to create novel single chain fragment variables (scFv) specific for DEC-205 fused with the EAE antigen MOG, in order to target non-activated DC *in vivo* to induce tolerance in EAE. The scFv were created through RT-PCR using degenerative primers on total RNA from a hybridoma cell line producing a monoclonal antibody for murine DEC-205. The isolated variable heavy and variable light regions were subcloned into an expression vector, fused with a sequence coding for MOG, and scFv-MOG fusion proteins were expressed in bacteria. Immunohistochemical staining of cytosins from isolated CD11c+ cells displayed a positive staining for scFv, which colocalizes with MHC class II. For functional testing scFv-MOG was injected into mice. Lymph node DC were prepared 3 days later and cocultured with MOG-specific T-cells derived from transgenic 2D2 mice. Here, strong proliferation was induced by DC obtained from mice injected with scFv-MOG, whereas DC from peptide-injected mice failed to do so. Thus, these data indicate that scFv-MOG efficiently targets DC *in vivo* and MOG is presented to T-cells. When further analyzing the T-cell populations *in vivo*, we found that the number of CD4+CD25+FoxP3+ Treg was enhanced (16% of CD4) after injection of scFv-MOG as compared to control animals (12% of CD4). Most importantly, when EAE was induced in either scFv-MOG-injected mice or in peptide-treated controls, none of the scFv-MOG injected mice developed any EAE symptoms. In contrast, all animals in the control group developed a severe EAE. Thus, these data indicate that targeting of MOG to steady state DC *in vivo* prevents EAE by a Treg-driven mechanism.

### P166 (V26) Rapid neutrophil activation by Mac-1-GPIIb/alpha interaction on tunable nanostructured surfaces under flow

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**Aim:** The integrin Mac-1 (CD11b/CD18) is critical for leukocyte adhesion and migration and for immune functions. It also mediates the firm adhesion and translocate migration of leukocytes on vascular thrombi via its interaction with the platelet surface receptor GPIIb/alpha. However, the biophysical parameters of this interaction are poorly characterized and its biological relevance is not well understood. Therefore, our aim was to create a novel, precisely tunable *in vitro* microfluidic model in which the interaction of neutrophilic leukocytes expressing the Mac-1 integrin with GPIIb/alpha could be investigated and the importance of biophysical parameters such as ligand density or hydrodynamic shear stress could be tested.

**Method:** In order to design surfaces with precisely tunable densities of the GPIIb/alpha biomolecule, nano-patterns of 6 nm gold nanoparticles were created by self-assembly of diblock copolymer micelles on glass substrates. With this method, the distance between gold nanoparticles can generally be adjusted between 25 and 300 nm, in our experiments 200, 100 and 60 nm interparticle spacings were used. GPIIb/alpha was then bound to the gold nanoparticles in a site directed manner. In contrast to conventional protein-adsorption methods on surfaces, this ensures biologically correct presentation of the binding epitopes. The glass substrates were then integrated into a flow chamber system in which hydrodynamic parameters could be controlled. Interaction of neutrophils with surfaces presenting GPIIb/alpha at different densities were then surveyed and quantitated.

**Results:** This novel approach to investigate ligand-receptor interactions allows the determination of important biophysical parameters of the Mac-1-GPIIb/alpha interaction, such as ligand density thresholds and maximum and minimum shear rate needed for receptor interactions. We found that, depending on shear conditions, Mac-1-GPIIb/alpha interaction is strongly dependent on interparticle spacing of GPIIb/alpha and that this interaction presents a threshold for the GPIIb/alpha intermolecule distance between 200 and 100 nm. Furthermore, we were able to show that neutrophils become activated very quickly on GPIIb/alpha surfaces and show a distinct migratory behaviour on this substrate.

### P167 Staphylococcal exotoxins are strong inducers of interleukin (IL)-22: a potential role in atopic dermatitis

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**Background:** Patients with atopic dermatitis (AD) and psoriasis are frequently colonized with Staphylococcus aureus (S. aureus) that produce staphylococcal enterotoxin B (SEB) and  $\alpha$ -toxin. In patients with AD, S. aureus colonization is positively correlated with the severity of their eczema. Moreover, IL-22 producing cells have been shown to accumulate in AD skin and to correlate with disease severity.

**Objective:** To assess IL-22 production in response to SEB and sublytic  $\alpha$ -toxin stimulation in patients with AD and psoriasis compared with healthy controls.

**Methods:** IL-22 induction was investigated in Peripheral Blood Mononuclear Cells (PBMCs), T cells and autologous cocultures of keratinocytes and T cells upon SEB and  $\alpha$ -toxin stimulation in a time and dose dependent manner at the mRNA and protein (ELISA and flow cytometry) level. Anti-IL-1R or anti-IL-6 antibodies were used in blocking experiments.

**Results:** SEB and sublytic  $\alpha$ -toxin concentrations induced IL-22 production in PBMCs and isolated CD4+ T cells. IL-22 secretion was enhanced by  $\alpha$ -toxin stimulation in autologous cocultures of keratinocytes and T cells. In T cells and PBMCs from AD patients IL-22 secretion was significantly enhanced upon  $\alpha$ -toxin stimulation compared to psoriasis patients and healthy controls.

**Conclusion:** Increased IL-22 secretion induced by staphylococcal exotoxins in the skin partially explains how skin colonization and infection with S. aureus can contribute to chronic skin inflammation in AD.

### P168 IL-10-modulated human dendritic cells: induction of anergic T cells by CD83/CCR7/HLA-DRhigh as well as CD83lowCCR7negativeHLA-DRlow subpopulations of tolerogenic DC

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Our studies previously demonstrated that IL-10-modulated tolerogenic human dendritic cells (IL-10DC) induce anergic regulatory CD4+ and CD8+ T cells. Flow cytometry analysis revealed the existence of two subpopulations of IL-10DC with distinct states of maturation, characterized as CD83highCCR7highHLA-DRhigh and CD83lowCCR7negativeHLA-DRlow IL-10DC. Here, we investigated these two populations of human tolerogenic IL-10DC in detail with regard to their phenotype and their tolerogenic capacity to generate anergic CD4+ T cells. For this purpose, we compared the expression of costimulatory and inhibitory molecules of the B7- and ILT-family between human mature DC (mDC) and both CD83high and CD83low IL-10DC subpopulations. As compared to mDC, the immature CD83lowCCR7negative IL-10DC subset exhibited a significantly reduced expression of costimulatory molecules (CD80, CD86, B7-H2, CD40) and lower upregulation of inhibitory molecules (B7-H1, ILT3, ILT4). In contrast, we observed minor changes in expression of costimulatory

molecules (CD80, CD86, B7-H2, CD40) and an upregulated expression of the inhibitory molecules (B7-H1, ILT3, ILT4) on the mature CD83<sup>high</sup>CCR7<sup>high</sup> subpopulation of IL-10DC, demonstrating significant differences in expression of costimulatory and inhibitory molecules (B7-H1, B7-DC) between the two IL-10DC subsets. FACS sorting of both subpopulation was performed with regard to the high or absent expression of CCR7 (representing CD83<sup>high</sup> or CD83<sup>low</sup> DC) (purity >95%). Subsequently, coculture experiments with naive CD45RA<sup>+</sup>CD4<sup>+</sup>CD25<sup>high</sup>- T cells were conducted. In contrast to mDC, both subpopulations of IL-10DC exhibited an inhibited T cell stimulatory capacity resulting in a significantly reduced T cell proliferation and Th1 and Th2 cytokine production (IFN- $\gamma$ , IL-5 and IL-13). Notably, restimulation experiments (using anti-CD3/anti-CD28-mAb) revealed that both subpopulations, regardless of their maturation state, induced an anergic CD4<sup>+</sup> T cells as evidenced by a significantly reduced T cell proliferation and significantly diminished Th1 and Th2 responses (IFN- $\gamma$ , IL-5 and IL-13). In conclusion, both, phenotypic mature CD83/CCR7/HLA-DR<sup>high</sup> and immature CD83<sup>low</sup>CCR7<sup>negative</sup>HLA-DR<sup>low</sup> IL-10DC subpopulations display properties of tolerogenic human DC which may be used for the development of novel therapeutic approaches for allergies, autoimmune disease or transplant rejections.

### P169 Langerhans cell-selective deletion of EpCAM (CD326) attenuates langerhans cell motility and migration *in vivo* and enhances contact hypersensitivity reactions

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After activation, Langerhans cells (LC), a subset of dendritic cells, migrate from epidermis to regional lymph nodes where they influence the magnitude and quality of immune responses initiated in response to epicutaneously-applied antigens. LC migration that occurs in the absence of skin perturbation may contribute to peripheral tolerance. Modulation of adhesion of LC to keratinocytes is likely to be central to regulation of LC migration. LC are distinct from other dendritic cells in that they express high levels of EpCAM (CD326), a cell surface protein that is characteristic of some epithelia and many carcinomas, and that has been implicated in intercellular adhesion and metastasis. Because EpCAM knockout mice die *in utero*, we generated mice with EpCAM-deficient LC (LC/EpCAM cKO mice) to determine functional consequences of EpCAM expression by LC. LC/EpCAM cKO mouse epidermis contained two-fold increased numbers of LC that expressed normal levels of MHC Class II and costimulatory molecules and exhibited normal T cell stimulatory activity *in vitro*. Studies of skin explants revealed that migration of LC/EpCAM cKO LC was inhibited, while chemotaxis of dissociated LC was not. The ability of contact allergen-stimulated EpCAM-deficient LC to exit epidermis *in vivo* was delayed, and fewer hapten-labeled LC accumulated in regional lymph nodes. Attenuated LC migration in LC/EpCAM cKO mice caused enhanced contact hypersensitivity responses equivalent to those seen in LC-deficient mice. Intravital microscopy revealed dramatically reduced LC translocation and dendrite motility (dSEARCH) *in vivo* in contact allergen-treated LC/EpCAM cKO mice. Our results suggest that LC EpCAM facilitates LC disengagement from keratinocytes, and promotes LC emigration from skin. These studies also validate the concept that LC trafficking from skin to draining lymph nodes is essential for normal LC function.

### P170 (V28) Apis mellifera (honey bee) toxin activates the AIM2 inflammasome in primary keratinocytes

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Pattern recognition receptors (PRR) are some of the body's means of danger surveillance. Inflammasomes are danger recognition systems and different types of inflammasome complexes recognize an array of signals that range from biological and chemical macromolecules to changes in the microenvironment (e.g. during injury). Subsequently, inflammasome activation leads to release of pro-inflammatory mediators and initiation of an immune response.

The toxin of *Apis mellifera* (honey bee venom) is cytotoxic and most people react to bee venom (BV) intoxication with local inflammation, pain and swelling whereas major life threatening conditions due to allergic reactions affects approximately 2% of the population. How BV initiates innate immunity at sites of exposure and hence might prime allergic reactions in predisposed individuals is not known.

In an *ex vivo* bee sting model, we found that bee venom induces caspase-1 activation and IL-1 $\beta$  release in human skin. In cultured keratinocytes the BV component initiating the inflammasome dependent IL-1 $\beta$  release was found to be melittin. Melittin induced the activation of the AIM2 but not the NALP3 inflammasome in primary epidermal keratinocytes. AIM2 is a cytosolic DNA receptor that initiates inflammasome association and activation upon DNA binding resulting in IL-1 $\beta$  release. The trigger of AIM2 inflammasome activation upon BV/melittin intoxication was not extracellular DNA as DNase treatment of primary keratinocytes showed no effect. However, isolated mitochondrial DNA or genomic DNA from primary keratinocytes activated the AIM2 inflammasome *in vitro*. Finally, we could show that mitochondrial DNA as well as genomic DNA is present in the cytosol of BV/melittin treated primary keratinocytes. Additionally, BV/melittin induced cellular cytotoxicity in keratinocytes and fluorescence microscopy and FACS analyses revealed melittin mediated destruction of both cell membrane and mitochondrial membranes which subsequently might lead to DNA leakage from the nucleus and mitochondria.

These data indicate that upon bee venom exposure keratinocytes are involved in a primary immune response by activation of the AIM2 inflammasome and subsequent IL-1 $\beta$  release. IL-1 $\beta$  in turn is a potent activator of both innate and adaptive immune cells and may also mediate allergic sensitization towards bee venom.

### P172 Murine beta-defensin-14 exerts its immunosuppressive effects via affecting T cells but not antigen presenting cells

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Beta-Defensins are small cationic antimicrobial peptides (AMP) playing an important role in the innate defense of the skin. Since ultraviolet radiation (UVR) which suppresses the adaptive immune response was found to induce AMPs we initially asked whether AMP might be involved in this process and thus exert immunosuppressive functions as well. In fact, we could demonstrate that the UVR-inducible AMP murine beta-defensin-14 (mBD-14) switches CD4<sup>+</sup>CD25<sup>-</sup> T cells obtained from UVR-tolerized mice into a regulatory phenotype. This switch which involved the expression of Foxp3 was functionally relevant since injection of these T cells inhibited sensitization of the recipients. Furthermore, comparable to UVR mBD-14, when injected intravenously (i.v.), suppressed the induction of contact hypersensitivity and induced antigen-specific regulatory T cells (Treg). Since UVR causes immunosuppression via affecting antigen presenting cells (APC), we asked whether mBD-14 induces Treg via a similar mechanism. Therefore, bone marrow-derived dendritic cells (BMDC) were incubated overnight with

mBD14 and MHC class II expression was evaluated by FACS analysis. In contrast to UVR, mBD-14 did not affect the expression of MHC class II on BMDC. BMDC were coupled with dinitrobenzenesulfonic acid-sodium salt (DNBS) in the absence or presence of mBD-14. Subcutaneous injection of BMDC which were not exposed to mBD-14 resulted in pronounced sensitization as indicated by a specific ear swelling response upon antigen-specific challenge with dinitrofluorobenzene (DNFB). A similar sensitization response was observed upon injection of BMDC which were pretreated with mBD-14. Accordingly, induction of Treg by mBD-14 was only observed upon i.v. but not upon subcutaneous injection of mBD-14. Together, these data indicate that mBD-14 like UVR inhibits the induction of contact hypersensitivity and induces Treg. However, mBD-14 might achieve this effect via different mechanisms. In contrast to UVR which primarily affects APC, mBD-14 might exert its immunosuppressive effects via directly modulating T cells rather than APC.

### P173 Size and sulfation of hyaluronan- how do they affect macrophage functions?

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The implantation of biomaterial coated with hyaluronan (HA) has become a suitable solution for enhanced body integration. Especially sulfated HA has been identified to exert immunomodulatory capacities to innate immune cells. We could show recently that primary inflammatory macrophages (iM) are able to switch towards a regulatory phenotype at the presence of artificial extracellular matrix (aECM) composed of collagen I and high sulfated HA (hsHA). During inflammation HA is normally degraded by hyaluronidases, therefore implanted hsHA would probably also be exposed to its own fragmentation at inflamed site of implantation. It is well documented that native HA is capable to push immune cells towards anti-inflammatory, immunosuppressive functions whereas small fragmented HA does exert pro-inflammatory effects. Thus, it is important to assess how small sized soluble hsHA affect immune cell functions. Here, we address this by investigating the modulation of macrophage activities that are key for the resolution of inflammation at implantation sites. Therefore, functions of iM including cytokine response, pathogen uptake and killing and immune cell attraction will be examined in the presence of different sized sulfated and non-sulfated (control) HA. We hypothesize that enzymatic fragmentation of implanted hsHA would influence its immunomodulatory capabilities towards resolution of inflammation and initiation of wound healing. In fact we assume that immunomodulatory effects of hsHA are due to its sulfation. We want to confirm this hypothesis by examination also of sulfated polyglycerols which have been shown recently to induce anti-inflammatory functions of leukocytes.

### P174 The immunomodulatory tripeptide K(D)PT, related to the C-terminal sequence of $\alpha$ -melanocyte-stimulating hormone, ameliorates ongoing psoriasis by inducing tolerogenic DC

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Alpha-melanocyte-stimulating hormone ( $\alpha$ -MSH), as well as its C-terminal tripeptide KP $\nu$ , is known to exhibit potent anti-inflammatory and immunomodulatory effects *in vitro* and *in vivo*. In addition, K(D)PT a tripeptide derived from an inhibitory loop of IL-1 $\beta$  and structurally related to KP $\nu$  was shown to have similar immunomodulatory effects, which most likely are mediated by a reduction of nuclear factor  $\kappa$ B (NF- $\kappa$ B) activation and translocation. To elucidate the mechanisms underlying K(D)PT-induced immunomodulation we investigated whether K(D)PT affects dendritic cell (DC)-T cell communication since especially this interaction plays a role in the regulation of immune responses. DC stimulated with K(D)PT showed a reduced expression of co-stimulatory molecules compared to PBS-stimulated controls but demonstrated an increased secretion of IL-10, a marker that has previously been associated with the induction of tolerogenic DC and the DC-mediated expansion of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells (Treg). To investigate whether K(D)PT-stimulated DC were indeed able to induce functional Treg, bone marrow-derived DC (bmDC) were treated with K(D)PT and co-cultured with CD4<sup>+</sup> T cells. Notably, CD4<sup>+</sup> T cells from co-cultures with K(D)PT-stimulated bmDC showed an enhanced expression of markers characteristic for Treg, like Foxp3, Neurophilin-1 or CTLA-4, and suppressed the proliferation of CD4<sup>+</sup>CD25<sup>-</sup> effector T cells *in vitro*. To test whether K(D)PT might induce tolerogenic DC, capable of expanding immunosuppressive Treg *in vivo* we induced a psoriasis-like skin inflammation in mice by topical application of imiquimod since in psoriasis disease progression has been associated with dysfunctional Treg. Subsequently, mice were injected with PBS or K(D)PT. Interestingly, K(D)PT treatment resulted in a significant reduction of epidermal ridge thickness and furthermore, decreased the levels of pathogenic Th17 cells in regional lymph nodes, which was paralleled by increased numbers of Treg. Next, we analyzed whether tolerogenic DC generated *in vitro* by stimulation with K(D)PT might induce functional Treg in psoriatic mice and therefore, injected PBS and K(D)PT-stimulated bmDC into imiquimod-treated mice. Whereas the transfer of PBS-stimulated bmDC did not result in the amelioration of skin inflammation, recipients of K(D)PT-treated bmDC showed a decreased epidermal thickness. Of note, this was associated with increased numbers of Treg and down-regulated levels of pathogenic Th17 cells in lymph nodes draining cutaneous lesions. To scrutinize whether Treg induced by the adoptive transfer of K(D)PT-stimulated bmDC were indeed functional CD4<sup>+</sup> T cells as well as Treg were purified from mice injected with PBS- or K(D)PT-stimulated bmDC and co-cultured *in vitro*. While substantial amounts of Th17 cells were detectable in co-cultures with Treg from recipients of PBS-treated bmDC the numbers of IL-17 and IL-22 secreting T cells were significantly reduced in recipients of K(D)PT-stimulated bmDC indicating that K(D)PT-stimulated tolerogenic DC induced functional Treg *in vivo* which efficiently suppressed the activity of pathogenic Th17 cells. Together, these data indicate that the tripeptide K(D)PT is able to ameliorate ongoing psoriasis and suggest that K(D)PT might represent a potential therapeutic option for the treatment of patients with moderate to severe psoriasis.

### P175

### Thymic stromal lymphopoietin (TSLP) is highly upregulated in epidermis of scurfy mice as a potential differentiation factor for the strong Th2-deviation in autoimmune skin inflammation of scurfy mice

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Scurfy mice spontaneously develop autoimmune inflammation in multiple organs including the skin due to the lack of functional regulatory T cells. The disease is clearly CD4<sup>+</sup> T cell-mediated since isolated Scurfy CD4<sup>+</sup> T cells transfer disease in RAG-/- recipients after i.v. injection. We performed analysis of the inflammatory infiltrate in Scurfy skin and the cytokine profile of skin-infiltrating autoreactive CD4<sup>+</sup> T cells particularly with regard to Thymic Stromal Lymphopoietin (TSLP) as potential differentiation factor.

CD4+ T cells and granulocytes are the predominant cell types in inflamed skin as revealed by FACS-analysis. As determined by intracellular FACS-analysis after *in vitro* restimulation with PMA/Ionomycin CD4+ T cells isolated from inflamed Scurfy skin secreted high levels of Th2-cytokines (IL-4 and IL-5) and low levels of the Th1-cytokine interferon- $\gamma$ . Next we analysed the expression of TSLP, a soluble factor known to mediate Th2-differentiation of CD4+ T cells: Scurfy mice show high TSLP-serum levels in contrast to WT animals as measured by ELISA. Since TSLP is secreted by epithelial cells under stress-conditions, we analysed TSLP expression in inflamed Scurfy skin by immunohistochemistry and found strong TSLP expression in Scurfy epidermis in contrast to WT epidermis. Next we performed FACS-analysis of TSLP-receptor-expression to determine if skin-infiltrating CD4+ T cells can respond to TSLP: CD4+ T cells in Scurfy but not WT skin show high TSLP-receptor expression. In summary we show that skin-infiltrating CD4+ T cells in Scurfy mice spontaneously develop a Th2-phenotype with overexpression of the Th2-differentiation factor TSLP in Scurfy epidermis and serum as potential driving factor for this Th2-deviation.

#### P176

##### Bisphenol A regulates PPAR gamma expression in immune cells

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Bisphenol A (BPA) belongs to the group of endocrine disruptors and is used for production of polycarbonate plastic and epoxy resins. Previous mouse studies revealed that exposure to BPA upregulates mRNA expression of retinoic acid receptors. BPA as well as retinoic acid receptor ligand retinoic acid (RA) can modulate immune cell function. Therefore, BPA may exert its effects by regulating RA receptors. An assumed correlation of low dose BPA exposure and RA receptor family expression in dendritic cells and T cells was investigated.

The influence of BPA and RA on expression levels of RA receptors in human monocyte-derived dendritic cells (MoDC) and human activated naive CD4+ T cells (TC) were analyzed by quantitative Real-time PCR. BPA significantly reduced the RA receptor family member Peroxisome proliferator-activated receptor gamma (PPAR gamma) mRNA in MoDC. In contrast, BPA treatment in TC enhanced mRNA levels of PPAR gamma and its heterodimerization partner RXR alpha. In further experiments it was investigated if the changed mRNA expression affected PPAR gamma signalling. It is known that PPAR gamma agonists regulate surface marker expression on MoDC, but this effect is not influenced by the BPA-reduced PPAR gamma expression.

Although no changed PPAR gamma signalling in MoDCs after BPA treatment was detectable, it is still possible that BPA may disrupt PPAR gamma signal transduction.

#### P177

##### Gain-of-function human STAT1 mutations in patients with chronic mucocutaneous candidiasis

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Chronic mucocutaneous candidiasis (CMC) constitutes a selective inability to clear infection with the yeast *Candida albicans* resulting in persistent debilitating inflammation of skin, nails, and mucous membranes and is maybe caused by autosomal dominant IL-17F deficiency or autosomal recessive IL-17RA deficiency. Here, heterozygous germline mutations were identified in CMC patients using whole-exome sequencing and DNA binding activity of STAT1 was investigated. Furthermore, differentiation to and effector-functions of Th17 cells was analyzed. DNA isolated from 2 CMC patients and their healthy relatives was investigated for STAT1 mutation using whole-exome sequencing and IFN- $\gamma$  stimulated PBMCs of these patients were analyzed in a STAT1 TransAM system. PBMCs of six CMC patients and 12 healthy controls were stimulated with Candida, PHA and aCD3/aCD28 for 72 h. Naive T cells of both groups were differentiated to Th17 cells; cytokine production quantified in supernatants by ELISA. Whole-exome sequencing revealed that both CMC patients carried the STAT1 mutant alleles Q271P and R274W whereas none of these mutations were found in any of the healthy relatives tested. Functional characterization of the CMC-causing STAT1 alleles revealed that STAT1-dependent cellular responses to IFN- $\alpha/\beta$ , IFN- $\gamma$ , IFN- $\lambda$  and IL-27 – cytokines that inhibit Th17 development – are increased. These STAT1 gain-of-function STAT1 alleles affect the coiled-coiled domain and impair the nuclear dephosphorylation of activated STAT1. Treatment of PBMCs of these patients with IFN- $\gamma$  resulted in an increased DNA binding activity compared to healthy controls in the TransAM system. Importantly, T cells from CMC patients secreted significantly lower amounts of Th17-associated cytokines IL-17A and IL-22 in response to Candida compared to healthy controls and differentiated Th17 cells showed a reduced ability to produce Th17-associated cytokines. These data indicate that the inability to clear the yeast *Candida albicans* in CMC patients can be caused by gain-of-function STAT1 alleles which might impair IL-17 immunity by the enhancement of STAT1-mediated cellular responses to STAT1-dependent repressors of IL-17 producing T cells.

#### P178

##### Keratinocyte derived mediators and their influence on T cell effector functions

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Regarding the important role of human skin in host defence, a clear knowledge about the crosstalk of keratinocytes and infiltrating immune cells is of great relevance. Especially the secretion of mediators may provide keratinocytes a tool to influence specifically the fate of T cells. Recent data demonstrated that co-cultures of keratinocytes and CD4+ T cells result in a differed cytokine profile and proliferation potential. However, nothing is known about possible individual effects on the CD4+ T cell subtypes. This prompted us to study the modulating effect of keratinocyte derived mediators on three different T cell lines Th1, Th2, and Th17. For this purpose primary human keratinocytes were cultured under steady state as well as under inflammatory conditions provoked by IFN- $\gamma$  stimulation (300 U/ml). Cell free supernatants were collected and to eliminate donor specific effects the supernatants of three different keratinocyte donors were mixed. For generation of T cell lines, naive CD4+ T cells were isolated by microbead untouched technique from human blood and subsequently polarised to Th1, Th2 and Th17 cells within 7 days. Keratinocyte supernatants were added to the fully polarised T cell lines and after 72 h of incubation T cell supernatants were taken to quantify cytokine production by ELISA (IFN- $\gamma$ , IL-10, IL-4, IL-17 and IL-22). To determine the proliferation potential of the T cells 3H thymidine incorporation assays were performed.

Especially in the case of supernatants generated by unstimulated keratinocytes the results showed a strikingly clear inhibitory effect on IL-10 as well as IL-22 production in all T cell lines. For the Th2 cell line an inhibition of IL-4 secretion was observed as well as for Th1 cells the tendency of a decreased IFN- $\gamma$  secretion was seen when co-incubated with keratinocyte supernatants. In contrast secretion of IL-17 by Th17 cells seems to be supported by the presence of keratinocyte supernatant. In conclusion, keratinocytes are critically involved in defining the threshold of inflammatory processes in the skin by inhibiting T cell proliferation and cytokine production.

#### P179 (V30)

##### Sulfonated- and phosphonated-polystyrene nanoparticles enhance stimulatory capacity of human dendritic cells

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Abstract Cell type-specific drug delivery by nanoparticles represents a promising tool in immunotherapy regarding specificity, stability and efficacy. Investigating the influence of nanoparticles which feature the capability for clinical applications on immune cells is of great importance. Based on cell type-specific targeting molecules, surface hydrophobicity and charge of the nanoparticles cell maturation or differentiation of distinct cell types may be affected differently.

In the present study, the impact of sulfonate- and phosphonate-functionalized polystyrene nanoparticles on human monocyte-derived dendritic cells (DC) representing the most efficient antigen-presenting cells of the immune system was analyzed. The fluorescence-labeled sulfonate- and phosphonate-polystyrene NP with negative zeta potential revealed a size of 230 and 280 nm, respectively. Independently of their maturation status, immature (iDC) and mature DC (mDC) exhibited a high uptake of sulfonate- and phosphonate-functionalized polystyrene nanoparticles resulting in 95% positive cells at 75 g/ml NP concentration which was measured via flow cytometry analysis. Confocal laser scanning microscopy confirmed the intracellular uptake of nanoparticles. The overall uptake was time- as well as dose-dependent without any toxic effects. Cultivation of DC with incorporated nanoparticles for up to 7 days revealed high stability of the particles within the cells without altering cell viability. In order to characterize DC with incorporated nanoparticles, the immunophenotype of the cells was analyzed. mDC showed neither an alteration in the immunophenotype or cytokine production nor did mDC induce an increased T cell proliferation after polystyrene nanoparticle treatment. In contrast, NP-treated iDC induced an enhanced maturation and increased stimulatory capacity confirmed by significant upregulation of the maturation marker CD83 as well as of the co-stimulatory molecules CD80 and B7H2. In addition, the production of the cytokines IL-6 and TNF- $\alpha$ , which trigger DC maturation- and T cell activation, was increased in the supernatant of NP-treated iDC. Compared to control iDC, an enhanced stimulatory capacity of NP-treated iDC was detected by a significantly increased proliferation and elevated IFN- $\gamma$  production of co-cultured allogeneic CD4+ T cells.

The current study showed that sulfonated- and phosphonated-polystyrene nanoparticles are non-toxic for DC at the concentration used. Beyond that, we could demonstrate a significant increase in iDC maturation accompanied with an elevated stimulatory capacity induced by incorporated nanoparticles.

#### P180

##### The pathogenic effect of autoantibodies in anti-p200 pemphigoid patients is not mediated by reactivity to the C-terminus of laminin gamma 1

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Anti-p200 pemphigoid is a subepidermal blistering skin disease characterized by autoantibodies against a 200-kDa protein (p200) of the dermal-epidermal junction. Recently, the laminin  $\gamma$ 1 chain has been identified as the target antigen and the C-terminus of laminin  $\gamma$ 1 was described as an immunodominant region of this protein. However, the pathogenic relevance of autoantibodies to laminin  $\gamma$ 1 has not been demonstrated yet. To address this question, we used a cryosection assay, an *in vitro* model involving cryosections of human skin incubated with patients' autoantibodies and leukocytes from healthy donors. We showed that anti-p200 pemphigoid sera ( $n = 7$ ) induced dermal-epidermal separation in a time-dependent manner. Furthermore patients' autoantibodies, affinity-purified against a recombinant form of C-terminal fragment of human laminin  $\gamma$ 1 (hLAMC1-cterm), failed to induce dermal-epidermal separation in the cryosection assay. Similar results were obtained by using a eukaryotic form of hLAMC1 and the E8 fragment of laminin 111 (truncated C-terminal portions of  $\alpha$ 1,  $\beta$ 1, and  $\gamma$ 1 chains) as well as rabbit IgG generated against hLAMC1-cterm. In contrast, patients' sera depleted of reactivity against hLAMC1-cterm retained their blister-inducing ability to the same extent as the non-depleted, original sera. Here, we demonstrate for the first time that serum autoantibodies of anti-p200 pemphigoid patients are pathogenic *ex vivo*. Interestingly, this pathogenic effect is not mediated by antibodies against the C-terminus of laminin  $\gamma$ 1. Future studies will explore the pathogenic potential of autoantibodies to p200 using mouse models.

#### P181

##### Ligands of the danger receptor RAGE are highly upregulated in psoriatic lesions

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Psoriasis is a common complex genetic disease of the skin characterized by hyperplasia and chronic inflammation. The cause of psoriasis is unknown, however, the relative contributions of epidermal cells and the immune system to disease initiation and maintenance remain unclear. Recently, we have shown that mice deficient for the receptor of advanced glycation end-products (RAGE) are resistant to experimental chronic inflammation and that RAGE expression on myeloid cells is essential for sustaining a pro-inflammatory microenvironment. Here, we aim at elucidating the role of RAGE and its ligands S100A8, S100A9, S100A12, S100B, and HMGB1, so called alarmins or members of the damage-associated molecular pattern (DAMP), on myeloid cells for cutaneous inflammatory responses regarding quality and quantity of the immune response using skin biopsies from psoriatic patients. Here, we show that RAGE ligands are highly upregulated in human skin biopsies of psoriatic lesions and unaffected skin using immunofluorescence confocal microscopy and immunohistochemistry. Moreover, RAGE is found to be expressed by keratinocytes, endothelial cells as well as dermal inflammatory cells, e.g. CD11c-positive dendritic cells (DC), in psoriatic lesions suggesting an epidermal-dermal cross-talk of RAGE signaling involving CD11c positive-DC. Our data hint towards a central role of RAGE in the inflammatory process initiating and maintaining a psoriatic plaque. Using conditional knock-out models, human skin biopsies, and state-of-the-art *in vitro* models we will study whether RAGE represents a central regulatory molecule linking innate and adaptive immunity and therefore might represent a novel therapeutic target in psoriasis.

P182

**Mast cells control chronic type IV allergic skin inflammation in mice**V. A. Gimenez-Rivera, F. Sichenhaar, M. Metz and M. Maurer *Department of Dermatology and Allergy, Charit Universitätsmedizin, 10117 Berlin, Germany*

Mast cells (MC) have been shown to modulate skin inflammatory responses to contact allergens. However, the role of mast cells in chronic skin inflammation, the clinical picture in patients with allergic contact dermatitis, has not been investigated in detail. Here, we have studied the role of mast cells in skin inflammatory responses to repeated exposure to the contact allergen oxazolone. Mast cell-deficient C57BL/6-KiW-sh/W-sh (Sash) mice showed increasingly enhanced skin inflammation upon repeated challenge with oxazolone, as assessed by measuring ear thickness (AUC values: wild type = 80, sash = 230; [0.01 < P]). The adaptive transfer of mast cells to challenge sites completely repaired this phenotype (AUC Values: Sash = 230, Mast cells reconstituted mice = 93; [0.01 < P]). Interestingly, enhanced inflammatory skin responses to contact allergen exposure resulted in markedly increased CD4/CD8 double positive T Cell populations in the draining lymph nodes of mast cell deficient mice. These data point to a crucial role of mast cells in the prevention and/or down regulation of type IV allergic skin inflammation induced by repeated allergen challenge, possibly by modular effects on pro-inflammatory T cell populations.

P183

**Gene gun treatment with NC16A prevents autoimmunity towards hBPAG2 in a skin grafting model**M. Ettinger<sup>1</sup>, D. Peckl-Schmid<sup>1</sup>, C. Gruber<sup>1</sup>, M. Laimer<sup>1</sup>, J. Thalhammer<sup>2</sup>, H. Hintner<sup>1</sup>, I. K. Gratz<sup>1</sup> and J. W. Bauer<sup>1</sup> *<sup>1</sup>Division of Molecular Dermatology and EB House Austria, Department of Dermatology, Paracelsus Medical University, 5020 Salzburg, Austria; <sup>2</sup>Department of Molecular Biology, University of Salzburg, 5020 Salzburg, Austria*

Immune responses to a therapeutic gene product are potentially serious complications in somatic gene therapy of autosomal recessive genetic skin diseases. Due to the high immunogenicity of human bullous pemphigoid antigen 2 (hBPAG2), the induction and maintenance of tolerance towards this neo-antigen is critical for the success in the treatment of genetic skin blistering disease epidermolysis bullosa junctionalis. Therefore, NC16A, the immunodominant domain of BPAG2, was used in a mouse model to *in vivo*-transfect skin of graft recipients prior to grafting to prevent immune reactions towards transplanted hBPAG2 expressing donor grafts. In contrast to control mice, 80% of wild-type mice gene gun transfected with hNC16A showed indefinite survival of skin grafts from mice expressing hBPAG2 in the epidermal basement membrane. Immunological tolerance was stable and transferable by lymphocytes of tolerant mice. Graft acceptance was associated with a dense Foxp3+ regulatory T cell infiltrate and lack of inflammation. Depletion of regulatory T cells lead to graft rejection identifying them as potential mediators in the mechanism of tolerance induction. These mechanisms are relevant for patients undergoing gene therapy and has a potential impact on the treatment of autoimmune blistering skin diseases.

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**Interferon- $\alpha$  induced reduction of suppressor activity of human CD4+CD25high regulatory T cells is associated with loss of cAMP and altered immuno-phenotype**N. Bacher<sup>1</sup>, T. Bopp<sup>2</sup>, C. Becker<sup>1</sup>, E. Graulich<sup>1</sup> and K. Steinbrink<sup>1</sup> *<sup>1</sup>University Medical Center Mainz, Department of Dermatology, Johannes Gutenberg-University, Mainz, Germany; <sup>2</sup>Institute for Immunology, Johannes Gutenberg-University, Mainz, Germany*

Administration of Interferon- $\alpha$  (IFN- $\alpha$ ) represents the only approved adjuvant therapeutic approach in stage II-III melanoma patients. Previously, we have shown that IFN- $\alpha$  inhibits suppressor activity of human CD4+CD25high regulatory T cells (Treg) *in vitro* and *in vivo*. In this study, we analyzed alterations in the phenotype and activation of Treg after IFN- $\alpha$  treatment in order to reveal underlying mechanisms of IFN- $\alpha$ -induced inhibited Treg function. Treg and CD4+CD25low effector T cells (Teff) were isolated from buffy coats of healthy volunteers, treated with 10<sup>4</sup> U/ml IFN- $\alpha$  and subsequently activated by anti-CD3/anti-CD28 mAb. Alterations of the immuno-phenotype of Treg and Teff were analyzed by flow cytometry. In order to exclude IFN- $\alpha$ -induced toxicity, rate of apoptosis was assessed by annexin-V staining. Of note, IFN- $\alpha$  did not alter apoptosis rates in Treg as well as Teff. Characterization of Treg-associated markers revealed an unchanged expression of the IL-7 receptor, CD39 and CTLA-4, important markers for homeostasis and function of Treg, as well as the Treg-associated transcription factor FoxP3 after IFN- $\alpha$  treatment of Treg. Engagement of the glucocorticoid-induced tumour necrosis factor receptor (GITR) in Treg proliferation and function has been reported. After IFN- $\alpha$  treatment GITR was significantly decreased in Treg as well as Teff. Activation of Treg by IFN- $\alpha$  was indicated by a significantly enhanced expression of the early activation marker CD69 on Treg but not on Teff. As reported recently, function of human Treg is dependent on upregulation of endogenous cAMP. Assessment of cytosolic cAMP concentrations revealed that IFN- $\alpha$  significantly repressed cAMP upregulation in activated human Treg, indicating that IFN- $\alpha$  inhibits the function of Treg by depletion of cAMP. Differential responsiveness of Treg to IFN- $\alpha$  was indicated by significantly elevated levels of the type I IFN receptor chain-2 (IFNAR-2) when compared to Teff. In addition, IFN- $\alpha$  treatment significantly decreased the expression of IFNAR-2 on Teff but not on Treg. These findings suggest an increased susceptibility to IFN- $\alpha$ -mediated effects on Treg induced by IFN- $\alpha$  treatment when compared to Teff. Thus, we postulate that IFN- $\alpha$  mediates inhibition of Treg function via disruption of cAMP homeostasis and alteration of surface molecules important for suppressor activity of Treg.

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**Alterations in regulatory T cell population in atopic-dermatitis-like inflammation in mice**V. Martinz, C. H. Tripp, M. Schmuth and S. Dubrac *Department of Dermatology and Venerology, Innsbruck Medical University, Innsbruck, Austria*

Repeated high dose of topically applied vitamin D3 (VitD3) upregulates the expression of thymic lymphopoietin (TSLP) in mouse keratinocytes and results in a Langerhans-cell-dependent atopic dermatitis (AD)-like inflammation. Although abnormal immune reactivity plays an important role in the multifactorial pathogenesis of atopic dermatitis, the complex network of the AD immune system remains still unclear. We here present data from a VitD3-induced mouse model of AD-like skin inflammation with hyperkeratosis, epidermal hyperplasia and dermal inflammatory infiltrates revealing enhanced mRNA expression of TSLP, interleukin-13 (IL-13), IL-1, IL-10 and TARC/CCL17. Mice with AD-like inflammation revealed greater numbers in skin draining lymph node cells with an increase in numbers of emigrated Langerin+ dendritic cells (DC), while numbers of emigrated Langerin- DC were not affected in this mouse model of AD. ICOS ligand-carrying skin DC in draining lymph nodes were significantly increased in VitD3-treated mice, when compared to control mice. Furthermore, VitD3-induced AD increased CD4+ CD25+ FOXP3+ regulatory T cells with appearance of a second, CD4+ CD25high FOXP3+ regulatory T cell population (Treg). Upon induction of AD-like inflammation, Treg cells upregulated expression of activation markers CTLA-4 and ICOS, as compared to the vehicle-

treated control. While Treg cells seem upregulated and activated, defective regulatory function cannot be excluded in this mouse model of AD as reported in various cohorts of AD patients.

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**Antibodies to the von Willebrand Factor A domain of type VII collagen induce strain-dependent subepidermal blistering in mice**H. Iwata<sup>1</sup>, S. Leinweber<sup>2</sup>, U. Samavedam<sup>1</sup>, A. Shimizu<sup>3</sup>, A. Ishiko<sup>3</sup>, K. Seeger<sup>2</sup>, E. Schmidt<sup>1</sup>, D. Zillikens<sup>1</sup> and R. J. Ludwig<sup>1</sup> *<sup>1</sup>Dermatology, University of Luebeck, Luebeck, Germany; <sup>2</sup>Chemistry, University of Luebeck, Luebeck, Germany; <sup>3</sup>Dermatology, Faculty of Medicine, School of Medicine, Toho University, Tokyo, Japan*

Epidermolysis bullosa acquisita (EBA) is a subepidermal blistering disease associated with antibodies to type VII collagen (COL7). Epitopes recognized by the majority of EBA sera were mapped to the non-collagenous I domain of COL7, including the von Willebrand Factor A sub-domain (vWFA), which is an important interaction site with type I collagen. Mutations within vWFA were shown to result in congenital dystrophic epidermolysis bullosa. However, the pathogenic relevance of autoantibody binding to the vWFA is unclear. We here challenged the assumption, that the vWFA domain is pathogenically relevant in EBA. To test this hypothesis, rabbit antibodies specific to murine vWFA were generated. These antibodies induced dermal-epidermal separation when incubated with cryosections of murine skin in the presence of neutrophils. Injection of purified anti-vWFA IgG into C57BL/6 (B6) mice duplicated clinical, histological and immunopathological findings in human inflammatory EBA. Interestingly, mice of different strains (B6, BALB/c and SKH-1) demonstrated distinct susceptibility to induction of skin disease. While B6 mice developed a severe clinical phenotype, BALB/c mice showed moderate skin lesions, and SKH-1 mice were completely protected from EBA induction, although same levels of circulating rabbit IgG to vWFA were detected in all mice. Furthermore, disease induction was completely dependent on the expression of activating Fc receptors: FcR gamma chain-deficient mice were resistant to EBA induction. In addition, C5-deficient mice had a significantly reduced phenotype, but still developed mild disease pointing to a contribution, but not complete dependency, of complement activation in the pathogenesis of EBA. Taken together, our data clearly demonstrates that autoantibodies to the murine vWFA domain of COL7 are pathogenic. Furthermore, strain dependency of induction of skin lesions indicates a genetic control of tissue injury after binding of autoantibodies to their skin target.

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**A critical role for ROS production and fragmentation of hyaluronic acid in full DC activation by contact allergens and CHS responses**P. R. Esser<sup>1</sup>, U. Woelfle<sup>2</sup>, T. Jakob<sup>1</sup>, C. M. Schempp<sup>2</sup> and S. F. Martin<sup>1</sup> *<sup>1</sup>Department of Dermatology, Allergy Research Group, University Freiburg Medical Center, 79104 Freiburg, Germany; <sup>2</sup>University Freiburg Medical Center, Competence Center Skintegral, 79104 Freiburg, Germany*

Contact allergens trigger innate immune and stress responses involving pattern recognition receptors and the production of reactive oxygen species (ROS). The underlying signalling pathways strikingly resemble the signalling involved in pathogen defense. Further understanding of the molecular mechanisms involved in contact allergen responses is a crucial pre-requisite for the development of reliable *in vitro* test systems for the identification of chemicals with skin sensitizing potential. Moreover, modulation of these responses will help to prevent the inflammation that is crucial for the sensitization to contact allergens and should, therefore, result in new therapies for allergic contact dermatitis. We have previously shown a role for the Toll like receptor (TLR) 2 and -4 signalling in the mouse contact hypersensitivity (CHS) model in germ-free mice ruling out a role for pathogen associated molecular patterns (PAMPs). Thus, we aimed at elucidating the role of endogenous danger signals in contact allergen induced TLR signalling. One candidate are low molecular weight fragments of hyaluronic acid (HA), a highly abundant extracellular matrix (ECM) component which can trigger TLR2 and -4 signalling in sterile inflammation. In addition, we sought to analyse the mechanisms leading to fragmentation of ECM. To this end, we analysed the generation of oxidative stress after contact allergen stimulation as well as the induction of ECM degrading enzymes.

The induction of ROS and of HA degradation was studied *in vitro* and in murine skin. Antioxidants and enzyme inhibitors were used to analyse the functional role of ROS and hyaluronidases for CHS. Generation of ROS and degradation of HA as a result of ROS formation was studied in the skin by immunohistology. In addition we have used TLR reporter cells to shed further light on the role of ROS induced HA degradation products for the activation of this signalling pathway. We demonstrate a role for ROS production after contact allergen stimulation and its potential influence on the oxidative degradation of hyaluronic acid. Furthermore we provide evidence for the indirect activation of TLRs by contact allergen induced production and degradation of HA in the inflammatory skin milieu. In the CHS model we demonstrate the *in vivo* potential of inhibitors of HA metabolism and of antioxidants to prevent CHS responses when used in a short time window before or after sensitization and elicitation.

Innate immune receptor signaling can be indirectly induced by contact allergens either by production or release of endogenous danger signals in the skin microenvironment. Here we demonstrate analogies between innate immune and stress responses to contact allergens and infections. We show a direct link between ROS production and breakdown of ECM components indirectly triggering TLRs. Taken together we point out future strategies for causative therapies of allergic contact dermatitis by targeting innate immune and stress responses.

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**The flavonoid luteolin inhibits the autoantibody-induced granulocyte activation and tissue damage in experimental bullous pemphigoid**E. Oswald<sup>1,2</sup>, A. Sesarman<sup>2</sup>, C. W. Franzke<sup>2</sup>, C. M. Schempp<sup>3</sup>, L. Bruckner-Tuderman<sup>1</sup>, T. Jakob<sup>1</sup>, S. F. Martin<sup>1</sup> and C. Sitaru<sup>2</sup> *<sup>1</sup>Allergy Research Group, Department of Dermatology, University Medical Center Freiburg, 79104 Freiburg, Germany; <sup>2</sup>Molecular Dermatology, Department of Dermatology, University Medical Center Freiburg, 79104 Freiburg, Germany; <sup>3</sup>Department of Dermatology, University Medical Center Freiburg, Competence Center Skintegral, 79104 Freiburg, Germany*

Autoantibody-induced inflammatory tissue damage is a major pathogenetic mechanism in blistering skin diseases. Bullous pemphigoid (BP) is a prototypical autoimmune skin disease associated with autoantibodies against the hemidesmosomal proteins BP180/collagen XVII and BP230 at the dermal-epidermal junction (DEJ) and subepidermal blistering. Recently, we established a new passive transfer model of BP in adult mice. Here immunization against rabbit antibodies transferred 3 days before injection of rabbit anti-mouse BP180 autoantibodies results in spontaneous inflammatory blistering of the skin. In our model, the predominant inflammatory cells infiltrating the lesional skin of mice are neutrophils. Our previous data from an *ex vivo* cryosection model of BP show that the NADPH oxidase-dependent respiratory burst in granulocytes is a prerequisite for autoantibody-induced dermal-epidermal separation. Therefore, the aim of our present study was to investigate the effects of anti-inflammatory compounds with potent antioxidative properties to alleviate blister formation in BP. For this purpose, we have used luteolin (3,4,5,7-tetrahydroxyflavone), a flavonoid widely distributed in plants with potent anti-inflammatory and antioxidative properties. In a first set of experiments, we observed that depletion of neutrophils using a Ly6G-specific monoclonal antibody in mice ( $n = 5$ ), starting at day 8 after immunization, significantly inhibits skin blistering *in vivo* when compared with mice treated with a mock antibody ( $n = 5$ ). Since our *in vitro* experiments demonstrated that luteolin

inhibited the production of reactive oxygen species by human and mouse leukocytes stimulated with BP180-specific immune complexes, we tested its effects on skin blistering in a further set of experiments. Luteolin inhibited the pemphigoid autoantibody-induced granulocyte-dependent dermal-epidermal separation of mouse skin in the *ex vivo* cryosection model of subepidermal blistering. Importantly, BALB/c mice treated with luteolin intraperitoneally ( $n = 5$ ) showed significantly decreased induction of experimental BP when compared with the group that received vehicle alone ( $n = 5$ ). In conclusion, our results show for the first time that granulocyte-dependent oxidative stress responses are required for full-blown inflammatory pemphigoid disease in mice. The flavonoid luteolin available in both systemic and topical preparations is a protective compound in experimental BP and may offer a significant therapeutic benefit in patients with granulocyte-mediated autoimmune inflammatory diseases.

**P189 (V31)**  
**Direct recruitment of slan (6-sulfo LacNAc) dendritic cells to immune complexes in a model of physiologically relevant fluid shear stress**

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The recognition of immune complexes (IC) by dendritic cells (DCs) is regarded as an important pathogenic event in autoimmune diseases. Among the distinct subsets of blood DCs we previously identified the population of slanDCs, a human DC subset characterized by pronounced expression of the low affinity Fc gamma receptor III (CD16). SlanDCs are highly proinflammatory and serve as a major and early source of IL-12 and TNF- $\alpha$ . We show that expression of CD16 equips slanDCs with a unique capacity to capture soluble ICs. In addition, we asked whether immobilized ICs, as found in the vasculature in autoimmune diseases, can serve as an early signal to recruit proinflammatory slanDCs. To this end we applied a flow chamber adhesion assay to measure the arrest function of slanDCs to ICs. Interestingly, we could show that small glass surface-coated ICs alone are highly efficient in mediating the arrest of slanDCs under conditions that match venous blood flow. By specifically blocking the Fc gamma receptors of slanDCs, we were able to show that adherence to immobilized ICs is largely dependent on CD16. In contrast, blocking of CD32 (Fc gamma R II) only slightly inhibited the adherence of slanDCs. In line with this, blocking of both CD16 and CD32 only marginally increased inhibition of slanDC arrest compared to blocking of CD16 alone. To confirm and extend these findings, we employed a second model. Here, microslides were seeded with human endothelial cells and incubated with anti-endothelial IgG antibodies prior to measuring the arrest function of slanDCs under physiologic shear stress. These studies confirmed our hypothesis that slanDC can be recruited under physiologic shear stress to antibodies or ICs deposited on endothelial cells. Taken together, we identified CD16 as the critical structure for the capture of soluble ICs and reveal that immobilized ICs can recruit slanDCs under defined shear stress conditions. Our data provide first evidence for slanDCs as being a cell type that meets important functional criteria for an involvement in the initiation of IC-mediated inflammation.

**P190 (V36)**  
**Mda5 activation and type I interferon signaling in the microenvironment seems to be more important for melanoma therapy with cytosolically targeted isRNA than direct induction of apoptosis in tumor cells**

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Activation of pattern recognition receptors with immunostimulatory RNA (isRNA) emerges as a new treatment option for cancer. The cytosolic RNA receptors Mda5 and Rig-I may represent a more promising target than the Toll like receptors (TLRs) 3 and 7 because they are more ubiquitously expressed in immune, stroma and tumor cells. It has been shown, that cytosolically targeted isRNA can promote apoptosis directly in tumor cells in addition to activating innate and adaptive antitumor immunity. We observed, that synthetic polyinosinic:polycytidylic acid (pI:C) delivered to the cytosol by complexation with polyethyleneimine (PEI) impairs the growth of primary cutaneous melanomas in Hgf-Cdk4R24C mice more efficiently than naked pI:C.

Here we experimentally dissect the relative contribution of direct tumor cell-intrinsic versus indirect microenvironmental effects of pI:C PEI by transplanting newly established Hgf-Cdk4R24C cell lines (HCmel3 and HCmel12) in wildtype, Mda5 $^{-/-}$  and Ifnar $^{-/-}$  knockout mice.

*In vitro* experiments confirmed that cytosolic targeting of pI:C with PEI induces apoptosis and leads to secretion of the interferon-regulated chemokine CXCL10 as well as upregulation of MHC I on HCmel3 and 12. To analyze the importance of the direct effects on tumor cells *in vivo*, we transplanted these lines in syngeneic wildtype or Mda5 deficient hosts. In wildtype mice treatment with intratumoral injections of pI:C PEI delayed tumor growth more efficient than naked pI:C. Interestingly, the effectiveness of pI:C PEI was reduced in Mda5-deficient mice indicating that RNA recognition in the tumor microenvironment also plays an important role in tumor destruction. Characterizing the *in vivo* effects, we found that pI:C PEI induces a strong activation of intratumoral NK and myeloid cells. Because activation of immune cells by Mda5 is mediated in part by the type I interferon system, we also treated melanoma transplanted in mice lacking functional type I interferon receptors. In these mice the treatment efficacy of pI:C PEI was strongly reduced. This emphasizes the importance of an intact type I interferon system on the host as the tumor cells were still competent for the receptor.

Taken together, our data suggest that melanoma therapy with cytosolically targeted immuno-stimulatory RNA is more dependent on RNA recognition and functional type I IFN signaling in the tumor microenvironment than direct induction of apoptosis in the tumor cells.

**P191**  
**Autologous T cell stimulatory capacity and cell yields of peripheral blood mononuclear cells for dendritic cell-based immunotherapy differ between cryopreservation protocols**

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Availability of large quantities of functionally effective dendritic cells (DC) represents one of the major challenges for immunotherapeutic trials against infectious and malignant diseases. Insufficient DC numbers or a lack of important functional characteristics may, for the individual patient, result in premature termination of treatment and unsatisfying immune responses in clinical trials.

Recently it was shown that cryopreservation of monocytes with subsequent differentiation into DC is superior to cryopreservation of immature or mature DC in terms of resulting DC quantity and immuno-stimulatory capacity. The aim of our study was to establish an optimized protocol for the cryopreservation of highly-concentrated peripheral blood mononuclear cells (PBMC) for DC-based immunotherapy. Cryopreserved cell preparations were analyzed regarding quantitative recovery, viability, phenotype, and functional properties. Results were compared to DC of the same donor generated without prior cryopreservation.

In contrast to standard isopropyl alcohol (IPA) freezing, PBMC cryopreservation in an automated controlled-rate freezer (CRF) with subsequent thawing and differentiation resulted in significantly higher cell yields of immature and mature DC. Immature DC yields and total protein content after using CRF were comparable to results obtained with freshly prepared PBMC and exceeded results of stan-

dard isopropyl alcohol freezing by approximately 50%. DC generated from CRF-cryopreserved PBMC induced a significantly higher antigen specific IFN- $\gamma$  release from autologous effector T cells when used as stimulator cells in ELISPOT assays as compared to DC from standard freezing procedures. The analysis of further relevant phenotypic or functional characteristics including differentiation-markers, allogenic T cell stimulatory assays, viability tests, and microarray cytokine profiles revealed no significant differences.

In summary, automated controlled rate freezing of highly-concentrated PBMC represents a much improved freezing method capable of increasing DC yields and autologous T cell stimulatory capacity for immunotherapy.

**P192**  
**Therapeutic effect of TNF- $\alpha$  antagonists and fumaric acid esters in psoriasis is associated with increased peripheral and reduced lesional regulatory T cells**

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Psoriasis is considered a T cell dependent chronic inflammatory disease. Agents that block TNF- $\alpha$  may restore the capacity of regulatory T cells (Treg) and increase peripheral Treg count. Recently, we have shown that treatment with the TNF- $\alpha$  antagonists, adalimumab and etanercept, led to an increase of peripheral Treg which was associated with a marked reduction of proinflammatory T cells including Th17 cells in the psoriatic skin lesions. In addition, the suppressor function of the Treg was not altered by anti-TNF treatment. Since fumaric acid esters (FAE) have been shown to be effective in psoriasis through inhibition of TNF-induced gene expression in endothelial cells by a mechanism involving NF- $\kappa$ B, the aim of this investigator initiated study was to find out whether therapeutic efficacy of FAE is associated with a modulation of Treg. We investigated the dynamics of Treg populations in the peripheral blood and lesional skin of patients with moderate to severe plaque-psoriasis ( $n = 16$ ) who received FAE during the entire 28 week observation period. Peripheral blood samples were examined using flow cytometry and skin biopsies were analyzed by immunohistochemical staining. All patients showed a marked clinical response documented by a significant decrease of PASI and DLQI throughout the 28 week observation period. Treatment with FAE led to a significant increase of peripheral Treg within the subpopulation of CD4 $^{+}$  T cells, measured by surface marker expression of CD4, CD25, CD127, which was directly linked to a significant decrease of the proinflammatory skin infiltrate consisting of CD4 $^{+}$ , CD8 $^{+}$ , CLA $^{+}$  T cells and Th17 cells. Noteworthy, CD4 $^{+}$ CD25 $^{+}$ CD127 $^{low}$ - Treg were reduced in psoriatic skin as early as 8 weeks after treatment in all the 16 patients. Thus, we identified two potential effects of treatment with FAE, i.e. (i) restoration of the frequency of Treg cells and (ii) an inhibition of lesional T cell subsets which are presumably critical regulators of skin inflammation. In addition, these findings suggest that both, treatment with FAE and TNF blockers may target disease-modifying Treg.

**P193**  
**Dimethylfumarate protects from autoimmunity by preventing Th1 and Th17 immune responses**

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Experimental autoimmune encephalomyelitis (EAE) is a T helper (Th) cell-mediated inflammatory demyelinating disease of the central nervous system (CNS). In this model of multiple sclerosis CNS inflammation can be induced by active immunization with myelin peptide in complete Freund's adjuvant (CFA) and pertussis toxin or by adoptive transfer of myelin-specific Th cells. The disease-inducing autoreactive Th cells typically show an interferon (IFN- $\gamma$ )-producing Th1 and interleukin (IL)-17-producing Th17 phenotype. IL-4-producing Th2 cells recognizing myelin peptide do not induce EAE in wildtype mice. Thus, immune-modulating therapies may be feasible for this Th1/Th17-mediated autoimmune disease. Since dimethylfumarate (DMF) is effective for the treatment of Th1/Th17-dominated autoimmune disease of skin and joints, namely psoriasis, we asked whether this compound can be effective in EAE. *In vitro*, stimulation of nave CD4 $^{+}$  Th cells with APC and myelin peptide in the presence of DMF induced IL-4-producing Th2 cells. Th cells activated with APC and myelin peptide in the absence of DMF developed a Th1/Th17 phenotype. As expected, adoptive transfer of autoreactive Th1/Th17 cells induced severe EAE, while DMF-treated autoreactive Th2 cells did not induce clinical signs of neuroinflammation after adoptive transfer in nave mice. Next we studied the potential role of DMF on active EAE *in vivo*. Mice were fed with DMF or control water before immunization with myelin peptide in CFA and pertussis toxin. The expression of lineage-defining cytokines was analyzed by quantitative PCR and intracellular cytokine staining. Analysis of draining lymph nodes from DMF-fed mice after active EAE induction showed suppressed mRNA levels of proinflammatory cytokines such as IL-12p35, IL-12/IL-23p40 and IL-23p19 compared to control mice. In addition, in DMF-treated mice we found an inhibition of the expression of IFN- $\gamma$  and IL-17, and their corresponding transcription factors T-bet and ROR $\gamma$ t. In contrast, only mice treated with DMF showed an induction in GATA3 and IL-4 expression compared to control mice. As a consequence, DMF protected mice from developing severe EAE after active immunization with myelin peptide in CFA and pertussis toxin. Only control mice developed a severe course of inflammatory CNS disease. Thus, DMF is a potent immune modulator that protects from autoimmunity by preventing the development of autoreactive Th1 and Th17 cells and instead induces non-pathogenic Th2 cells.

**P194**  
**Diverse roles of curcumin and sodium benzoate on dendritic cells and autoimmunity**

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Food additives like curcumin (diferuloylmethane) and sodium benzoate have both been reported to have anti-inflammatory activities. However, the underlying mechanisms that could explain their anti-inflammatory activity are unclear. Here, we studied the effects of curcumin and sodium benzoate on cell metabolism and immune responses *in vitro* and *in vivo*. First, we analyzed the effects of curcumin and sodium benzoate on dendritic cell (DC) differentiation, maturation and apoptosis. The expression of surface receptors was only minimally affected by the substances, when using concentrations that do not induce apoptosis. Interestingly, we found significant differences in cytokine expression of TLR4 activated DC. The modulation of DC cytokine expression was dependent on the induction of oxidative stress in DC. Treatment with curcumin first induced reactive oxygen species (ROS) in DC and then resulted in a dose-dependent inhibition of the production of IL-12 and IL-23 upon TLR4 activation. In contrast, treatment of DC with sodium benzoate did not alter the ROS level and did not affect IL-12 or IL-23 production. However, IL-10 secretion was inhibited by this antioxidative food additive. Next we analyzed the effects of diets with curcumin or sodium benzoate on the course of T cell-mediated autoimmune disease in mice using a model of multiple sclerosis. During immunization for experimental autoimmune encephalomyelitis (EAE), curcumin protected the mice from disease, while diets with sodium benzoate had no protecting effect. In conclusion, ROS-inducing food additives like curcumin can suppress IL-12 and IL-23 production by DC and inhibit the development of severe T cell-mediated autoimmunity.



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**Heme oxygenase 1 regulates IL-23 transcription through interaction with NFB**

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Fumarates like dimethylfumarate improve the clinical course of autoimmune diseases like psoriasis and multiple sclerosis.

In mice fumarates protect from experimental autoimmune encephalomyelitis. IL-23 produced by dendritic cells (DC) is crucially involved in both of these autoimmune diseases. We have shown that fumarates generate type II DC that promote Th2 cells and suppress Th1 and Th17 cells. Type II DC result from fumarate-induced glutathione-depletion and induction of reactive oxygen species (ROS). Interestingly, fumarates induced transcription and protein expression of the ROS-sensitive heat shock protein HO-1, which is implicated in the regulation of inflammation. To analyze this association we studied the interaction between fumarate-induced HO-1 and IL-23 expression. Fumarate treatment induced HO-1 and inhibited IL-23 expression in DC *in vitro* and *in vivo*. As HO-1 is not a typical transcription factor we determined the direct impact of HO-1 induction on IL-23 production. Therefore we transfected DC with HO-1 siRNA prior to stimulation. HO-1 siRNA prevented HO-1 induction and more importantly, fully restored IL-23p19 expression in fumarate-treated DC. Since HO-1 has been reported to interact with NFKB, which is implicated in the transcriptional regulation of IL-23, we characterized the exact role of this possible interaction. Fumarate-treatment induced translocation of a truncated HO-1 protein into the nucleus. By co-immunoprecipitation we could show that fumarate-induced HO-1 binds to NFKB elements within the IL-23 promoter. This was associated with modifications of the IL-23 promoter locus as shown by decreased histone 3 acetylation after fumarate-treatment. In conclusion, fumarate-induced HO-1 specifically inhibits IL-23 expression and explains its therapeutic activity in Th17-mediated autoimmune diseases.

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**Zytapherese (Adacolumn® Otsuka Pharma GmbH) als neues Therapieverfahren bei Patienten mit einem Lupus erythematodes**

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Der Einsatz der Zytapherese in der Behandlung von Patienten mit chronischen Darmerkrankungen basiert auf dem Grundgedanken, Entzündungszellen, die die Darmschleimhaut infiltrieren, zu reduzieren. Durch die Adsorption aktivierter Granulozyten und Monozyten wird das Immunsystem moduliert, es werden weniger proinflammatorische Zytokine freigesetzt. Andererseits wird verstärkt antiinflammatorisch wirkender Interleukin-1-Rezeptor-Antagonist (IL-1ra) gebildet und die Apoptose von Granulozyten forciert.  
In 2010 konnte durch Decker et al. gezeigt werden, dass bei Patienten mit einem Lupus erythematodes ein gestörter Abbau von Nukleosomen (Reduzierte Aktivität von Komplement C1q +/-o des Enzyms DNaseI) vorliegt. Die Nukleosomen verursachen einen Zelltod der Lymphozyten (LE-Lymphopenie) und aktivieren gleichzeitig neutrophile Granulozyten, als Initiierung der Entzündungskaskade. Aktivierte Granulozyten produzieren viele proinflammatorische Mediatoren, u.a. auch das Interferon- $\alpha$ , dessen bedeutende Rolle in der Entwicklung eines Lupus erythematodes nachgewiesen ist.

Auf Grundlage der überzeugenden Daten aus der Anwendung der Zytapherese bei Patienten mit chronischen Darmerkrankungen und dem wissenschaftlichen Background, dass auch bei einem Lupus erythematodes gerade neutrophile Granulozyten nachweislich eine entscheidende ätiologische Rolle spielen, wurde die Zytapherese bei Patienten mit einer cutanen Manifestation eines Lupus erythematodes als adjuvantes Therapieverfahren eingesetzt. Das Absenken der Neutrophilen und Monozyten konnte eindrucksvoll dargestellt werden, parallel zeigten sich die entzündlichen Veränderungen an der Haut deutlich rückläufig. In den Kontrollen zeigten einige Patienten unter dieser Therapie ein Ansteigen der krankheitsinduzierten reduzierten Lymphozyten. Ob es sich hierbei um eine Umverteilung oder eine tatsächlichen quantitativen Anstieg der Zellzahlen handelt, muss bewiesen werden. Das klinische Gesamtbild der Patienten zeigte sich nachhaltig gebessert, der gebesserte Hautzustand blieb bestehen. Überraschend konnte im Nachbeobachtungszeitraum von 12 Monaten ein Abfall der ANA-Titer beobachtet werden. Die Wertigkeit dieses Titerverlaufs in Korrelation zur Krankheitsaktivität wird diskutiert. Möglicherweise kann die kurzzeitige Unterbrechung der zellulären Unterhaltung der Entzündung (Reduktion von Neutrophilen und Monozyten) als die Kollagenose kausal beeinflussend interpretiert werden.

P197

**Glycolipid antigen as adjuvant for skin immunization against melanoma**

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Glycolipid antigens are currently tested as adjuvant for immunotherapy as they are able to enhance T cell responses after being presented by dendritic cells (DC) to natural killer T cells. We were interested in examining the potential of glycolipid antigen as adjuvant for skin immunization against melanoma. In addition, we investigated if targeting antigen to skin dendritic cells with an antibody would improve T cell responses.

We measured T cell responses after intradermal immunization of mice with the synthetic glycolipid alpha-Galactosylceramide (alpha-GalCer) plus the model antigen Ovalbumin (OVA) conjugated to an antibody against the DC surface molecule DEC-205/CD205. OVA was used together with alpha-GalCer for immunization against murine OVA-expressing B16-melanoma (B16.OVA). The involvement of skin DC in this process was tested by removal of the immunization site and with transgenic mice. Intradermal immunization with alpha-GalCer plus OVA strongly enhanced endogenous CD8+ T cell responses. As a consequence the growth of transplanted B16.OVA melanoma cells was inhibited in mice. Skin DC were not involved since depletion of skin DC did not alter cytotoxic immune responses after immunization. Targeting antigen to skin DC with an antibody against DEC-205 allowed using 1000-times less antigen to obtain similar inhibition of tumor growth. Thus, alpha-GalCer is an useful adjuvant for intradermal immunization strategies and in combination with targeting of antigen to skin DC inhibits tumor growth even with small amounts of antigen.

P198

**A 5 years observational study of immunopathological characteristics and intermolecular epitope spreading in a case of anti-laminin  $\gamma$ 1 pemphigoid**

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Background: Anti-laminin  $\gamma$ 1 pemphigoid (anti-p200 pemphigoid) was first reported in 1996 as a new variant of the pemphigoid spectrum. To date more than 50 cases have been published and recently the autoantigen could be identified as laminin  $\gamma$ 1, which is contributing to the dermal-epidermal adhesion

outside the hemidesmosomes. Diagnosis of anti-laminin  $\gamma$ 1 pemphigoid is based on direct immunofluorescence with linear deposition of *in vivo* bound IgG and C3 along the basement membrane zone and on indirect immunofluorescence on 1 M NaCl-split skin, where IgG-autoantibodies bind to the dermal side of the artificial split. Furthermore can circulating anti-laminin  $\gamma$ 1 IgG antibodies specifically be detected by either western blotting with dermal extracts or a newly developed ELISA, using a recombinant monomeric C-terminal fragment of laminin  $\gamma$ 1.

Methods: We have followed up a patient with anti-laminin  $\gamma$ 1 pemphigoid for 5 years. After diagnosis and successful initiation of an immunosuppressive therapy she experienced a total of three generalised relapses, most likely because she discontinued her long-term therapeutic regime. This gave us the unique opportunity to study our patient's autoantibody profile longitudinally by analysing a total of 10 serum samples, which were taken during that period and stored at  $-20^{\circ}\text{C}$ . Immunopathological findings were then correlated with disease activity.

Results: There was a clear correlation of disease activity with circulating autoantibody concentrations against laminin  $\gamma$ 1 as measured by ELISA and IIF on salt split skin. Furthermore we could nicely demonstrate intermolecular epitope spreading by detecting IgG4 autoantibodies against the  $\alpha$ 3 chain of laminin 332 in the course of the disease. Measurements of autoantibody titres against laminin  $\gamma$ 1 and the  $\alpha$ 3 chain of laminin 332 in ELISA as well as in westernblot studies suggest that both autoantibody fractions contributed separately to disease flares at different time-points.

Discussion: For the first time it could be shown that autoantibody concentrations measured by ELISA parallel disease activity in anti-laminin  $\gamma$ 1 pemphigoid. Epitope spreading is a rare but well known phenomenon in autoimmune bullous diseases. In our case correlation studies suggest that both autoantibodies contributed separately to disease flares. Therefore in cases of recurrent autoimmune blistering diseases, which are difficult to manage, epitope spreading should be considered. Remarkably despite having autoantibodies against the  $\alpha$ 3 chain of laminin 332 – the autoantigen of one type of mucous membrane pemphigoid – our patient never showed any mucosal involvement.

P199

**Production modi of IL-22**

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Psoriasis is a common disease and is generally regarded as a model disease for other chronic immune-mediated disorders like rheumatoid arthritis and Crohn's disease. The latest results from other and our lab strongly suggest that IL-22 plays a crucial role in the induction of the epidermal alterations in diseased skin of psoriasis patients. Currently, it is assumed that Th17-cells are the main producers of this cytokine in humans. By this study, we aimed to further investigate the production modes of IL-22 using different human Th-subsets. Furthermore, we used SCID/beige, Rag1-, IL-2Rgamma-, CD1d-, IL-12p35-, and IL-23p19-deficient mice and psoriasis patient samples to confirm the *in vitro* results. First, we demonstrated that IL-22 can be produced both by T-cells following T-cell receptor (TCR) stimulation and by NKT-cells after activation with inflammatory cytokines with comparable levels. Interestingly, type-I interferons were able to potentially inhibit these productions. Among different human Th-cell subsets, Th22- and Th1-cells produced high amounts of IL-22, whereas the secretion by Th17-cells was very low. The relevance of Th1-cells as an important source of IL-22 in psoriasis lesions was substantiated by significant positive correlation between levels of IL-22 and IFN- $\gamma$ , but not of IL-22 and IL-17A in samples of diseased skin from psoriasis patients. The presence of TNF- $\alpha$ , but not IL-6, was most critical for the generation of IL-22-producing Th22-cells. For IL-22 production after inflammatory cytokine stimulation IL-23, but not IL-12 played an important role. In line with this observation we found strong significant positive correlation between IL-22 and IL-23p19 levels in samples of diseased skin from psoriasis patients. Taken together, our study suggests that in the psoriatic lesions both an antigen-specific activation via TCR and stimulation with inflammatory cytokines induced high IL-22 production and can explain the efficacy of anti-TNF- $\alpha$ - and anti-p40-therapy in psoriasis.

P200

**Novel dendritic cell-based *in vitro* system for the detection of autoreactive CD4+ T cells in pemphigus**

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Background: Pemphigus vulgaris (PV) is an autoimmune blistering disease affecting the skin and mucous membranes characterized by IgG auto-antibodies (auto-ab) against the desmosomal glycoprotein desmoglein 3 (Dsg3). Autoreactive CD4+ T and B cells are considered to be the key players in the autoimmune response finally leading to the production of Dsg3 specific auto-ab. Although Dsg3-reactive CD4+ T cells have been previously identified in PV patients, it remains a technical challenge to define their epitope specificity and to characterize these cells. Dendritic cells (DCs) are the most potent professional antigen presenting cells known to prime T cells. *In vitro* generated BMDCs could be used to identify specific T cell subsets using known antigens.

Aim: The aim of this study was to establish a novel *in vitro* system for the detection and characterization of Dsg3-reactive CD4+ T cells in PV patients using bone marrow derived dendritic cells (BMDCs) from HLA-classII-transgenic mice. These humanized mice carry the PV-associated human HLA-DR0402/DQ8 alleles.

Methods and Results: In order to establish the *in vitro* stimulation system, we first generated dendritic cells (DCs) from the bone marrow of wild type mice (wt) as antigen presenting cells and cultured them with CD4+ T cells from ovalbumin (OVA)-specific TCR-transgenic OT-IIg mice. *In vitro* generated immature dendritic cells were routinely checked for their purity (>97%) by flow cytometry using the DC marker CD11c. Immature BMDCs generated by this method expressed intermediate levels of MHC-II (approximately 78%) and low levels of CD80/CD86 (approximately 69%) co-stimulatory molecules for T-cell activation. For antigen pulsing, immature DCs were incubated with 10  $\mu\text{g}/\text{ml}$  of either the OVA 323–339 peptide or control peptides in the presence of 0.1  $\mu\text{g}/\text{ml}$  of LPS for 16 h. For the BMDC-CD4+ T cell co-culture experiments, antigen-pulsed BMDCs were cultured with CFSE (1  $\mu\text{M}$ )-labelled murine splenocytes from OT-IIg mice for 5 days. We observed 15–20% peptide-specific proliferating-CD4+ T cells by CFSE dilution using flowcytometry. In the next step the same experimental approach was applied to BMDCs derived from the humanized HLA-DR/DQ transgenic mice. These cells were pulsed with Dsg3-peptides or control peptides in the presence of LPS. Sixteen hours later the pulsed DCs were cocultured for 5 days with either PV-patient-derived PBMCs or an isolated PV specific T cell clone. The PBMCs as well as the CD4+ T cells were labelled with CFSE to follow their proliferation. With this experimental setting we could observe a 0.2% Dsg3-peptide specific CD4+ T-cell proliferation. Thus we established a novel dendritic cell based *in vitro* system for the detection of peptide specific CD4+ T cells which allows further characterization of these cells and evaluation of therapeutic interventions.

P201

**Antimicrobially active HPLC-fractions of stratum corneum extracts contain N-terminal profilaggrin-peptides**

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Healthy human skin is markedly resistant towards microbial infection. We hypothesized that keratinocytes derived antimicrobial peptides (AMPs) are produced within the stratum granulosum and subse-

quently stored and/or released within the stratum corneum (SC). To test this hypothesis we previously analysed SC-extracts for antimicrobial activity against *E. coli* and identified, psoriasis (S100A7) as well as RNase-7 (R7) as the principal AMPs in SC extracts. Cation-HPLC- and reversed phase-HPLC-analyses however revealed the presence of additional AMPs in SC extracts. To identify these additional AMPs we focused on very polar, cationic peptides. Electrospray-ionisation-mass spectrometric analyses (ESI-MS) of prominent AMP-activity-containing HPLC fractions revealed the presence of multiple peptides, which, due to similarities in their physicochemical properties could not be separated further. To characterize these peptides the mixtures were digested with trypsin and the resulting tryptic fragments were sequenced by MS/MS. This way we constantly identified peptide fragments corresponding to defined peptides of the Profilaggrin N-terminal region (PFLG 91–100 and PFLG 162–173) in these AMP-activity-containing polar HPLC fractions. Not all peptide masses fitted well with defined unmodified PFLG sequence motifs. Subsequent mass analysis considering potential posttranslational modifications resulted in the identification of putative flaggrin phosphopeptides identified as those with masses 80 Da (or multiples of 80 Da) greater than the predicted unphosphorylated masses. Among them a phosphopeptide containing the phosphorylated Flaggrin Tyr-164, Ser-165 and Thr-167 residues was identified. Because the HPLC-fractions under study contained known antimicrobial peptides, further studies with recombinant peptides are needed to clarify, whether these PFLG-peptides display antimicrobial activity.

#### P202 Progressive skin fibrosis in systemic scleroderma is accompanied by the induction of MMP and cathepsin expression

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Systemic scleroderma (SSc) is characterized by an excessive overproduction and accumulation of collagen and extracellular matrix proteins (ECM), resulting in thickening of the skin and fibrosis of affected internal organs. It is increasingly acknowledged, that not only wound healing and tumor invasion but also the development of fibrosis is accompanied by a continuous tissue turnover and remodelling. For instance, we and others could recently demonstrate that in patients with SSc serum levels of the matrix metalloproteinases MMP-7 and MMP-9 were significantly elevated. As the skin is a potential source of the elevated MMP levels it was the aim of this study to analyze the expression of several MMPs as well as Cathepsins known to be involved in ECM degradation of the skin.

Skin samples were obtained from the forearms of seven SSc patients with progressive skin fibrosis and four healthy controls as frozen skin tissue and skin tissue processed in formalin and embedded in paraffin. A novel protocol was developed to be able to isolate RNA from paraffin embedded material using consecutive 25 µm sections. Thereafter mRNA expression levels in skin biopsies were determined using semi-quantitative PCR for α(I)collagen, MMP-2, MMP-7, MMP-9 and Cathepsins (B, L, K) using S26 as a control. Integrity of isolated RNA by the novel protocol was demonstrated by ethidium bromide stained agarose gels. In all SSc biopsies induction of type I collagen expression was found when compared to matched controls. The expression of MMP-7, and MMP-9 were markedly increased in skin biopsies (frozen, formalin stored and paraffin embedded) from all patients with SSc compared to healthy controls. In contrast, MMP-2 mRNA levels were unchanged in comparison to healthy controls. In addition, mRNA expression of the acidic cathepsins B, L, K was elevated to varying degrees in SSc skin.

We established a novel protocol to isolate and analyze RNA directly from paraffin embedded skin tissue. Our results demonstrate that MMP-7 and MMP-9 as well as Cathepsin-B, -K and -L are involved in the process of fibrotic tissue remodeling of SSc. These data further support the idea that the fibrotic process in SSc is characterized by a complex dysbalance of both synthetic and degradative processes of connective tissue.

#### P203 Slan-dendritic cells are a major population of human dermal dendritic cells in healthy skin and psoriasis

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The skin immune system bears a network of resident dendritic cells (DCs) consisting of epidermal Langerhans cells and different subsets of dermal DCs, which are not well defined. We recently reported on a new population of dermal DCs, called slan (6-sulfoLacNAc+) DCs, which have a potent capacity to stimulate Th17/Th1 T cell responses.

Staining healthy skin for DCs revealed that slanDCs (CD1a+, CD1c-, CD11c-, CD14-, CD163-) can be found in healthy human dermis at a similar frequency compared to CD1c+, CD11c+, CD1a+, CD14-, CD163- DCs that were previously regarded as the major population of resident DCs. In the prototypic inflammatory disease psoriasis the frequency of slanDCs and CD1c+ DC doubled and slanDCs now expressed CD11c. In depth analysis of DCs in psoriatic dermis by four color immunofluorescence analysis revealed that in addition to slanDCs and CD1c+ DCs the pool of CD11c+ cells can be further subdivided into CD11c+ CD14+ CD163- DCs and CD11c+ CD163+ CD14+ macrophages. In conclusion, we provide evidence that slanDCs, initially described as large population of proinflammatory DCs in blood, are a novel major part of the resident dermal myeloid DC-network in healthy skin and inflammation.

#### P204 Mast cells are key promoters of contact allergy that mediate the adjuvant effects of haptens

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A prominent feature of sensitizing environmental compounds that cause allergic contact dermatitis is the rapid induction of an innate inflammatory response that seems to provide danger signals for efficient T cell priming. Using novel mouse models of mast cell-deficiency, mast cell-specific gene inactivation and intravital imaging in mast cell-reporter mice, we show that these adjuvant effects of contact allergens are mediated by mast cells and histamine. Mast cell-deficiency resulted in impaired emigration of skin DCs to the lymph node and contact hypersensitivity was dramatically reduced in the absence of mast cells. In addition, mast cell-specific inactivation of the IL-10 gene did not reveal any role for mast cell-derived IL-10 in the regulation of contact allergy. Collectively, we demonstrate that mast cells are essential promoters of contact hypersensitivity highlighting their potential to promote immune responses to antigens entering via the skin.

#### P205

##### Paraoxonase 2 (PON2) functions as a quorum sensing quenching factor in human keratinocytes

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Expression of several bacterial virulence factors is controlled by quorum sensing, a bacterial cell-to-cell signaling system. Quorum sensing is mediated by the production of autoinducers, small molecules that activate or repress bacterial gene expression upon reaching a certain minimal threshold concentration. Inactivation of autoinducers reflects a host strategy to interfere with bacterial communication. We could show that human keratinocytes express paraoxonase 2 (PON2), a lactonase which is able to degrade the important autoinducer *N*-(3-oxododecanoyl)-L-homoserine lactone (3OC12-HSL), one of the major autoinducers produced by Gram-negative bacteria. To assess the functional relevance of PON2 expression in keratinocytes we incubated primary keratinocytes with 3OC12-HSL. After 3 h incubation the presence of 3OC12-HSL was analyzed by the use of the biosensor strain *Pseudomonas putida* F117 (pAS-C8) which expresses GFP in the presence of 3OC12-HSL. This revealed that keratinocytes are able to inactivate 3OC12-HSL. Inactivation of 3OC12-HSL requires contact with the keratinocytes indicating that a cell-associated activity rather than a secreted factor inactivates 3OC12-HSL. To analyze whether PON2 contributes to the capability of keratinocytes to inactivate 3OC12-HSL we used PON2 specific siRNA to downregulate expression of PON2. Keratinocytes transfected with two different PON2 siRNAs showed a significant decrease in their capacity to inactivate 3OC12-HSL as compared to the cells transfected with a control siRNA. In addition, PON2 downregulation led to increased attachment of *P. aeruginosa* to the keratinocytes. These data indicate that keratinocytes are able to inactivate the bacterial autoinducer 3OC12-HSL and that this activity is in part mediated by PON2. Thus, PON2 acts as a quorum sensing quenching factor in keratinocytes and may play an important role in cutaneous defense against bacterial infections.

#### P206

##### Lack of CD14 contributes to the failure of *P. aeruginosa*-derived LPS to efficiently induce human beta-defensin-2 (hBD-2) expression in keratinocytes

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Human skin is able to produce several antimicrobial peptides to control cutaneous growth of microorganisms and to protect itself against infection. Human beta-defensin-2 (hBD-2) is an important skin-derived antimicrobial peptide and low concentrations of hBD-2 are able to rapidly kill bacteria and fungi. The expression of hBD-2 in keratinocytes can be induced by proinflammatory mediators and upon contact with bacteria or bacterial products. The Gram-negative bacterium *Pseudomonas aeruginosa* is a strong inducer of hBD-2 expression in keratinocytes. Since the role of lipopolysaccharide (LPS) to activate the innate immune response of human keratinocytes is still emerging and controversial we investigated the capacity of LPS derived from *P. aeruginosa* to induce the expression of hBD-2 in HaCaT and primary keratinocytes. Only very high, supraphysiological concentrations (1–10 microgram) of LPS dose-dependently induced hBD-2 gene expression in keratinocytes. However, the addition of human serum markedly increased the capacity of LPS to induce hBD-2 expression in keratinocytes. Since it is known that CD14 is an essential mediator for LPS-signalling we hypothesized that serum-derived CD14 may contribute to the pronounced LPS-mediated hBD-2 induction upon addition of serum. Indeed, treatment of the cells with a CD14-blocking antibody decreased the LPS-mediated induction of hBD-2 in keratinocytes cultured in serum-containing medium. In summary, our data indicate that LPS alone is not able to efficiently induce the expression of hBD-2 in keratinocytes. The lack of sufficient CD14 amounts may be one reason for this hyporesponsiveness towards LPS.

#### P207

##### Prospective comparison of a specially composed biochip mosaic with the conventional multistep procedure in the serological diagnosis of autoimmune bullous diseases

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Although direct immunofluorescence microscopy (IFM) is still the gold standard for the diagnosis of autoimmune bullous diseases in the great majority of patients, today serological diagnosis is sufficient in most cases. Here, a BIOCHIP mosaic was designed for indirect IFM that allows simultaneous testing for autoantibody reactivity against a broad spectrum of relevant substrates and target antigens, including primate salt-split skin, monkey esophagus, tetrameric BP180-NC16A, and human HEK293 cells transfected either with the ectodomains of desmoglein 1 and 3, or the C-globular terminus of BP230. The biochip mosaic was probed with sera from well characterized patients with pemphigus vulgaris (PV, *n* = 65), pemphigus foliaceus (PF, *n* = 50), bullous pemphigoid (*n* = 55), non-inflammatory skin diseases (*n* = 97), and healthy blood donors (*n* = 154). While using PF, PV, and BP sera, sensitivities of the desmoglein 1-, desmoglein 3-, and NC16A-specific substrates were 100%, respectively, BP230 was recognized by 44% of the BP sera. In all cases, specificities were >98%.

Results were compared with those of the diagnostic standard procedure, including conventional IFM, immunoblotting and ELISA, using various tissue substrates as well as cell-derived and recombinant target antigens. This prospective study included 454 consecutive sera that had been sent to the dermatology autoimmune laboratory with suspicion of an immunobullous disorder. Biochip mosaics and standard procedure provided similar results in the diagnosis of BP (*n* = 77), PV (*n* = 12), and PF (*n* = 4). In 326 sera (71.8%), no autoantibody reactivity was found. In 22 sera (4%), the standard multistep procedure resulted in the diagnosis of anti-p200-pemphigoid (*n* = 5), anti-laminin 332 mucous membrane pemphigoid (*n* = 6), dermatitis herpetiformis (*n* = 2), linear IgA disease (*n* = 6), and pemphigoid gestationis (*n* = 3). These diagnoses could not be exactly specified with the biochip mosaic, which did not (yet) contain the corresponding antigen substrates. In additional 25 (5.5%) sera, biochip reactivity was observed while the standard multistep approach was negative.

The biochip technology facilitates the serological diagnosis of BP and pemphigus, incubations are highly standardized and easy to conduct, and the IFM tests are highly sensitive and specific. The novel biochip mosaic will be enhanced by adding further target antigens to allow the diagnosis of the entire spectrum of autoimmune bullous diseases.

#### P208

##### CD40L induces a vitamin D-dependent antimicrobial pathway in human macrophages

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The vitamin D-dependent induction of cathelicidin and human beta-defensin 2 (DEFB4) antimicrobial peptides in human keratinocytes, monocytes and macrophages has been described as an important host defense mechanism against microbial pathogens. T cells induce cathelicidin and DEFB4 antimicrobial peptides in human monocytes and macrophages via secretion of the Th1 cytokine IFN- $\gamma$ . However, it is unclear if T cell-mediated contact-dependent mechanisms contribute to host defense by inducing vitamin D-dependent antimicrobial activity. Here, we show that activation of human macrophages via CD40L, which is expressed on T cells, alone and in combination with IFN- $\gamma$  induces the CYP27B1-hydroxylase, responsible for the conversion of 25-hydroxyvitamin D (25D) to the bioactive 1,25-dihydroxyvitamin D (1,25D), and the generation of cathelicidin and DEFB4 antimicrobial peptides. Finally, CD40L-mediated activation of human macrophages cultured in 25D sufficient human serum resulted in antimicrobial activity against intracellular *M. tuberculosis* infection. In summary, our data suggest that contact-dependent and contact-independent T cell-mediated mechanisms provide protection against intracellular infection in human macrophages that is dependent on 25D.

## P209

### Old mice accumulate phenotypically abnormal T cells associated with distinct immune dysfunctions of immunosenescence

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Aging of the immune system (immunosenescence) is characterized by a functional decline leading to an onward immunodeficiency. But regulatory mechanisms also decline resulting in an inefficient or even harmful over-activation of the immune response. This in turn increases the risk for diseases such as autoimmunity and chronic inflammation. Particularly, a senescent T cell repertoire, phenotype and function deeply impact immunity in the elderly. Although certain features of T cells in the aged have been determined, the functional role of age-dependently accumulating, phenotypically suspicious and, thereby, potentially harmful T cells remains unclear. Similar T cell subsets seem to occur also in immunodeficient individuals and systemic chronic inflammation. Recent reports indicate that imbalanced levels of reactive oxygen species (ROS) contribute critically to driving chronic inflammation and, potentially, also immunosenescence in an ambiguous fashion, especially in T cells.

To study the effects of aging on T cell differentiation in mice, we here sought to identify distinct aged-related T cell populations in peripheral lymphoid organs of young, adult and old mice (3–6, 9–12 and 18–24 months-old C57BL/6). Therefore, high-throughput six-channel fluorescence FACS was performed to allow a precise phenotypic characterization combining panels of markers that were suggested to be altered on age-related and/or dysfunctional T cells (such as CD5, CD27, CD28, CD45RB, CD71, CD127, p-Gp, V-beta chain repertoire) in a first step.

We found a marked age-dependent shift in the frequency of CD4+ T cells towards unconventional CD4–CD8– DN T cells in murine lymph nodes while the frequency of CD8+ T cells was not changed. However, CD8+ T cells showed a pronounced down-regulation of both CD27 and CD28 co-stimulatory receptors, which strongly suggests a state of anergy. Also CD5 was reduced on these cells indicating homeostatic expansion, which typically occurs due to a reduced thymic T cell output in age. Interestingly, the here identified unconventional CD4–CD8– DN T cells phenotypically resemble a peripheral T cell subset previously shown to accumulate rapidly in lymph nodes of a mouse model of leukocyte-adhesion deficiency syndrome 1 (LAD1), a primary immunodeficiency syndrome.

Our data reveal a substantial increase of distinct T cell subsets in a highly detailed phenotypic profile showing strong characteristics of dysfunction in old mice. It offers the opportunity for a FACS-based isolation for further functional analyses as well as investigation of ROS biology in these subsets, which is currently undertaken. This may help clarifying important aspects of an age-associated dysfunction of the immune system and also give insight into pathomechanisms that unite or separate immunosenescence from exhaustion by chronic inflammation and/or immunodeficiency.

## P210

### Analysis of single nucleotide polymorphisms in the IL7R and IL7 gene in patients with systemic sclerosis

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Systemic sclerosis (SSc) is characterized by vascular damage, autoimmunity and fibrosis and especially the interplay between these processes is likely to be pivotal to pathogenesis of SSc. Clinical heterogeneity is a hallmark of SSc and it is likely that this is determined at least in part by genetic factors. Several genome-wide and/or candidate gene studies have already implicated multiple genetic factors, increasing the risk of SSc susceptibility. In several other rheumatic diseases IL7 and its receptor plays an important role in immune response and regulation and single nucleotide polymorphisms (SNPs) in the gene coding interleukin-7 receptor (IL7R) have been reported to be associated with T cell activation and immunopathogenesis, but no detailed studies of genetic alterations of IL7 and its receptor (IL7R) in SSc have been undertaken. SSc represents a prototypic, chronic, non-relapsing, progressive autoimmune disease; we investigate whether genetic alterations and differences in IL7 serum levels also exist in SSc patients that may associate with clinical phenotypes.

In this cohort study we have focused on candidate SNPs of the IL7 receptor (IL7R) and IL7, showing an association in other rheumatic diseases, in patients with SSc in comparison to healthy controls and determines the association with specific clinical and serological characteristics.

Patients with SSc ( $n = 728$ ) and healthy controls ( $n = 260$ ) were genotyped for 15 SNPs in the gene region of IL7R (rs11567685, rs11567714, rs11567751, rs11567761, rs11567762, rs1353251, rs2292232, rs3194051, rs41270321, rs6891095, rs6893142, rs6897932, rs7718919, rs987106 and rs987107) and seven polymorphisms in the region of IL7 (rs112311844, rs16906063, rs2717548, rs2919935, rs7828417, rs78570944 and rs894221). All patients and controls were UK Caucasian and we grouped our SSc patients according to the autoantibody status and organ involvement. Genotyping was performed by the KASPar system (allele specific PCR; K Biosciences, UK).

The statistical analysis was performed using logistic regression analysis to compare the distribution of IL7R/IL7 polymorphisms. IL7-serum levels of SSc patients were compared to healthy controls by using a commercial enzyme immunoassay kit (Quantikine; R&D Systems).

We could find a significant difference between SSc patients being positive versus negative for anti-topoisomerase I antibodies (ATA) in four SNPs located in the IL7R region, rs11567685 ( $P = 0.0075$ , odds ratio [OR] for CC genotype 1.469, 95% confidence interval [CI] 1.11–1.95), rs11567751 ( $P = 0.007$ , OR for TT 1.467, 95% CI 1.11–1.94), rs987107 ( $P = 0.0081$ , OR for TT 1.456, 95% CI 1.10–1.92) and rs3194051 ( $P = 0.0072$ , OR for GG 1.466, 95% CI 1.11–1.94). Significant increased median serum IL7 levels were found in patients with diffuse SSc compared to patients suffering from the limited form of SSc.

Here we report that homozygous carriers of the minor allele in four SNPs of the IL7R gene region were significantly stronger associated with a positive anti-topoisomerase antibody status in SSc patients. This gene has been described to be associated with immune regulation in other autoimmune diseases opening a possibility of a common autoimmune genetic pathway. This is the first study which reports a potential association of IL7R gene in SSc susceptibility. Additional independent cohorts

should be analyzed to confirm our findings. In addition it is possible that IL-7 expression of signalling may prove a useful candidate biomarker in SSc classification.

## P211 (V35)

### Impaired wound healing in CD18 deficient mice is due to reduced activation of the phagocyte NADPH oxidase NOX2 in wound macrophages

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Mutations in the gene encoding the common beta-chain (CD18) of the beta2 integrins family of adhesion molecules, results in severe wound healing disturbances in humans EUR<sup>1</sup> patients suffering from Leukocyte Adhesion Deficiency Syndrome type 1 (LAD1) EUR<sup>1</sup> and the CD18 deficient murine LAD1. Previously we showed that this defect is due to the impaired formation of the phagocytic synapse between apoptotic neutrophils (PMN) and macrophages leading to impaired oxidative burst and reduced release of active TGF-beta1 at wound sites. So far we could identify the guanine nucleotide exchange factor Vav3 and the GTPase Rac2 as downstream targets of beta2 integrins which are activated in macrophages upon phagocytosis of apoptotic PMN and control TGF-beta1 activation. However, active GTP-Rac2 is an essential component of the phagocyte NADPH oxidase NOX2 which is required to kill engulfed cells and bacteria by secretion of high concentrations of reactive oxygen species.

Here we set out to investigate whether the impaired phagocytic activation of NOX2 in macrophages with the subsequent insufficient formation of oxygen radicals drive the reduced release of active TGF-beta1 at the wound site and the impaired wound healing in CD18 deficiency.

First, we found that the strong release of active TGF-beta1 in co-cultures of wildtype macrophages with apoptotic neutrophils was significantly reduced by the co-incubation with scavengers of oxygen radicals superoxide dismutase (SOD) and catalase. Furthermore, in a full thickness excisional wound healing model we could show that targeting the wound macrophages by injection of the superoxide anion scavenger SOD or the hydrogen peroxide scavenger Ebselen encapsulated in polyelectrolyte capsules around the wounds significantly delayed the wound healing of wildtype mice when compared with mice injected with empty capsules, confirming that the release of reactive oxygen species (ROS) by macrophages is required for TGF-beta1 activation *in vitro* and *in vivo*.

By contrast, injection of the superoxide anion inducer Rotenone around the wound margins of CD18-/- mice virtually rescued their wound healing defect to wildtype levels, suggesting that a reduced oxidative burst causally impairs wound healing in CD18 deficiency.

To specifically assess the role of NOX2 in oxidative TGF-beta activation by macrophages we then analyzed the activation of NOX2 in wildtype and CD18-/- macrophages during phagocytosis of apoptotic PMN using a novel imaging method based on the fluorescence lifetime of NADPH (FLIM). We found that NOX2 was strongly activated in wildtype macrophages upon phagocytosis of wildtype or CD18-/- apoptotic PMN, whereas the assembly of NOX2 in CD18-/- macrophages under the same conditions was remarkably reduced. Importantly, similar to CD18-/- mice, NOX2-/- mice presented with severely delayed healing of full-thickness wounds when compared to wildtype control mice. Moreover, injection of wildtype, but not of CD18-/- macrophages in wound margins of NOX2-/- mice completely significantly restored the impaired wound healing of NOX2-/- mice, suggesting NOX2 to be activated downstream of CD18 in wound macrophages.

Modulation of oxidative burst and of active TGF-beta1 release in macrophages by targeting NOX2 may prove promising to modulate macrophage functions in macrophage-dependent inflammatory disorders.

## P212 (V07)

### Mesenchymal stem cells improve wound healing and reduce scar formation in a murine full-thickness excisional wound model via the release of TNF-stimulated gene 6

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Evolution has optimized adult wound healing for fast closure of wounds with a prominent inflammatory phase to counteract detrimental bacterial infection at the price of scar formation. In contrast, fetal wound healing – in the absence of inflammation – proceeds scarless. The inflammatory response of macrophages has been suggested to causally contribute to scar formation in adult wound healing and fibrosis in a variety of disorders. We here set out to address the questions whether (i) local delivery of mesenchymal stem cells (MSCs) to acute wounds may control macrophage activation and scar formation during tissue repair and if so (ii) to identify the underlying mechanisms and key molecules (iii) which longterm may hold considerable promise to improve tissue repair and scar formation *in vivo* in translational regenerative medicine. In a first attempt to address these questions, using *in vitro* co-cultures of bone marrow-derived MSC with activated macrophages (LPS and IFN $\gamma$ ), we observed a significantly diminished release of pro-inflammatory TNF $\alpha$  and IL-12, and increased release of anti-inflammatory IL-10. By means of real time PCR and Western Blot analysis a marked increase in the synthesis and release of TNF-stimulated gene 6 (TSG-6) from MSC was observed. TSG-6 was identified to be responsible for this anti-inflammatory effect on macrophage activation as its silencing in MSCs via TSG-6 targeted siRNA completely abrogated the anti-inflammatory response. Complementary to these *in vitro* data, an increase in TSG-6 both on mRNA and protein levels was detected following MSC injection around murine full thickness wounds compared to almost no TSG-6 in PBS-injected wounds of C57BL/6 mice. In addition, the increase in TSG-6 in MSC injected wounds correlated with reduced macrophage activation in the wound margins as assessed by reduced TNF $\alpha$  expression at day 3 after wounding. Interestingly, under the same conditions accelerated wound healing and reduced scarring was detected by quantitative assessment of wound areas, the organization of collagen bundles and the depths of scars at different time points following wounding. These favorable effects were completely abrogated when TSG-6 silenced MSC were injected around wounds, while MSC transfected with non targeted scrambled siRNA maintained their anti-inflammatory, anti-scarring properties and accelerated murine wound healing. Similar favorable effects were observed when rhTSG-6 was directly injected in the wound margins. Notably, concentrations of the pro-fibrotic active Transforming Growth Factor- $\beta$ 1 (TGF $\beta$ 1) were reduced in both MSCs and rhTSG-6 treated wounds when compared to PBS-injected control wounds at day 3 and 5 after wounding. By contrast, active TGF $\beta$ 3, the TGF $\beta$  family member which has previously been shown to reduce scar formation was significantly up-regulated on protein level following injection of either MSCs or recombinant TSG-6 into wound margins. Taken together, these data very much suggest that TSG-6 released by MSCs improve wound healing by dampening macrophage activation and at the same time reduce scar formation by increasing the relative ratio of TGF $\beta$ 3 to TGF $\beta$ 1. We here identified a master molecule which – upon delivery either by cell-based therapy or as a recombinant protein – may be particularly useful in the treatment of acute wounds in dermatology and regenerative medicine.

P213

### Tumor-derived myeloid-derived suppressor cells change immune response in experimental leishmaniasis

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Recent studies have described the so-called myeloid-derived suppressor cells (MDSC) in various tumors. They suppress anti-tumor CD8<sup>+</sup> T cell responses and represent a newly detected important immune escape mechanism of tumors. In mice they are concentrated among those phagocytes which express CD11b, Gr-1 and IL-4 receptor alpha (CD124).

We demonstrated the presence of MDSC in a murine tumor model of B16 melanoma. B16-induced MDSC express MHCII molecules on their surface, thus they are able to present antigens, but they down-regulate antigen-specific CD4<sup>+</sup> T cell responses.

We now investigated if the presence of tumor-derived MDSC not only down-regulates anti-tumor immune responses, but also immune responses directed against infectious agents. Therefore we transferred MDSC from melanoma-bearing mice (C57/Bl6) or control monocytes from healthy mice into C57/Bl6 mice that had been infected with *Leishmania major* (*L. major*). Subsequently, those C57/Bl6 mice which had received MDSC showed a higher parasite load than infected C57/Bl6 mice which had received control monocytes. To further analyse their immune response we performed *in vitro* syngeneic T cell stimulation and found that splenic CD4<sup>+</sup> T cells from mice that received MDSC showed a significant weaker proliferative response to *L. major* antigen presented by dendritic cells. These T cells also showed a Th2 profile (down-regulation of IFN $\gamma$ , up-regulation of IL-13) in comparison to T cell reactions from mice that had received control monocytes (Th1 profile). This phenomenon is critical since an effective immune reaction against *L. major* depends on a Th1 T cell response.

Our data show that the existence of MDSC might be a critical mechanism of immunosuppression in tumor patients hampering their immune response against concomitant infections.

P214

### IFN- $\alpha$ treatment induces TRAIL expression on NK cells and myeloid cells present in the circulation of melanoma patients

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Recombinant IFN- $\alpha$  is the only adjuvant treatment for stage II/III melanoma patients that was shown to increase disease-free and, when used at high doses, also overall survival. The effect of IFN- $\alpha$  is attributed to its direct antiproliferative and cytotoxic activity on melanoma cells and its indirect immunostimulating properties. Its exact mode of action, however, is not clear. We and others have previously shown that IFN- $\alpha$  induces the upregulation of the cytotoxic molecule TNF-related apoptosis-inducing ligand (TRAIL) on immune cells, which both in its membrane-bound and soluble form lyses tumor targets *in vitro*. In melanoma patients undergoing IFN- $\alpha$  treatment we now observed that TRAIL is significantly upregulated on peripheral blood NK cells, monocytes and, in some cases, also on myeloid dendritic cells (DCs), as assessed by flow cytometry. These changes were paralleled by an increase of soluble TRAIL in the serum (ELISA). Phenotypic analysis showed that plasmacytoid DCs of melanoma patients express higher levels of the activation markers CD40 and CD86 when compared to healthy controls. Myeloid DCs, in contrast, exhibit elevated levels of CD62L, CCR6 and CXCR4, and a down-regulation of CCR7, compared to healthy controls. These changes were even more pronounced during IFN- $\alpha$  therapy, albeit not at statistically significant levels, and were consistent with phenotypic properties of DCs endowed with the ability to migrate into (tumor) tissue. Indeed, we observed a significant reduction in the relative frequency of myeloid DCs, but not plasmacytoid DCs, in the peripheral blood of patients receiving IFN- $\alpha$  when compared to numbers obtained before treatment. Our findings demonstrate that IFN- $\alpha$  treatment induces the expression of TRAIL on immune cells present in the circulation of melanoma patients. This effect may contribute to the anti-melanoma activity of adjuvant IFN- $\alpha$ -treatment in high-risk melanoma patients.

P215

### CD11c+ inflammatory dendritic cells and CD163+ macrophages are the main source of TNF-alpha in chronic plaque-type psoriasis

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The spectrum of tumor necrosis factor (TNF)-alpha-producing cells as well as their fate during treatment with TNF-alpha-antagonists is not clearly defined in psoriasis. The elucidation of these questions should allow us to better understand the mode of action, efficacy and, perhaps, also the risks of an anti-psoriatic treatment with TNF-alpha-blockers.

Using conventional immunofluorescence methods, we were not able to reproducibly detect TNF-alpha in sections of lesional psoriatic skin, but by the application of a tyramide signal amplification system, which allows the detection of even trace amounts of antigen, we obtained reproducible and firm stainings.

TNF-alpha was exclusively found on dermal leukocytes co-expressing CD11c and HLA-DR and, to a much lesser extent, CD163. This marker profile is consistent with that of mDCs and macrophages.

In addition, we found corresponding populations of TNF-alpha-producing mDCs and monocytes in PBMCs of psoriatic patients. TNF-alpha + mDCs of the peripheral blood were of the 6sulfu LacNAc (slan) rather than the BDCA-1 or BDCA-3 subtype, and their number closely correlated with disease activity. Furthermore, the total amount of PBMC-derived slanDCs was increased in psoriatic patients compared to healthy controls with a concomitant decrease of BDCA-1 + DCs. Isolated slanDCs produced high amounts of TNF-alpha, IL-1beta, IL-6, IL-12 and IL-23 upon stimulation with LPS or peptidoglycan. We could not find detectable levels of these cytokines in BDCA-1 + DCs. However, they produced high amounts of IL-10.

Early after *in vivo*-infusion of the TNF-alpha-antagonist infliximab in psoriatic patients, we were not able to detect complement killing or apoptosis of TNF-alpha-producing cells in lesional skin. However, we observed a rapid decrease of key proinflammatory molecules (IL-12p40, IL-1beta, CCL20) in psoriatic lesions within hours after initiation of therapy. Strikingly, the decrease of these molecules might not only reflect the impact of TNF-alpha on epidermal cells which are the source of e.g. CCL20. Infliximab is likely to interfere with an autocrine effect of TNF-alpha on inflammatory mDCs and macrophages, as *in vitro* blockade of TNF-alpha strongly inhibits production of IL-12 as well as IL-23 in these cells. These effects are boosted by a concomitant decrease of IL12RB1 (CD212), the common subset of the IL-12- as well as the IL-23-receptor, in lesional psoriatic skin.

Our data strongly suggest that certain myeloid cells (slanDCs, monocytes/macrophages) are the main source of TNF-alpha and other proinflammatory molecules in chronic plaque-type psoriasis and therefore serve as a key target of TNF-alpha-antagonists.

P216

### Exploring the feasibility of future combinatorial approaches of chemotherapy with immunotherapy for melanoma – influence of chemotherapeutic drugs on the functions of immune cells

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Classical cytotoxic anticancer agents and also the new class of more specific kinase inhibitors are able to shrink large tumor masses in only a short time in many patients, however often success is not durable. Immunotherapy has shown long-time responses though time to show those effects is long. Neither of these two types of treatment by itself has been sufficient to cure cancer, but, combining chemotherapy with immunotherapy may allow improving therapeutic results and expanding their durability.

The effects of different chemotherapeutic agents on immune cells were studied at different timepoints *ex vivo* in melanoma patients currently under chemotherapeutic treatment and in cells of healthy donors after *in vitro* preincubation. The peripheral blood mononuclear cells (PBMC) were exposed to different chemotherapeutic agents then the cell viability and phenotype were analyzed by eight-color flow cytometry. Effects of the drugs on antigen-specific T-cell function were analyzed by specific cytokine release, proliferation and cytotoxicity activity using two-color fluorospot (fluorescent ELISpot), multiplex cytokine-bead array and multifunctional T cell assay (MFTC).

At concentrations up to twofold of the plasma level found in patients, most chemotherapeutic agents showed only marginal effects on lymphocyte viability and T cell effector functions in most cases. However, differential kinetics and effects on preexistent memory T cell responses, priming of naive T cells or release of suppressed T cells by therapeutic antibodies show the requirement of further finetuning of treatment schedules.

P217

### Kinetics of antibodies and clinical response following immune adsorption (TheraSorbTM-Ig-flex<sup>®</sup>) of patients with severe atopic dermatitis

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**Introduction:** Severe Atopic Dermatitis (AD) often leads to the need for systemic immunosuppressive therapy over years with concomitant drug related relevant adverse effects. Since adsorber columns allowing strong reduction of immunoglobulins (Ig) in the plasma are available, immune apheresis (IA) removing plasma components with pathophysiological relevance are proposed as new therapeutic option in the treatment of i.e. autoimmune diseases. Zillikens et al. were able to demonstrate clinical and histological efficacy by removing IgE in AD patients by short term IA with recrudescence of IgE-levels after therapy. Therefore we tested the suitability of long term IA at patients with severe AD and correlated the IgE-levels with clinical symptoms. Another aim was to answer the question whether an absolute level or the relative decrease (%) of IgE is responsible for clinical benefit of the Ig-apheresis. **Methods:** Six patients with severe AD with IgE > 750 IU/ml, age between 18 and 80 year. Every patient underwent >1 series of immune adsorption over 5 day (TheraSorbTM-Ig-flex<sup>®</sup>; Miltenyi-Biotec, Bergisch-Gladbach, Germany) with intervals of 4 weeks between the series. Daily, plasma IgE were measured by ELISA (IU/ml) before and after IA and the levels correlated with SCORAD. **Results:** IA was clinical beneficial for all patients with mean total-SCORAD lowering to 40% (mean SCORAD lowering per series 16%). Prolongation of IA (>2 series) was associated with further SCORAD improvement.

After at least three accomplished (=3 x 5 day) series ongoing clinical benefit despite recrudescing IgE-levels (no systemic corticoids + saving of local ointments) was observed. The data show that relative IgE reduction and not an absolute IgE-level seems responsible for clinical benefit. **Conclusion:** IA seems suitable for severe AD and can avoid systemic immunosuppressive and reduce local medication. The therapy is well tolerated by the patients and at least three up to five series proved efficacy and may become a therapeutic option in the treatment of severe AD.

P218

### IL-24 expression during early wound healing phase is depended on an inflammatory environment

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Cutaneous wound healing is characterized by a precisely regulated action of different growth factors and cytokines. Its disturbance can lead to an impaired healing response, which represents a great medical problem. We demonstrated previously, that the novel IL-10 cytokine family members IL-22, IL-20, and IL-24, play a major role in psoriasis and skin homeostasis. By using an *in vivo* mouse model we demonstrated, that IL-20 was constitutively expressed, whereas no IL-22 expression was observed. In contrast, IL-24 was massively upregulated upon wounding. Immunohistochemistry revealed keratinocytes and CD3-positive cells as the main source of IL-24 in the early wound healing phase. By *in vitro* experiments we could show, that among different T-cell populations, Th17-cells are the most potent IL-24 producers. Thereby, Th17-cells rely on the presence of TGF- $\beta$  and IL-1 $\beta$  to upregulate IL-24. Additionally, primary human keratinocytes secrete IL-24. The IL-24 expression level was elevated after 24 h stimulation with IL-4, IL-1 $\beta$ , IL-6, TNF- $\alpha$  and TGF- $\beta$ . We next investigated the expression of these mediators in non injured mouse skin as well as wound biopsies at indicated time points upon wounding. T-cell derived cytokines were hardly induced in early wound healing phase. TGF- $\beta$  was constitutively expressed throughout the healing response. IL-1 $\beta$ , IL-6 and TNF- $\alpha$  were highly upregulated during the inflammatory phase and showed a similar expression kinetic as IL-24, which emphasizes a potential role for IL-24 upregulation. Interestingly, among these cytokines, IL-24 along with IL-1 $\beta$  were the highly expressed mediators relative to unwounded skin during the inflammatory phase.

P219

### Susceptibility of *Staphylococcus aureus* sepsis strains to different epithelial antimicrobial proteins

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*Staphylococcus aureus* is a major human pathogen and the most important cause of life-threatening bacterial infections like bacteremia and sepsis. These infections are often caused by *S. aureus* derived from the own epithelial microflora. Antimicrobial proteins (AMP) such as human beta-defensin-3 (hBD-3) and RNase 7 protect the skin and other epithelia against infections and are highly active against staphylococci. The aim of this study was to determine whether *S. aureus* strains from sepsis patients are less susceptible against various antimicrobial proteins than strains derived from age and sex matched *S. aureus* colonizers (control strains).

*S. aureus* strains derived from sepsis patients ( $n = 14$ ) as well as control strains were tested in parallel for their susceptibility to different AMP (hBD-3, RNase 7, psoriasis, lysozyme) by agar diffusion assay and microdilution assay. In only two cases, the sepsis strains were less susceptible to hBD-3, RNase 7 and psoriasis compared to the *S. aureus* strains derived from *S. aureus* colonizers. In the remaining cases, the *S. aureus* strains from sepsis patients showed equal or even more susceptibility to the tested AMP.

In summary, our data indicate that *S. aureus* strains causing sepsis are not more susceptible to the investigated AMP than *S. aureus* strains derived from *S. aureus* colonizers. Although we can not exclude a potential relevance of other AMP not tested here, our results suggest that the onset of sepsis may not be linked to a different susceptibility of *S. aureus* to skin-derived AMP.

## P220

**Distinct immune responses induced by lipoteichoic acid from *S. aureus* or *S. epidermidis* despite common pattern recognition pathways**

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Evolutionary highly conserved microbe associated molecular pattern (MAMP) are sensed by pattern recognition receptors (PRR). Innate immune sensing by PRR is especially relevant for the integrity of surface organs such as the skin. It is well known that colonization of atopic dermatitis (AD) skin with *S. aureus* leads to inflammation and disease exacerbation whereas other staphylococci, especially *S. epidermidis*, are part of the endogenous human skin microflora not leading to inflammation under physiological circumstances. It has not been characterized how innate immune sensing of different *Staphylococcus* species and subsequent induction of adaptive immune responses differs based on the presence of fundamentally the same MAMPs in different *Staphylococcus* species. To investigate innate immune sensing of pathogenic and non-pathogenic *Staphylococci*, highly purified lipoteichoic acid (LTA) from *S. epidermidis* and *S. aureus* was prepared and immune consequences of PRR ligation by LTA were characterized. As expected, LTA of both *Staphylococcus* strains induced maturation of dendritic cells (DC) as observed by upregulation of MHC II and B7 molecules. Moreover, DC activation was dependent on TLR2 and MyD88 as TLR2-deficient and MyD88-deficient DC remained immature following LTA contact. In contrast to consistent DC maturation, marked differences were seen in regard to cytokine production: *S. aureus* LTA induced large amounts of IL-12p70 in DC, only low IL-12p70 levels were elicited in response to *S. epidermidis* LTA. Interestingly, production of the anti-inflammatory cytokine IL-10 was similar in response to both LTA preparations. To mimic the micro-environment present in early lesions of atopic dermatitis, DC were activated in the presence of IL-4. IL-4 significantly enhanced IL-12p70 levels induced by *S. aureus* LTA in DC. In sharp contrast, low IL-12p70 levels induced by *S. epidermidis* LTA were suppressed in the presence of IL-4. This immune modulation mediated by IL-4 was dependent on STAT6 signaling as STAT6 knock-out DC failed to regulate LTA induced IL-12p70 levels in response to IL-4.

To investigate consequences of different innate immune sensing on adaptive immune responses, T-cell cocultures were set up either with DC stimulated with LTA derived from *S. aureus* (sa-DC) or with DC activated by *S. epidermidis* LTA (se-DC). In accordance with the pre-dominant role of *S. aureus* LTA in inducing pro-inflammatory cytokines, CD4+ T helper cells primed with sa-DC produced large amounts of the Th1 hallmark cytokine IFN- $\gamma$ . In contrast, IFN- $\gamma$  levels of CD4+ T helper cells primed with se-DC were more than fivefold lower.

Our data demonstrate a hitherto undescribed functional difference between LTA from *S. aureus* and from *S. epidermidis*. It is of special interest that the innate immune sensing of LTA is mediated by TLR2 and MyD88 in both cases, however, this pathway lead to marked differences in regard to the induction of pro-inflammatory cytokines especially under the influence of the typical AD cytokine IL-4. Consequently, adaptive immune responses elicited by sa-DC or se-DC differed significantly with *S. aureus* LTA being superior in induction of Th1 cells. These data provide an explanation how (i) *S. aureus* leads to exacerbation and chronicification of skin inflammation in AD mediated by Th1 cells and how (ii) *S. aureus* mediates a Th2 to Th1 shift as detected when comparing acute and chronic AD lesions, and why (iii) *S. epidermidis* is well tolerated on the skin surface.

## P221 (V09)

***Leishmania major* susceptibility is determined by the genetic background of leukocytes, not by stromal cells**

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Susceptibility to *Leishmania major* in BALB/c mice is due to predominant Th2 development, whereas protection in resistant C57BL/6 mice is mediated by Th1 cells. Previously, we reported that this is – in part – a result of differences in DC cytokine production, e.g., IL-1 $\alpha/\beta$  and IL-12p80 homodimer. Others also recently demonstrated the importance of cytokine production by stromal cells, e.g., keratinocytes. Applying a bone marrow chimera model we aimed to determine the key cell population(s) responsible for this strain-dependent dichotomy. Lethally irradiated BALB/c or C57BL/6 mice were reconstituted with either their own or the other strains bone marrow. Six to ten weeks later, engraftment was analyzed by flow cytometry of blood samples. Successfully reconstituted mice were infected with  $2 \times 10^5$  *L. major* promastigotes intradermally into both ears and lesion development was measured weekly. After infection, control BALB/c wild type (WT) mice showed rapid lesion development and disease progression, while corresponding C57BL/6 WT mice exhibited self-healing lesions as expected. Interestingly, C57BL/6 mice reconstituted with BALB/c BM showed lesion development according to the BM origin, while lesions of BALB/c mice with C57BL/6 BM showed a peak in lesion volumes 5 weeks after infection with slightly declining, but non-healing lesions. Unfortunately, some mice died from allogeneic graft-versus-host disease (GVHD) after BM transfer. To avoid this, we next used BALB/c  $\times$  C57BL/6 F1 mice and again reconstituted with either BALB/c or C57BL/6 BM. Interestingly, the course of disease in infected mice reflected the origin of the BM in both circumstances: Mice reconstituted with BALB/c BM showed rapid disease progression. On the other hand, mice reconstituted with C57BL/6 BM developed the largest lesions at week 4 and were healed in week 15 after infection. These results show that the cell populations determining the differences between BALB/c and C57BL/6 in *L. major* infection must be of hematopoietic origin. To further narrow down these key cell populations, we applied a mixed bone marrow chimera model. This enabled us to have mice that only differed in T and B cells being either BALB/c or C57BL/6. Everything else, including the hematopoietic compartment, was uniformly BALB/cRag1 $^{-/-}$   $\times$  C57BL/6Rag1 $^{-/-}$  F1. This experiment showed surprisingly clearly, that the presence of BALB/c-derived T and B cells led to susceptibility, while the presence of C57BL/6 T and B cells is sufficient for protection. To further investigate the relevance of the genetic background of individual cell populations in this important disease, we are planning to reconstitute mice leading to the concomitant presence of, e.g., C57BL/6 T cells and BALB/c B cells and vice versa. The method of mixed bone marrow chimeras offers various opportunities to test the effects of individual cell populations in syngeneic and allogeneic matters in *L. major* infections as well as several other diseases.

## P222

**Characterization and identification of antigens derived from the human pathogenic parasite *Leishmania major***

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Healing of cutaneous leishmaniasis, which is caused by the species *Leishmania major*, requires interferon (IFN) $\gamma$  secretion of both antigen specific CD4+ Th1 and CD8+ Tc1 cells. Dendritic cells (DC) infected

with the amastigote life form of *L. major* represent very potent antigen-presenting cells. An effective vaccine does not exist at present, but attempts for vaccination of mice showed promising results using selected recombinant *Leishmania* proteins in combination with adjuvants. Draining lymph node (dLN) T cells isolated from resistant, infected C57BL/6 mice typically show a protective IFN $\gamma$ /high/IL-4 low cytokine profile after restimulation with parasite lysate (soluble *Leishmania* antigen, SLA). To identify immunogenic antigen-specific proteins of *L. major*, we separated soluble proteins derived from *L. major* promastigote SLA from other parasite components (such as nuclei, mitochondria, membrane proteins) by differential centrifugation. dLN cells restimulated with the soluble protein fraction displayed a dominant Th1 cytokine profile similar to the cytokine profile observed after restimulation with total SLA. Next, a fractionation of the soluble proteins was performed by anion exchange chromatography; sub-fractions were subsequently tested for their T cell restimulating ability *in vitro*. We identified several immunoreactive fractions of SLA, capable of inducing an increased release of proinflammatory IFN $\gamma$ , which we additionally tested in T cell proliferation assays. As expected, antigen-specific expansion of CD4+ T cells dominated over a CD8+ T cell response using these proteins. To further reduce the complexity of *L. major* fractions and ensure identification of abundant antigens in these fractions, selected pools of SLA promastigote fractions were further fractionated applying a shallower gradient during anion exchange chromatography. Antigenic fractions exhibiting high capacity to induce a Th1/Tc1 response *in vitro* will next be analyzed by label-free quantitative mass spectrometry. Additionally, another attempt will be the comparative antigen characterization of both disease-propagating amastigote life form and promastigote life forms, which is possible for the first time since the *in vitro* generation of axenic *L. major* amastigotes (free of host proteins) was established. This comparison will aim to identify and characterize immunogenic stage-specific proteins, which could constitute potential sources for protective T cell epitopes. In future experiments, selected epitope candidates will be analyzed for their capacity to induce a Th1/Tc1 immune reaction both *in vitro* and in immunization studies *in vivo* to contribute to a better understanding of T cell-mediated protection against cutaneous leishmaniasis.

## P223

**CD11c-DTR bone marrow chimeras as a tool to study the immunological function of dermal dendritic cells in experimental cutaneous leishmaniasis**

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Skin dendritic cells (DC) play an important role in immune reactions against infections such as the intracellular parasite *Leishmania major*. In particular, they are thought to initiate a T helper type-1 (Th1) dominated response, which is crucial for parasite clearance and healing of the skin lesions. Previously, we showed that epidermal Langerhans cells (LC) act as negative regulators of the anti-*Leishmania* response. Selective depletion of LC *in vivo* led to smaller ear lesions and reduced parasite burdens associated with enhanced Th1 and reduced regulatory T cell (Treg) activity. To dissect the role of dermal DC (dDC) populations in *L. major* infections, we used a transgenic mouse model in which CD11c high expressing dDC can be transiently depleted. Here, CD11c-DTR mice carry the primate diptheria toxin receptor (DTR) under the control of the murine CD11c promoter. Treatment with DT leads to transient depletion of CD11c high cells. We established a chimeric mouse model to selectively deplete dDC only. C57BL/6 mice were lethally irradiated and transferred with CD11c-DTR bone marrow (BM). Thus, the chimeras reconstitute their hematopoietic cells with donor-derived cells with the exception of epidermal LC, which are radioresistant. In these chimeras, DT treatment leads to depletion of dDC, but not of host-derived C57BL/6 LC. Chimerism was assessed by FACS analysis. Intradermal infection of dDC-depleted chimeras with physiological low doses (103) of metacyclic *L. major* promastigotes led to clearly larger lesion volumes ( $38 \pm 8$  vs  $19 \pm 5$  mm<sup>3</sup> E3 in PBS-treated control mice 7 weeks post infection,  $n \geq 6$ ,  $P = 0.06$ ) paralleled with increased lesional parasite burdens, and – interestingly – IL-12 levels which were reduced by 60% compared to untreated control mice, suggesting impaired induction of the protective Th1 response. Next, we assessed if dDC are vital for the success of an anti-*Leishmania* vaccination approach. Previously, we demonstrated that vaccination of C57BL/6 mice with the fusion protein TAT-LACK resulted in smaller lesion sizes due to preferential CD8 T-cell priming. Similarly, CD11c-DTR bone marrow chimeras that were vaccinated with TAT-LACK in the presence of CpG developed smaller skin lesions as compared to PBS-treated chimeras. In contrast, mice depleted of dDC during TAT-LACK vaccination displayed reduced protection in subsequent infections. In summary, CD11c-DTR chimeras are a helpful tool to study the role of dDC during infection with *L. major* and in vaccination approaches. Our data suggest that dDC are required to initiate an effective Th1 response against *Leishmania major* parasites and appropriate targets to establish immunization protection against cutaneous leishmaniasis.

## P224

**Role of skin mast cells for the T cell-dependent anti-*Leishmania* response**

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Mast cells (MC) are key effector cells in type I hypersensitivity reactions or in responses to parasites and play an important role in the regulation of protective adaptive immune responses against pathogens. Strategically located in the skin they contribute to the control of parasitic skin infections by *Leishmania major*. In experimental infection models using *L. major*, acquired resistance results from activation of CD4+ T cells which secrete IFN gamma after recognition of antigen presented by dendritic cells (DC) in the draining lymph node. To analyze the impact of MC to protect against *L. major* and to investigate their functions *in vivo* we used KitW-sh/KitW-sh mice, which exhibit a profound deficiency in MC in all tissues including skin, but normal levels of major classes of other differentiated hematopoietic and lymphoid cells. In these mice, intradermal inoculation of physiologically relevant low doses of *L. major* resulted in significantly worsened disease with larger lesions in MC-deficient KitW-SH/KitW-SH compared to infected C57BL/6 control mice. This was correlated to enhanced parasite burdens in ears and spleens in KitW-SH/KitW-SH mice. Additionally, skin draining lymph node (LN) cells from infected KitW-SH/KitW-SH and C57BL/6 mice were isolated and pulsed with soluble *Leishmania* antigen to analyze antigen-specific cytokine production. Here, IL-10 production was significantly increased in MC-deficient mice, which strongly supports a shift towards a Th2 response. To confirm these results we isolated CD4+ T cells from infected KitW-SH/KitW-SH mice and C57BL/6 mice and restimulated both with *L. major*-infected C57BL/6 DC *in vitro*. In line, CD4+ T cells from MC-deficient mice produced enhanced levels of IL-4 and IL-10. In addition, numbers of CD4+, CD8+ and regulatory T cells were significantly higher in infected ears of KitW-SH/KitW-SH mice. Finally, numbers of neutrophils in the infected ears were strongly increased. These experiments show that MC-deficiency leads to impaired development of T cell-dependent protective immunity against *L. major* and future studies will show, if this is a defect in direct MC-T cell interactions or indirectly e.g. via DC.

## P225

***Leishmania major*-specific epitopes: identification by *in vitro* and *in vivo* analysis**

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Healing of *Leishmania (L.) major* infections is based on Th1/Tc1 immunity and requires secretion of interferon (IFN) $\gamma$  by CD8+ and CD4+ T cells. No vaccine exists against this human pathogen so far. Unfortunately, it is still unclear which peptides or proteins are presented towards different T cells during an effective immune response by relevant antigen-presenting cells, such as dendritic cells (DC) and macrophages. Using mass-spectrometry, we identified the most abundant proteins expressed by both life-forms of *Leishmania major*, the metacyclic promastigotes and the intracellular amastigotes. Those proteins were then predicted for their immunoreactivity using the computer-based algorithm SYFPEITHI. Peptides that had a SYFPEITHI-score  $\geq 25$  were chosen for further analysis. As the score is a relative indicator for the binding affinity to MHC class-I molecules, we randomly set a cut-off of 25 for SYFPEITHI to identify potentially immunodominant epitopes. Next, we chose 300 peptides for further analysis. Those peptides were then tested in *in vitro* assays: peptide loaded DC from C57BL/6 mice were co-cultured with primed CD8+ T cells from infected mice. After 48 h the supernatants were analysed for the amounts of secreted IFN $\gamma$ , IL-4 and IL-10. We were able to identify ~20 peptides which induced CD8+ T cells to secrete high amounts of IFN $\gamma$ . In proof-of-concept experiments, we randomly selected ~16 peptides, either secreting high amounts of IFN $\gamma$ , IL-4 or IL-10, for *in vivo* analysis. Nave C57BL/6 mice were immunized with 10  $\mu$ g peptide plus CpG as adjuvant in one ear. In week 3, mice were infected with  $1 \times 10^6$  *L. major* in the alternate ear. Lesion volume was measured weekly. Surprisingly, only one peptide that *in vitro* induced high secretion of IFN $\gamma$  protected mice against challenge, as they had smaller lesions compared to PBS-control mice. But interestingly, we were able to identify three peptides which *in vitro* induced high secretion of IL-4. Mice immunized with these peptides had smaller lesion volumes compared to PBS-control mice. Mice immunized with peptides, that *in vitro* induced high secretion of IL-10 did not protect against challenge with *L. major* as they had similar lesion volumes compared to PBS-control mice. We also tested peptide pools as they might enhance protection against *L. major* infections. Pools consisted of either four best peptides or four worst. But compared to PBS control mice, groups immunized with pools did not have smaller lesions at all. In summary, identifying peptides or proteins which protect mice against challenge with *L. major* would finally lead to a required vaccine against this human pathogen. And tetramer development with *L. major* specific peptides would elevate the understanding of T cell priming during parasite infection.

## P226 Host-pathogen interaction during *Staphylococcus aureus* induced skin infection

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*Staphylococcus aureus* (*S. aureus*) is a major human pathogen causing several bacterial skin infections, but also often found on normal skin and especially in ulcers or chronic wounds. However, it is not known when *S. aureus* remains a harmless dweller in wounds and when it becomes the cause of soft tissue infection.

In our mouse model of soft tissue infection, we study the contribution of host factors and bacterial virulence factors to the etiology of soft tissue infection. To this end we use different inbred strains of mice and different isolates of *S. aureus* with different pathogenicity in humans, i.e. form human non-infected and from soft tissue infection with different degrees of severity.

We have previously described that higher susceptibility of C57BL/6 mice to *S. aureus* correlated with significantly higher foot swelling, increased dissemination of bacteria into inguinal lymph nodes and kidneys as well as lower influx of polymorphonuclear leukocytes than in resistant BALB/c mice.

We now studied the relevance of various clinical *S. aureus* isolates which were obtained from a clinical spectrum ranging from colonized wounds to abscesses, osteomyelitis or fasciitis. These *S. aureus* isolates were analysed for certain virulence factors such as the invasiveness to host cells, the expression of alpha-toxin and the induction of cell death. They were then injected subcutaneous in foot of C57BL/6 mice.

We found that *S. aureus* isolates with high pathogenicity in humans caused a significantly more severe course in mice than isolates with low pathogenicity. Highly pathogenic strains caused significantly higher foot swelling than low pathogenic *S. aureus* strains. This higher aggressiveness correlated with the capability to invade host cells, to express alpha-toxin and to induce cell death.

Conclusions: (i) Both host factors and staphylococcal virulence factors are decisive for the outcome of *S. aureus* skin infection. (ii) Virulence factors with relevance in mouse models are e.g. expression of alpha-toxin mice. (iii) Our mouse model of soft tissue infection allows studying the complex host-pathogen interaction in case of *S. aureus* induced skin infection.

## P227 Is there a modulatory role for the aryl hydrocarbon receptor in experimental leishmaniasis?

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In experimental *L. major* infection a Th1 response in C57BL/6 mice results in resistance while a Th2 response is associated with susceptibility in BALB/c mice. The development of Th1/Th2 immune responses is driven by the early cytokine milieu in the infected tissue. We have previously shown that both keratinocytes and phagocytes contribute considerably to the immunological events preceding those in lymph nodes. Skin macrophages are among the first cells to come into contact with *L. major*. To analyze their contribution to the early cytokine milieu we studied *L. major* induced gene expression in macrophages 24 h after *in vitro* infection. Differences in the skin cytokine milieu between resistant and susceptible mice strains are responsible for several aspects of resistance to *L. major*, most notably for signals from the innate part of the immune response that determine Th- cell differentiation. We found that the transcription factor aryl hydrocarbon receptor (AhR) is considerably stronger upregulated in C57BL/6 macrophages than in Balb/c macrophages infected with *L. major*. AhR is involved in several processes in the skin, for example reaction to UV radiation, retinoic acid signaling and mediating dioxin toxicity. It has recently been reported to be involved in several aspects of innate immunity such as in regulation of macrophage response to LPS as well as in maturation and functionality of DC. This gives strain specific differences the potential to influence development of resistance to infection, e.g. by modifying the early cytokine milieu in *L. major* infected skin.

Therefore we analyzed the cytokine secretion of infected macrophages in response to neutralization, deletion and stimulation of AhR via cytometric bead assay and realtime PCR. (i) AhR-antagonist CH-223191 (30 nM) reduced secretion of TNF $\alpha$  and Mip1 $\beta$ , and reduced apoptosis (elicited by M-CSF starvation) synergistically with the antiapoptotic effect of *L. major*. (ii) Macrophages from AhR-/- mice also showed a reduced production of TNF $\alpha$ , Mip1 $\beta$ , IL1 $\beta$  and Cxcl2. (iii) Treatment of C57BL/6 macrophages with AhR agonist ITE led to higher secretion of TNF $\alpha$  and Mip1 $\beta$  and higher transcript levels of Cox2 and Cxcl2.

Thus, AhR is involved in Leishmania major induced effects in (skin resident) macrophages. We therefore analysed the possible effects of antagonizing AhR in C57BL/6 mice *in vivo*: co-treatment with AhR antagonist CH-223191 during infection with *L. major* caused a reduction in transcript levels of Mip1 $\beta$ , IL1 $\beta$ , Cxcl2 and several other genes, while co-treatment with AhR agonist ITE upregulates several cytokines. Our experiments show that AhR influences the expression of several important cytokines early after infection.

P228

## Cutaneous *Staphylococcus aureus* derived lipoteichoic acid directly induces temporary T cell unresponsiveness

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The interplay between microbes and surface organs such as the skin can result in pathways of inflammation and immune defense in response to pathogenic bacteria. Specific sensitization allows the immune system to control or even avoid infections upon later contacts. To this end, memory T cells orchestrate immunosurveillance in different organs with the skin. However, pathogenic and non-pathogenic bacteria share many antigens but minor amounts of pathogenic bacteria as well as non-pathogenic bacteria should not elicit immune defense mechanisms and inflammation. Over the last decade, mechanisms of specific immune tolerance have been elucidated that are functional to control unwanted inflammation. In this study we wondered whether there are also direct ways for bacteria to control unwanted T cell mediated inflammation. Limited application of *Staphylococcus aureus* derived lipoteichoic acid (LTA) during a second contact to the week hapten fluorescein isothiocyanate (FITC) significantly suppressed lesional T cell cytokine expression such as IL-4 and IFN- $\gamma$ . Moreover, proliferation of T cells from draining lymph nodes of LTA-treated skin was also highly reduced. Hypothesizing a direct effect on T cells, we analyzed CD4+ T cell activation *in vitro* in the absence of other cell types by polyclonal activation. In contrast to other known TLR2 ligands that acted as T cell costimulators, LTA significantly suppressed T cell proliferation and cytokine production. Interestingly, those T cells were still viable and did not show more Annexin V staining than control. Moreover, restimulating these T cells in the absence of LTA led to marked T cell proliferation and cytokine production indicating LTA induced temporary T cell paralysis. *In vivo*, LTA significantly reduced FITC-induced contact dermatitis following adoptive transfer of FITC-specific T cells. As we previously showed that TLR2 ligands amplify cutaneous inflammation, we wanted to dissect TLR2-dependent from TLR2-independent effects of LTA. To this end, we investigated OVA dermatitis as AD-like mouse model. In this model, we adoptively transferred OVA-specific Th2 cells and antigen to the ear skin of previously untreated wild type and TLR2-/- mice and monitored the ear swelling in presence or absence of LTA. Interestingly and confirming our hypothesis, in absence of TLR2, LTA profoundly suppressed OVA-specific dermatitis. Thus, we identified a new mechanism of how bacterial compounds temporarily and directly modulate the adaptive immune system by paralyzing T cell responses. This new mechanism of T cell paralysis may be a double edged sword: LTA induced T cell paralysis could function to avoid unwanted immune responses to non-pathogenic bacteria and to terminate inflammation. However, LTA induced T cell paralysis could also be a mechanism of immune evasion allowing pathogenic bacteria to invade and spread. Understanding this additional level of immune regulation in the complex interplay between microbes and the host may lead to new therapeutic concepts for inflammatory skin diseases and infections.

P229

## Causative pathogens of soft tissue infections

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Introduction: Soft tissue infections (STI) are frequently found in inpatient care. Terms or entities such as erysipelas, cellulitis, phlegmonas or necrotising fasciitis are all included under the term STI. However, exact definitions encompassing also the causative agents are lacking.

Commonly STI result from a barrier defect of the skin, such as an ulcer. In the clinical setting, antibiotic treatment is initiated according to isolation and susceptibility testing of all bacteria obtained by wound swabs. However, it has never been clarified, which of the various bacteria found in wounds do really invade the soft tissue and are the actual cause for the infection. As such *Pseudomonas aeruginosa* is often detected and treated, but we hypothesized that it would not be detected in the tissue as causative agent.

Methods: From patients with STI not yet treated with antibiotics biopsies were taken from the infected tissue for microbiological analysis; they were taken 1–2 cm from the margin of the wound and compared with results from the swabs from the wound

Results: Bacteria from the infected skin could be isolated in  $n = 9$  cases of  $n = 14$  examined patients with STI.

While the wounds contained various and multiple bacteria such as *enterobacter* sp., *streptococcus* sp. or *pseudomonas* sp., the most frequent bacteria detected in the skin was *S. aureus*, which was found in  $n = 6$  cases. In other cases we detected either another *staphylococcus* sp. than *S. aureus*, or, in one case, propionibacterium acnes.

Conclusion: In uncomplicated soft tissue infections *S. aureus* is the most frequent causative agent, independent from the multiple bacteria in the wounds, while *Pseudomonas* was not detected. Thus, treatment should be directed against *S. aureus* and may neglect complex antibiotics with adverse side effects.

P230

## Evaluation of systemic immune responses against cutaneous wart-associated alpha-Papillomaviruses in solid organ transplant recipients

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Cutaneous Human Papillomaviruses (cHPV), predominantly of genus alpha species 4 (HPV2, 27, 57) and 2 (HPV3, 10, 77), are of major importance among viral skin infections. The increased prevalence of HPV infection and associated disease among immunosuppressed populations, including solid organ transplant recipients (OTR), documents the importance of the immunological control of HPV infection. To date, the natural course of immunological cHPV-specific responses during systemic immunosuppression is poorly investigated.

Thus, we analyzed natural systemic immune responses against the major capsid protein L1 of HPV types 2, 27, 57, 3, 10 and 77 in the blood of OTR before and after initiation of iatrogenic immunosuppression compared to immunocompetent individuals (IC). HPV-L1-specific antibodies were detected by bead-based multiplex serology (Luminex) and L1-specific TH1, TH2 and cytotoxic T cell responses by flow cytometry. Among OTR, we observed a significant 42% decrease in humoral L1-specific immune responses during the induction phase of iatrogenic immunosuppression, comparing median values 30 day before and 30 day after initiation of immunosuppressive therapy. In contrast, direct comparison of L1-specific antibody responses in sera of individual OTR before and after long-term (>1 year) immunosuppression showed no significant alterations. The predominant cellular L1-specific immune response was of type TH1 (CD4+ CD40L+ IL-2+ IFN- $\gamma$ +). Consistent with the detected L1-specific antibody titers, L1-specific TH1 responses were unchanged in long-term immunosuppressed OTRs compared to IC. We conclude that the systemic immune responses against cHPV reflect the degree of iatrogenic immunosuppression indicating a higher susceptibility for cHPV infection among OTR during the early phase after organ transplantation.

### P231 Cold plasma antiseptics for skin and wounds: a new antimicrobial concept in dermatology

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**Introduction:** Plasma medicine has become an emerging field in medical science since cold plasma has demonstrated potent anti-inflammatory as well as antimicrobial effects. In the light of worldwide increasing resistance of many pathogens like methicillin-resistant *Staphylococcus aureus* (MRSA), cold plasma therapy with a complete different mode of action could constitute an alternative to conventional antibiotic and antiseptic therapy. Before implementing new antimicrobial therapies however, susceptibility of normo- like multiresistant isolates of relevant bacteria has to be evaluated.

**Methods:** As plasma susceptibility data of human skin and wound pathogens are not available, we tested the susceptibility of relevant clinical species and isolates (50 MRSA, 50 MSSA, 20 *Pseudomonas aeruginosa* and 10 *Streptococcus pyogenes*) from dermatologic patients of our clinic towards low temperature atmospheric pressure plasma (APPJ device) and dielectric barrier discharge plasma (DBD device) *in vitro*. The efficacy was tested treating suspensions of the strains plated on semisolid agar and evaluating the dimensions (diameter in mm) of the obtained killing zones. A strain is claimed susceptible if the diameter reaches >95% of the reference diameter of the control strains (evaluated in prior investigations).

**Results:** Plasma treatment proved high effectiveness in eradicating all tested strains including MRSA (hospital-associated ha-MRSA), community-associated ca-MRSA, and livestock-associated la-MRSA). To prove clinical significance, we tested the eradication of a recalcitrant MRSA colonization from the groin skin of a patient with exfoliative dermatitis (no effect after conventional antiseptic eradication) by treatment over 60 s with DBD plasma. This treatment was eradicating MRSA on the treated area and the treatment was well tolerated by the patient who had given former written consent to the treatment.

**Conclusion:** Cold plasma treatment exhibited strong and rapid effects against relevant clinical wound and skin pathogens including MRSA *in-vitro* and *in-vivo* and may constitute a safe and effective alternative antiseptic in Dermatology.

### P232 OATP5A1 a solute carrier transport protein with non-classical function is expressed in mature dendritic cells and melanoma cell lines

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Organic anion transporting polypeptides (OATPs) function as influx transporters and play a critical role in the bioavailability of drugs. While most members of the OATP family are well characterized, little is known about the expression and function of the transmembrane protein OATP5A1 (hSLCO5A1). Therefore gene expression of this transporter was analyzed in different human cell types and functional studies were performed in human T-Rex-Hela cells and *Xenopus laevis* oocytes. RT-PCR studies revealed the presence of hSLCO5A1 in renal cells, melanoma cell lines and monocyte-derived dendritic cells but not in monocytes, macrophages or lymphocytes. Interestingly, hSLCO5A1 is constitutively expressed in immature dendritic cells (iDCs) and expression significantly increases during maturation. Benzylpenicillin, which is known to be a substrate of OATPs, down-regulates the expression of hSLCO5A1 in iDCs. To further characterize the function of OATP5A1 a cell system was generated expressing the wildtype and a natural mutant (aa33, L→F) of OATP5A1 in *Xenopus laevis* oocytes. Transport assays with Tritium-labeled xenobiotics revealed an enhanced uptake of benzylpenicillin and arachidonic acid mediated by the mutant OATP5A1-L33F. Other known substrates of OATPs such as dehydroepiandrosteron-3-sulfate, taurocholate, oestrone-3-sulfate, oestradiol-17 $\beta$ -glucuronide, leukotriene C<sub>4</sub>, prostaglandin E<sub>2</sub>, ouabain, methotrexate, digoxin and [D-penicillamine] enkephalin were not transported by OATP5A1.

In summary, these studies revealed a strong expression of the influx transporter OATP5A1 especially in antigen presenting cells which might suggest a putative role of this protein in immune reactions such as drug allergy. Further studies, however, are required to elucidate the substrate specificity, cellular location and specific function of this novel transport protein.

### P233 UVB and singlet oxygen- potential new pathways of cell damage?

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UV radiation is already known as initiator and promoter of carcinogenesis in skin. Until now the cellular UV damage has been clearly differed into UVA (320–400 nm) and UVB radiation (280–320 nm) caused damage. UVB is absorbed in proteins and DNA and causes direct DNA damage. In contrast, UVA radiation generates reactive oxygen species such as singlet oxygen, which can initiate a variety of cellular damages. Singlet oxygen is generated after absorption of UVA radiation in various endogenous photosensitizers. The objective of the present study is to investigate whether and to which extent UVB radiation is additionally able to generate singlet oxygen plus how UVB radiation can alter endogenous photosensitizers during irradiation.

Therefore potential endogenous photosensitizers such as different vitamin molecules and unsaturated fatty acids are irradiated with monochromatic UVB radiation at 308 nm (Xe-Cl-laser). Singlet oxygen is directly detected and quantified by its luminescence in the near infrared spectrum at 1270 nm. In addition, absorption spectra of the photosensitizer solutions were recorded before and after UVB irradiation to investigate the photostability of the endogenous photosensitizers.

For all investigated endogenous photosensitizers a clear time and spectral resolved singlet oxygen luminescence signal could be obtained. Time- and spectral resolved. For most of the investigated molecules, a clear luminescence signal could be obtained by time-resolved measurements. By comparison with well-known photosensitizers the singlet oxygen quantum yields of endogenous photosensitizers could be estimated ranging from 5 up to 40%. Furthermore UVB radiation altered the photosensitizer molecules during irradiation yielding a change of absorption in the entire UV spectrum (280–400 nm). The absorption of UVB radiation in the investigated endogenous photosensitizers can lead to the generation of singlet oxygen that in turn changes the absorption of those molecules. The effect of UVB absorption and hence singlet oxygen production is either reduced or increased. The outcome of this effect needs further observation.

### P234 The AIM2 inflammasome is active in UVB treated primary human keratinocytes and in skin in patients with polymorphic light eruption (PLE)

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UVB exposure can cause sunburn and triggers cutaneous inflammation. People who are very sensitive to UV exposure may also present with polymorphic light eruption (PLE) which is characterized by significant cutaneous itching and the presence of vesicles and papules. The mechanisms of UVB induced skin inflammation are not completely understood.

Recently, it was demonstrated that in human keratinocytes the NLRP3 inflammasome is involved in UVB induced inflammation with activation of the cysteine protease caspase-1 and subsequent interleukin-1 beta (IL-1beta) release.

In our studies we could confirm that UVB irradiation of skin biopsies from healthy volunteers leads to caspase-1 activation and IL-1beta secretion. Furthermore, we observed that the recently discovered cytosolic protein absent in melanoma 2 (AIM2) inflammasome is also involved in UVB induced inflammation. In primary human keratinocytes (NHEK) UVB triggered IL-1beta release was reduced after RNAi-mediated knockdown of AIM2.

As AIM2 is a cytosolic receptor for double-stranded (ds)DNA and DNA is only present in the nucleus of eukaryotic cells and in mitochondria, we wondered how AIM2 could be activated after UVB irradiation. As a mechanism, we could show that genomic DNA was present in the cytosol of keratinocytes after UVB irradiation by PCR analyses.

In this current study we observed that AIM2 is also overexpressed in keratinocytes of skin samples of patients with PLE and that IL-1beta is present in affected skin. Furthermore, the active form of caspase-1 (p20) could also be detected in keratinocytes of PLE patients *in vivo* indicating inflammasome activity.

Since interleukin-18 (IL-18) is another pro-inflammatory cytokine which is cleaved into its active form by caspase-1 after inflammasome activation, we investigated whether its release is also activated after UVB irradiation. However, although we observed UVB triggered IL-1beta release in primary human keratinocytes upon UVB irradiation, we were not able to detect IL-18 release.

In sum, in this study we show that in addition to UVB induced cutaneous inflammation such as sunburn also in PLE the AIM2 inflammasome is activated. The trigger for the activation of the AIM2 inflammasome is not known to date. Since intense UVB radiation leads to cell death we suggest that an uptake of DNA from dying cells or an endogenous release of DNA from damaged mitochondria and/or the nucleus triggers the presence of cytosolic DNA. This DNA could then bind to AIM2 and lead to inflammasome activation and IL-1beta release.

### P235 Investigation of *C. albicans* heat shock response to photodynamic therapy

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*Candida albicans* is a commensal fungus in healthy individuals; however, in immunocompromised hosts, this microorganism may penetrate the epithelium causing bloodstream and systemic infections. Only a few drugs are available for treating this kind of infection, and the emergence of antifungal resistance has decreased the efficacy of conventional therapies. Antimicrobial photodynamic therapy (APDT) consists in an effective modality for the selective destruction of pathogenic microorganisms. Superficial and localized infected tissues, such as mucocutaneous candidiasis, are readily accessible for the topical delivery of photosensitizer and light. Heat shock proteins (HSPs) are a group of ubiquitous chaperone proteins responsible for the refolding, repair and recycling of damaged proteins and stabilization of lipid membranes during cellular stress. Moreover, upregulation of HSPs during oxidative, antibiotic, osmotic and acid stress is associated with resistance to these stresses, and upregulation of HSPs prior to subsequent stress probably enables *Candida* cells to acquire 'tolerance' to the photodynamic stress. Therefore resistance to APDT can arise by activation of Heat Shock Proteins (HSPs), which may help cells to recover from PDT damage.

Thus, the aim of this work was to investigate whether (i) APDT induces the 70 kDa heat shock protein (Hsp70s) expression and (ii) if HSPs are associated with rescue response of *C. albicans* cells after APDT. Using a sub-lethal concentration of the photosensitizer TMPyP and blue light, *C. albicans* cells did not exhibit an increase in HSP70 expression determined by Westernblot analysis. Pretreatment of the *Candida* cells with 45°C for 30 min to induce Heat shock induction and subsequently photosensitization of these samples did not reduce APDT action on *C. albicans* cells. Survival of both heat-shock induced and untreated *C. albicans* cells decreased upon incubation with TMPyP and light to  $\geq 5$  log<sub>10</sub> steps (>99.999% killing efficacy).

This study effectively demonstrated that the expression pattern of the Hsp 70 protein of *C. albicans* cells was not influenced by the photodynamic process as determined by immunoblotting. Heat pretreatment did not influence the *C. albicans* cells killing by the antimicrobial photodynamic process. Overall, the presence of a low-level stress signal, like Heat shock, did not influence the sensitivity of *C. albicans* to APDT with TMPyP and blue light.

### P236 Investigations of singlet oxygen detection in different environments for a better insight into aPDT

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Singlet oxygen is a highly reactive oxygen species that is involved in various processes in biology and medicine. Using special IR photomultipliers, singlet oxygen can be directly detected in solutions by its extremely weak luminescence at 1270 nm. In case of cells and bacteria, however, the luminescence signal can provide information about the environment of the generated singlet oxygen. These information can lead to a better insight into the mechanism of action of PDT and aPDT.

Therefore first experiments in aqueous solutions with and without biomolecules were done and after that the luminescence signals of singlet oxygen generated in HT29-cells and *S. aureus* were investigated.

Out of the first experiments it could be shown, that the oxygen concentration in solutions decreases dramatically when singlet oxygen can interact with biomolecules like unsaturated fatty acids or proteins.

For cells and bacteria it is to regard, that the oxygen concentration inside is naturally lower than in solutions and the meaning of the rates in singlet oxygen luminescence signals is changed.

Out of the experiments with TMPyP-incubated cells and bacteria it can be shown, that singlet oxygen is quenched, more in cells than in bacteria, and the oxygen concentration in bacteria and cells can be estimated.

The results can lead to an exploration of better working photosensitizers or to a possibility for the measurement of oxygen concentration in bacteria biofilms.

### P237 Detection of singlet oxygen luminescence in *Candida albicans* biofilm and phototoxic efficacy of the photodynamic inactivation

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The fast development of multiresistant patterns against antibiotics of many species of bacteria has led to novel antibacterial strategies like the antibacterial photodynamic therapy (aPDT). A lot of work has been done to find and investigate molecule structures and their derivatives that are able to generate reactive oxygen species (ROS) which are the active agent for killing microorganisms. The search for photo-

sensitizers (PS) for aPDT has led to the synthesis of a lot of porphyrin structures and their photophysical and chemical investigation. The phototoxicity of the porphyrin TMPyP has already been investigated and this PS is used for cell staining in order to investigate the effect of singlet oxygen ( $^1O_2$ ) during photosensitization. XF73 is a newly synthesized porphyrin that already revealed a huge bactericidal effect against both Gram-negative and Gram-positive bacteria. Different PS are considered to localise in different compartments of the cells due to their number of positive charges and side chain structure. In order to clarify the localisation of newly synthesized PS and therefore the site of  $^1O_2$  generation the fluorescence microscopy can be used. In case of microorganisms exact localisation of a given PS does not work due to the limited resolution of a light microscope. The direct measurement of  $^1O_2$  luminescence at 1270 nm might be a candidate for an alternative measurement, because the rise and decay time of  $^1O_2$  depend critically on the surrounding media, which consists of quenching agents.

The antifungal activity of  $^1O_2$  generated by the two photosensitizers 5,10,15,20-Tetrakis(1-methyl-4-pyridinio)-porphyrin tetra(p-toluenesulfonate) (TMPyP) and 5,15-bis-[4-(3-Trimethylammonio-propyl-oxyl)-phenyl]-porphyrin (XF73) is investigated in free-floating *Candida albicans* cells as well as in *C. albicans* biofilm. For this purpose a setup was built in order to measure  $^1O_2$  directly in *C. albicans* cells by its luminescence at 1270 nm in near-backward direction respect to the exciting beam using an infrared-sensitive photomultiplier.  $^1O_2$  was generated in *C. albicans* cells that were incubated with TMPyP and XF73. Planktonic cell suspensions as well as for the first time biofilms of *C. albicans* in polystyrene Petri dishes were investigated by this method and  $^1O_2$  was detected within the planktonic cells and also the biofilm. Additionally, phototoxicity tests were done with TMPyP and XF73-incubated *C. albicans* cells by illuminating the incubated cells. An effective killing has been detected with using XF73, which was  $6 \log_{10}$  with c(XF73) = 0.5  $\mu\text{M}$  (15 min incubation, 15 min illumination, 12.1  $\text{J}/\text{cm}^2$ ) for the planktonic cell solution and  $4 \log_{10}$  in case of the biofilm with c(XF73) = 1  $\mu\text{M}$  (12 h incubation, 1 h illumination, 48.2  $\text{J}/\text{cm}^2$ ), respectively.

The phototoxicity tests in combination with luminescence measurements give a possibility to compare the effectiveness of different photosensitizers and may help finding a way to describe the location of the photosensitizers and the site of  $^1O_2$  generation.

### P238

#### Fast and effective: intense pulse light photodynamic inactivation of bacteria

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In the last decade, only a few antibiotics with new mechanisms of action were approved by the European Medicines Agency or by the FDA. In parallel, the resistance of *S. aureus* increased substantially: Nowadays a total of 64.4% of *S. aureus* isolates in the U.S. and 22.6% in Germany were resistant to methicillin. These data indicate that there is a substantial need for the development of new antibacterial strategies.

The antimicrobial photodynamic process is a new approach to inactivate efficiently pathogenic bacteria. Bacteria are incubated with a photoactive dye (photosensitizer) that is subsequently irradiated with visible light. Both, short times of incubation and irradiation are of importance where time-consuming processes must be avoided like either in industrial or medical disinfection. Recently, intense pulsed light sources (IPLs) have been developed for different dermatological procedures and treatments, for instance rejuvenation of photo damaged skin, removal of port-wine stains, and aesthetic challenges such as hair removal. IPLs are high power flash lamps that can emit millisecond pulses at high radiant emittance ( $\text{mW}/\text{cm}^2$ ).

By using such short IPL light pulses and short incubation times of a few seconds, we investigate to achieve a fast and effective photodynamic killing of bacteria yielding more than 3  $\log_{10}$  steps ( $\geq 99.9\%$ ). Therefore different Gram-positive and Gram-negative bacteria strains, *S. aureus*, MRSA, and *E. coli*, were incubated with different concentrations of TMPyP (5, 10, 15, 20-Tetrakis(1-methylpyridinium-4-yl)-porphyrin tetra p-Toluenesulfonate) for 10 s as the respective photosensitizer and illuminated with short pulses (ms) of an intense pulse light source (wavelength range 490–750 nm) as the respective light source. The emission spectrum of this IPL closely matches the absorption peaks of TMPyP. A photodynamic killing efficacy of more than 5  $\log_{10}$  steps ( $\geq 99.999\%$ ) was demonstrated using a concentration range of 1–100 M TMPyP and multiple light flashes of 100 ms (from 20 up to 80  $\text{J}/\text{cm}^2$ ). All bacterial samples that were incubated without photosensitizers exhibited normal growth with or without illumination, demonstrating that the applied light doses of up to 80  $\text{J}/\text{cm}^2$  at the level of the illuminated samples alone had no antibacterial effect. Furthermore no dark toxicity of TMPyP was observed. We could demonstrate for the first time that a light flash of 100 ms is enough to generate sufficient amounts of reactive oxygen species upon photosensitizer activation to kill relevant key pathogens. Overall antimicrobial photodynamic inactivation seems to be a promising tool for industrial and clinical purposes where savings in time is a critical point to achieve efficient inactivation of microorganisms applied on animate or inanimate surfaces.

### P239

#### Photodynamic killing of enterohaemorrhagic *Escherichia coli* (EHEC) for the first time using TMPyP

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Enterohaemorrhagic *Escherichia coli* (EHEC) is an important zoonotic pathogen of humans, causing severe diarrhea (hemorrhagic colitis) and in a small percentage of cases, haemolytic-uremic syndrome (HUS). A novel strain of *Escherichia coli* 104:H4 caused a serious outbreak of food borne illness focused in northern Germany in May through June 2011, but also affected people in many other European countries. The RKI stated that 855 HUS diseases and 2987 EHEC gastroenteritis disease were diagnosed, therefore a total of 3842 cases were included to the outbreak. Antibiotic therapy has been discouraged after earlier experience indicating a danger of aggravating the disease due to induced or enhanced release of Shiga-toxin and Verotoxin which is pathophysiologically critical for the disease and its complications.

In this study, we revealed for the first time the possibility to inactivate EHEC very fast and efficiently using a new antimicrobial approach, called photodynamic inactivation of bacteria (PIB), which has a totally different mechanism of action compared to a standard antibiotic treatment. PIB is based on the concept that bacteria are incubated with a photoactive dye (photosensitizer) that is subsequently irradiated with visible light to induce an irreversible oxidative damage immediately during illumination. Therefore EHEC and the wildtype *E. coli* were incubated with different concentrations of TMPyP (5, 10, 15, 20-Tetrakis(1-methylpyridinium-4-yl)-porphyrin tetra p-Toluenesulfonate) for 10 s as the respective photosensitizer and illuminated with different time intervals of visible light (wavelength range 400–750 nm) at a dose of 50  $\text{mW}/\text{cm}^2$ . The emission spectrum of the used incoherent light source closely matched the absorption peaks of TMPyP. Already a concentration of 1 M of TMPyP and an applied light dose of 10.5  $\text{J}/\text{cm}^2$  achieved a photodynamic killing of 99.9% (3  $\log_{10}$  steps of reduction) of EHEC. Incubation with higher concentrations (up to 100 M) of TMPyP caused EHEC killing of  $\geq 5 \log_{10}$  ( $\geq 99.999\%$ ) after illumination. Incubation of wildtype *E. coli* with TMPyP at concentrations identical to those against EHEC revealed a reduction of  $\log_{10}$  number of CFU per ml in the same range as detected for EHEC upon illumination with 10.5  $\text{J}/\text{cm}^2$ . Both EHEC and wildtype *E. coli* which were incubated without TMPyP exhibited normal growth with or without illumination, demonstrating that the maximal applied dose of 10.5  $\text{J}/\text{cm}^2$  at the level of the illuminated samples alone had no antibacterial effects. Furthermore no dark toxicity of TMPyP was observed.

We could demonstrate for the first time that the photodynamic activation of TMPyP is able to inactivate enterohaemorrhagic *E. coli* efficiently *in vitro*. The fast and effective killing rate exhibited by this photosensitizer TMPyP encourages further testing for antimicrobial photodynamic applications against EHEC. Overall PIB seems to be a promising tool for industrial and clinical purposes where savings in time is a critical point to achieve efficient inactivation of pathogens applied on animate or inanimate surfaces.

### P240

#### Lipidomic analysis of oxidized phosphocholines generated by UVA irradiation in dermal fibroblasts

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Oxidized phospholipids (OxPLs) are increasingly recognized as signaling mediators that are not only markers of oxidative stress but also 'makers' of pathology relevant to disease pathogenesis. Understanding the biological role of individual molecular species of OxPLs requires the knowledge of their concentration kinetics in cells and tissues. In this work, we describe a simple and sensitive procedure for analysis of a broad spectrum of molecular species generated by oxidation of four most abundant species of polyunsaturated phosphatidylcholines (OxPCs). The approach is based on liquid-liquid extraction followed by reverse-phase core-shell HPLC coupled to ESI-MS/MS. More than 500 peaks corresponding in mobility to polar and oxidized PCs were detected within 8 min at 99 m/z values in extracts from human dermal fibroblasts. Importantly, 217 of these peaks were dose-dependently and statistically significantly increased upon exposure of cells to UVA irradiation, suggesting that these are genuine oxidized species. This method of semi-targeted lipidomic analysis may serve as a simple first step for characterization of specific 'signatures' of OxPCs produced by different types of oxidative stress in order to select the most informative peaks for identification of their molecular structure and biological role.

### P241

#### Dosimetric measurement of the UV exposure of an Austrian family at the seaside

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Summer vacations at the Mediterranean seaside are part of the leisure time behaviour of many Europeans. The ultraviolet (UV) doses acquired in such an environment contribute to the total annual UV dose. The goal of our study was to measure the UV dose a person is exposed to during a 6 day summer holiday on Island Hvar/Croatia (4309N/1639E) by electronic personal dosimeter.

We included an Austrian middle class family (father [P] 52 year, skin photo type I; mother [M], 51 year, III; elder daughter [F1], 18 year, III; younger daughter [F2], 14 year, I). None of the family members is accustomed to intended UV exposure neither to natural sunlight nor in tanning salons. Prior to the summer holidays [P] received narrow-band UVB photo therapy for polymorphic light eruption. We equipped each of our test persons with a calibrated electronic personal dosimeter (X-2000 1; Gigahertz Optik, Germany) which could be fixed to a loop on the front part of a peaked cap or a headband with the dosimeter tilted by 45 toward the horizon. Measuring started on the departure and was continued during the entire holidays. During the drive and inside the apartment a test person was allowed to take off the cap/headband, however, had to put it down in such a way that the dosimeter recorded the UV exposure of the test person. Another dosimeter [A1] was mounted on the flat roof of the apartment to measure the ambient (horizontal, free horizon) UV radiation from dusk to dawn. Dosimeter number 6 [A2] accompanied the family on the drives in the front of the car, on the walks to and from the shore always in a horizontal position. During the stay on the shore it was placed horizontally on an un-shaded rock. When sun bathing the test person took off the cap/headband and put it on the ground with the dosimeter directed to the sun. The dosimeters measured both UVA and UVB irradiance, temperature and erythemally-weighted UV dose with a resolution of 10 s and stored mean values over a period of 10 min. Each test person had to fill in a diary (activity, position, photo protective measures, vegetation of the surrounding, cloudiness) on an hourly base. We instructed our test persons to maintain their usual personal UV behaviour and to behave as naturally as possible.

The erythemally-weighted UV doses measured during vacation were as follows: [P] = 52.1 standard erythema doses (SED), [M] = 59.5 SED and [F1] = 56.9 SED. These values are equivalent to 36%, 41% and 39% of the ambient UV radiation of 146 SED recorded by dosimeter [A1]. On all their undertakings the family received between 85% and 98% of the dose (65 SED) measured by horizontally oriented dosimeter [A2]. During most of the day the ratio of personal exposure to ambient exposure was below 1.00. However, in the time after 4 p.m. this ratio increased up to 2.00. Unfortunately, dosimeter [F2] functioned only for 3 days (cumulative dose = 15.7 SED). On the 3 days when all dosimeters were functioning properly we recorded the following doses given here as SED and as equivalent minimal erythema dose (MED) for each participant: [P] = 16.3 SED (11 MED), [M] = 14.0 SED (4 MED), [F1] = 14.6 SED (4 MED), and [F2] = 15.7 SED (10.5 MED).

Our study shows that vacations in southern destinations contribute significantly to the total annual UV exposure. UV exposure both due to reflexion from the water surface and the beach and due to diffuse radiation appears to be underestimated. This is the reason why our fair-skinned test persons [P] and [F2] received relatively high doses although – as a consequence of their awareness of their UV sensitivity – spent a significant amount of time in the shadow of pine trees.

### P242

#### Targeting pruritus in lactase deficiency

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Chronic pruritus is associated with a broad spectrum of underlying diseases and is an interdisciplinary challenge in diagnostics and treatment. We analyzed 718 patients with chronic pruritus concerning concomitant lactase deficiency, demographic data, aetiology, duration and intensity of pruritus. A total of 154 patients (21.4%) were tested positive for lactase deficiency (H2 exhalation test). 38.3% showed a significant anti-pruritic response to a lactose-free diet (minimum 4 weeks, follow-up >3 months). The best results were observed in patients with pruritus of mixed or unknown origin ( $n = 91$ ). In this group, even 64.8% ( $n = 59$ ) had a significant effect on pruritic intensity. Lactase deficiency might elicit and maintain chronic pruritus via inflammatory cascades. To explain the mechanisms we performed a structure alignment analyses by NCBI-BLAST. A circumscribed region of Lactase-phlorizin hydrolase (LPH), the key enzyme of lactase metabolism, and NF-kappa B repressing factor (NRF) have a similar aminoacid sequence, which suggests themselves to be a common intracellular target within the pruritic inflammatory cascade. NRF might be the key mediator merging both pathways, as well as its ligand, NF-kappa B. These results and the statistical data of our study on a large collective of patients support a key role of lactase metabolism in pruritic, and possibly in a broader range of inflammatory diseases. Though *in vitro* studies are lacking, it might be helpful to define intracellular pathways involved in pruritogenesis via aminoacid sequence analysis to promote specific drug design.



In conclusion, screening for lactase deficiency is confirmed to be a rational step in the diagnostic work-up of chronic pruritus. Recommending a lactose-free diet to those with confirmed lactase deficiency is a simple, low-cost, highly efficient, yet specific therapy. Targeting intracellular inflammatory cascades shared in lactose metabolism might be a promising therapeutic attempt, even in other forms of pruritus.

#### P243

##### Mental itch induction in patients with chronic urticaria, patients with atopic dermatitis and healthy controls

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**Introduction:** To induce itch in experimental studies one cannot only use physiological methods like histamine-application, but also mental treatments like a presentation of audiovisual stimuli. It was shown that mentally induced itch is increased in patients with atopic dermatitis (AD) compared to healthy controls. Besides, in patients with AD increase in scratching behavior can be predicted significantly by psychological variables. So far these results have not been investigated in patients suffering from other skin-diseases like chronic urticarial (CU). Thus, the aim of the study was to investigate whether itch can also be induced mentally in patients with CU and whether mentally induced itch is increased in patients with CU compared to healthy controls and patients with AD. Additionally it is of interest whether psychological variables are predictors of itch-increase in patients with CU.

**Methods:** Thirty-six CU-patients, 36 AD-patients and 36 healthy controls, who are stratified according to age and gender, are video-recorded while watching three videos, which last 9:30 min each, in a randomized order: a video on crawling insects (AV), a video on skin-diseases (SV) and a control-video (CV), which deals with skin as a communication-organ. AV and SV are combined to an experimental video (EV). Counted scratch-movements and perceived itch are assessed as dependent variables. Furthermore psychological variables namely personality traits, private and public self-attention, depression and anxiety as well as appraisal of touching, shame and disgust were assessed using validated questionnaires.

**Results:** Preliminary results ( $n = 18$  per group; 13 female, five male) indicate that subjective itch can also be induced in patients with CU by the presentation of audio-visual stimuli ( $P < 0.05$ ). Counted scratch movements increased by trend in this patient-group ( $P = .066$ ). Patients with CU additionally do not differ from AD-patients and healthy controls in mentally induced itch- and scratch-increase. Interestingly an increase in scratching-behavior could be predicted by psychological variables in patients with CU. High anxiety scores as well as high private self-attention and low public self-attention are able to explain 71% of variance in scratch-increase in patients with CU. This could not be shown for healthy controls.

**Discussion:** So far this study showed that itch can also be induced in patients with CU by audiovisual stimuli. Besides, increase in scratch-movements is highly associated with psychological variables. This relationship could be of therapeutic use: One might assume that a reduction in e.g. anxiety by itching training programs could go along with alterations in sensitivity to itch-inducing situations. In future studies we will widen the spectrum of skin-patients by e.g. also including patients with psoriasis. It is also of interest to assess the scratching-behavior that occurred right after the videos ended, when subjects were not aware that they were still video-recorded, because in our impression subjects suppressed scratching while watching the videos, because they knew that they were video-recorded, but started scratching afterwards. One further step will also be to compare the intensity of mentally induced itch to the intensity of itch that is induced by histamine-iontophoresis.

#### P244

##### TrpV1 and TrpV3 are upregulated in chronic pruritus skin

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Patients suffering from chronic pruritus show a broad clinical variation in experiencing itch. Around 23% of patients experience additional thermal sensations (warm and cold). The present study was designed to investigate the cutaneous expression of temperature sensing transient receptor potential (TRP) channels in the skin of pruritus patients. Initially, pathological thresholds for cold and heat were determined by the method of quantitative sensory testing (QST). Skin biopsies of patients and healthy volunteers were subjected to RNA extraction and cDNA synthesis. Thermo-Trp channel expression was analyzed by the quantitative RealTime PCR technique including specific intron-spanning primers. Our investigation focused on general differences between healthy and pruritus patients and more particularly on a potential linkage between pruritus sub-phenotypes and distinct expression patterns. All thermo-Trp channels were detected in the analyzed skin biopsies with TrpV1 always showing the highest expression level. Comparative analysis revealed a weak but significant increase in TrpV1 and TrpV3 RNA transcript levels in pruritus patients over the healthy control group. However, expression profiles between both pruritus subgroups did not show significant differences. We therefore suggest that apart from TrpV1 also TrpV3 may be involved in the pathophysiology of the symptom pruritus at least in patients with abnormal thermal perception.

#### P245

##### Incidence of chronic pruritus and its determinants: results from a population-based study

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**Background:** While previous studies have showed a 12-month prevalence of chronic pruritus (>6 weeks) of 16.4% and a lifetime prevalence of 22% in the general population, data on its incidence and determinants in the community are lacking.

**Material and Methods:** A cohort of 1190 participants from a cross-sectional study on the prevalence of chronic pruritus (baseline study) participated in a follow-up study 1 year later. Participants completed a questionnaire covering the occurrence and characteristics of chronic pruritus, lifestyle variables, as well as social and psychological constructs. Incident chronic pruritus was defined as reported pruritus at follow-up in those free of disease at baseline. Cross-sectional analyses of data collected at follow-up were conducted to assess determinants of chronic pruritus. 1.3% ( $n = 15$ ) of the 1190 individuals died between baseline and follow-up. Of those alive ( $n = 1175$ ), 79.2% ( $n = 943$ ) participated in the follow-up study.

**Results and Conclusions:** The 1-year incidence of chronic pruritus in the general population was found to be 7.0%. Cross-sectional analyses revealed those reporting chronic pruritus to be significantly older than those who did not. Although more females than males reported incident chronic pruritus, the difference was not statistically significant. Those with chronic pruritus scored significantly lower on the social support and significantly higher on the neuroticism scale, the Hospital Anxiety and Depression Scale-Anxiety, the Hospital Anxiety and Depression Scale-Depression and the hypochondria scale. The prevalence of dry skin was also significantly higher in those reporting chronic pruritus. This is the

first study investigating the incidence of chronic pruritus and its determinants at the population level. These new data may help identified risk factors for the development of chronic pruritus.

#### P246

##### FAAH- and AMT- inhibitors exhibit anti-inflammatory and antipruritic effects: a role of the endocannabinoid system in atopic dermatitis

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Chronic pruritus in atopic dermatitis still constitutes a great therapeutic challenge. For some time the endocannabinoid system (ECS) in human skin is being investigated to determine the pathways through which it is involved in the sensation of pruritus. Endocannabinoids such as anandamide (AEA) are up-regulated in inflamed skin and mediate anti-inflammatory and antipruritic effects by binding to their receptors (cannabinoid receptors (CB) 1 and 2) on keratinocytes and mast cells. The duration of action is limited by the transport of AEA via a transporter protein (AMT) into the cells and subsequent degradation by the enzyme fatty acid amide hydrolase (FAAH). To increase the extracellular endocannabinoid levels in inflamed skin and to enhance their duration of action, we synthesized inhibitors of AMT and FAAH WO20100083-440 and WO20100083-479 which do not bind to CB. Starting point for the medicinal chemistry program was an alkylamide from purple coneflower (*Echinacea* spp.), which interacts with the ECS by activating CB2. We first investigated in primary cultured mast cells, isolated from human healthy skin, the effect of our compounds on the degranulation and the release of histamine after 30 min stimulation with substance P. Both substances WO20100083-440 and WO20100083-479 showed *in vitro* the potency to inhibit mast cell degranulation, as measured by histamine release, using an ELISA method. On RNA expression level the influence of the substances on the cannabinoid receptor 2 and the enzyme FAAH was assessed in a human mast cell line. The data suggest a regulation of the expression after stimulation with the novel compounds within the first 8 h. In keratinocytes isolated from human skin we analyzed the release of different cytokines after stimulation with substance P and with AEA and/or our compounds present. After 6 h, we found a decrease in the interleukin-1 alpha level compared to the controls in WO20100083-440 while WO20100083-479 showed no effects. In a disease specific animal model using BALB/c mice, the *in vivo* potency of both substances was evaluated. A contact dermatitis was induced by oxazolone (1% in 100 l acetone) with sensitization on day 0 and challenges on days 7, 9 and 11. Compounds WO20100083-440 and WO20100083-479 (1% in 100 l acetone) were applied from day 11 to day 18. A significant reduction of pruritus as measured by electronic scratch movement recording for 22 h after application of the substances was observed on day 11 but not on day 18. On day 19, all mice ears were subjected to histological investigation. Both substances reduced epidermal thickness and spongiosis, total ear thickness, and inflammatory infiltrate density in comparison to vehicle only treated mice. The reduction of the parameters, however, was less pronounced as seen for the positive control betamethasone dipropionate (0.05% in 100 l acetone).

Together, these data demonstrate that FAAH- and AMT- inhibitors inhibit substance P-induced mast cell degranulation *in vitro* and exhibit anti-inflammatory and anti-pruritic effects *in vivo* upon topical administration. Pruritus reduction occurred shortly after application of the substances but did not show a long-term effect, which is currently investigated.

#### P247

##### The TRPM8 agonist cooling compound®; improves chronic itch and quality of life in pruritic patients

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Cooling the skin by menthol quickly relieves itch and is often used by chronic pruritus patients. In a vehicle-controlled, double-blind study including 71 chronic pruritus patients with dry skin, 36 patients were treated with a lotion containing the newly developed TRPM8 agonist Cooling Compound® (CC) (group 1: 22 f, 14 m; mean age 54.3 years) and 35 patients with the same lotion without CC (group 2: 25 f, 10 m, mean age 56.9 years). The lotion was applied twice daily for 4 weeks followed by a follow-up period of 2 weeks without treatment. At every visit (V), next to clinical investigation and measurement of skin dryness with corneometry, patients filled in several self-reporting questionnaires including scales and questions concerning the cooling effect of the lotion, intensity of pruritus, quality of life (DLQI) and patient-relevant benefit, the last with the help of the Patient Benefit Index Pruritus (PBI-P) questionnaire. Eight patients dropped out of the study in group 1 (with CC) and three in group 2 (without CC). The drop outs were due to side effects ( $n = 2$ , one of each group) or without giving a reason (group 1,  $n = 7$ ; group 2,  $n = 2$ ). In total, 60 patients (group 1;  $n = 28$ ; group 2,  $n = 32$ ) patients completed the study.

At both V2 (2 weeks) and V3 (4 weeks), a significantly larger number of patients in group 1 reported a cold sensation ( $P < 0.05$ ). Comparison of the median duration of the cooling effect showed a longer duration of action in group 1 (median, 70 min at V2) in comparison to group 2 (median, 2 min at V2) at both visits. Concerning the overall rating of the effect, 76.9% (V3) to 90.9% (V2) of patients of group 1 rated the effect as being quite to very strong which was more than in group 2 (50.1–53.8%). At V3, significantly more patients of group 1 (with CC) reported an improvement of itch/burning and tightness of the skin compared to group 2 ( $P < 0.5\%$ ). Pruritus intensity (assessed by global rating, reduction in percent and a five-point Likert scale,  $P < 0.05$ ) showed in both groups significant improvement without significant group differences. According DLQI ( $P < 0.01$ ) a significant improvement in group 1 compared to the group without CC could be observed. The two groups did not differ significantly in PBI-P. Descriptively, the calculated total benefit score of 1.9 in group 1 (with CC) was higher than the score of 1.6 in group 2 (without CC).

Measurement of skin lubrication with corneometry revealed significant improvement ( $P < 0.001$ ) in each treatment group from V1 to V3 without a significant difference between the groups indicating improvement of dry skin with and without CC. The lotion with CC also improved but did not damage the skin barrier. In this study, high concentrations of CC were used. As a consequence, an intense cold sensation more often caused adverse side effects in group 1 (with CC) such as extreme feelings of cold and burning on mucosa (group 1: 68.6% vs group 2: 14.6%).

Overall, both groups tolerated the lotions well. In summary, treatment of dry, pruritic skin with Cooling Compound® reduces chronic pruritus of different origins and represents an interesting new adjunct treatment option in chronic pruritus.

#### P248

##### Dual role of cutaneous RANK-RANKL signaling during skin carcinogenesis

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The TNF-family receptor RANK and its ligand (RANKL, CD254) play a key role for the regulation of immune responses. In a transgenic mouse model (K14-RANKL tg) we could show that cutaneous overexpression of RANKL resulted in the peripheral expansion of regulatory T cells (Treg) via interaction

with RANK-RANKL activated antigen-presenting cells (APC). Since Treg are involved in the surveillance of anti-tumoral immunity, we investigated the role of RANK-RANKL signaling for skin tumor development comparing both, chemically induced and UV-induced cutaneous carcinogenesis. Interestingly, in a two-stage chemocarcinogenesis model consisting of a single DMBA treatment followed by repeated applications of TPA, K14-RANKL tg mice developed fewer tumors than wildtype (wt) controls. Whereas tumors of wt mice showed a high degree of dysplasia, tumors of tg mice presented as small papillomas with less invasive cells. Moreover, increased numbers of cytotoxic CD8+ T cells (CTL) expressing granzymes and perforin as well as the CTL-specific transcription factors Runx3 and Eomes, were detectable in tumor-draining lymph nodes from tg mice. Since during skin carcinogenesis cutaneous APC migrate from the skin to regional lymph nodes where they induce MHC class I-mediated anti-tumoral immune responses, we analyzed the phenotype of APC in skin-draining lymph nodes from wt and tg mice after DMBA/TPA treatment. Notably, APC from K14-RANKL tg mice showed a prolonged life-span, an up-regulated expression of activation markers, like CD80, CD86 and IL-12, as well as an increased T cell stimulatory capacity, which could explain the elevated numbers of CTL in tg mice and furthermore, might suggest that cutaneous RANK-RANKL signaling, in combination with the repeated application of the pro-inflammatory phorbol ester TPA, results in the up-regulation of MHC class I-mediated anti-tumoral immunity. However, upon chronic UV irradiation in a photocarcinogenesis model K14-RANKL tg mice developed significantly more skin tumors than wt controls. Analysis of tumor-draining lymph nodes revealed substantially increased numbers of Treg in UV-irradiated tg mice compared to wt controls. Notably, Treg from tg mice were characterized by reduced levels of neuropilin-1 and PD-1 as well as a decreased expression of Helios, a transcription factor that is present in thymic derived but absent in peripherally induced Treg. Together, these data point to the peripheral induction of Treg, which might suppress MHC class I-mediated anti-tumoral immune responses in K14-RANKL tg mice upon chronic UV irradiation. This hypothesis was strengthened by the observation that decreased numbers of CTL expressing granzymes, perforin, Runx3, and Eomes were detectable in regional lymph nodes from UV-irradiated tg mice compared to wt mice. To decipher the mechanism underlying UV-mediated Treg induction in K14-RANKL tg mice we analyzed the phenotype of cutaneous APC. Interestingly, in contrast to APC from wt mice, APC from tumor-draining lymph nodes of UV-irradiated tg mice were characterized by an up-regulated expression of CD205, CD209, and IL-10, markers which are associated with tolerogenic APC known to induce functional Treg. In summary, our results suggest a dual role of RANK-RANKL signaling during skin carcinogenesis. Treatment of K14-RANKL tg mice with DMBA/TPA resulted in the activation of cutaneous APC, the expansion of CTL, and subsequently, the induction of MHC class I-mediated anti-tumoral immune responses. On the other hand, chronic UV irradiation of K14-RANKL tg mice induced tolerogenic APC, which mediate the peripheral induction of Treg able to suppress MHC class I-mediated anti-tumoral immunity.

#### P249 (V10)

##### The subcompartment-specific distribution of differentially cycling tumor cells in melanoma is regulated by MITF

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Dysregulated proliferation is a hallmark of cancer progression. Thus, by outcompeting negative regulatory signals, cell division drives the growth of primary tumors and metastasis. The molecular mechanisms that uncouple malignant cells from regulatory cues have been subject to intense investigation – indeed, great insight has been attained into the genetics and molecular pathways that regulate the cell cycle in cancer cells, and this has led to the development of promising new drugs that interfere with these pathways. One aspect of cancer cell proliferation has, however, received little attention: what are the dynamics of cell division of individual malignant cells within the complex microenvironment of a tumor?

We have developed a model to visualize melanoma cell cycle dynamics in real-time *in vitro* and *in vivo*. Cells transfected with the fluorescence ubiquitination cell cycle indicator (FUCCI) appear red in G1, yellow in S and green in S/G2/M-phase with a fluorescence gap during cytokinesis. FUCCI-melanoma cells were grown as 3D-spheroids and implanted into a collagen matrix to mimic tumor architecture and microenvironment, or as xenografts in NOD/SCID mice.

In our 3D-spheroids, initially the ratio of red:green melanoma cells was roughly equal and distributed randomly. Within hours the interior cells became slow-moving and arrested in G1, while peripheral cells were cycling and highly motile, invading cell cycle phase-independently the collagen away from the spheroid. We sorted and cultured the interior cells in 2D. Live-cell imaging revealed that the G1 arrested population re-entered the cell cycle, however with a lag compared to the 'outer' population. This novel FUCCI-melanoma spheroid model has allowed us to mimic conditions that occur in tumors *in vivo* and determine their effects on cell cycle dynamics. Expression levels of microphthalmia-associated transcription factor (MITF) were higher in 'proliferative' melanoma cell lines. Using confocal microscopy we have determined that cells at the periphery of the spheroids derived from invasive (MITFlow) cell lines are cycling and highly motile, invading into the collagen surrounding the spheroid, while cells in the interior of the spheroid are mostly arrested in G1 and slow moving. In contrast, proliferative (MITFhigh) cell lines appear to show less invasion and a more random cell cycle progression pattern. Intravital multiphoton microscopy of FUCCI-melanoma xenografts visualizing cell motility relative to intact tumor vasculature in live mice revealed that the tumors indeed can be divided into two groups: Xenografts derived from MITFhigh melanoma lines proliferate heterogeneously throughout the tumor, while MITFlow lines proliferate predominantly at the tumor periphery and in close proximity to capillaries, while most cells further away from oxygen and nutrient supply arrest in G1.

Our data suggest that MITF expression dictates the subcompartment-specific distribution of differentially cycling tumor cells in melanoma, which may result in differential sensitivity to apoptosis and therefore may contribute to the resistance of melanoma to therapy.

#### P250

##### New strategies to overcome therapy resistance in melanoma-derived brain metastasis: Inhibition of the PI3K-AKT signaling pathway

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Brain metastases occur in over 70% of patients with metastatic melanoma and are the most common cause of death. Current therapy options are neurosurgery, radiosurgery, whole brain radiation, chemotherapy and supportive care. The median survival time for melanoma patients with brain metastasis ranges from 0.7 to 5 months. Therefore, new therapy strategies are mandatory.

In melanoma, activation of the RAF-MEK-ERK and PI3K-AKT-mTOR signaling pathways makes a decisive contribution to tumor progression and treatment resistance. Clinical studies suggest a transient effect of BRAF inhibitors in melanoma brain metastases. We asked if inhibition of these pathways would be a promising strategy for the treatment of melanoma brain metastases. We blocked both pathways at different levels and investigated the effects on viability/proliferation and survival/apoptosis of

>10 newly isolated cell lines derived from melanoma brain metastases. Furthermore, immunohistochemical analyses of brain metastases from >10 melanoma patients including matched extracerebral metastases for p-ERK, ERK, p-AKT, AKT and PTEN were performed. Growth inhibition was most pronounced with PI3K inhibitors achieving growth inhibition rates of up to 80%. Moreover, PI3K inhibitors potentially induced apoptosis in cerebral metastatic melanoma cells. Immunohistochemically, p-ERK was seen predominantly at the tumor periphery of both cerebral and extracerebral metastases. Interestingly, most melanoma brain metastases were highly positive for activated AKT, whereas matched extracerebral metastases in the same patients were weakly positive or negative for activated AKT. PTEN expression was downregulated in brain metastases. PTEN expression and AKT activation in the brain appeared to be independent of BRAF and NRAS mutation status. Together, these findings suggest that activation of AKT is relevant for the survival and growth of melanoma cells in the brain parenchyma and that inhibition of PI3K-AKT signaling may be a suitable strategy to enhance and/or prolong the antitumor effect of BRAF inhibitors in melanoma brain metastases.

#### P251

##### Characterizing the role of SOX9 and SOX10 in melanoma

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The transcription factors SOX9 and SOX10 are crucial for the embryonic development of melanocytes. SOX10 is expressed in melanoblasts *in vivo*, while SOX9 is upregulated in differentiating melanocytes. Previous studies showed that SOX9 and SOX10 activated the same target genes like microphthalmia-associated transcription factor (MITF) and melanogenic enzymes. However, it is not clear whether SOX9 acts independently or in parallel with SOX10. Recently, both transcription factors have been detected in melanoma and our group has demonstrated synergistic regulation of the intermediate filament nestin by SOX9 and SOX10. But the function and further downstream target genes of these factors in melanoma remain unclear.

We performed expression studies of both SOX9 and SOX10 in primary and metastatic melanoma cell lines. SOX9 showed varying expression levels in nine tested cell lines, while SOX10 was significantly lower in all melanoma cell lines compared to melanocytes. In the majority of melanoma cell lines inverse expression levels of SOX9 and SOX10 were observed.

We then analyzed putative target genes of SOX10 in melanoma. One candidate was the receptor tyrosine kinase ERBB3, which has been linked with SOX10 in neural crest derived cell lines and associated with metastatic progression in melanoma. Expression levels of ERBB3 paralleled those of SOX10 in different melanoma cell lines and downregulation of SOX10 by siRNAs resulted in reduced expression of ERBB3. Another candidate target gene was pleiotrophin, a growth factor which is overexpressed in melanoma. Pleiotrophin was shown to be a target gene of SOX10 in Schwannoma cells. In agreement with these results, our data suggest that pleiotrophin is regulated by SOX10 also in melanoma cells.

In summary, we identified two new target genes of SOX10 in melanoma, i.e. ERBB3 and pleiotrophin, both associated with tumor progression, and thus highlighting the key regulatory function of these transcription factors in melanoma.

#### P252

##### Loss of Noxa delays melanomagenesis in a predisposed, genetically-defined mouse melanoma model

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Metastatic melanoma is highly therapy-resistant and standard chemotherapy has failed in clinical trials. Resistance to chemotherapeutics is often attributed to defective apoptosis pathways. Common mutations in melanoma include constitutive activation of the MAPK and PI3K pathways and loss of the CDKN2a gene which encodes for the p16INK4A and p14ARF (p19ARF in mouse) proteins. These mutations lead to aberrations in the intrinsic apoptosis pathway. Studies linking the intrinsic apoptosis pathway to melanomagenesis and drug resistance have only been shown *in vitro* but not yet *in vivo*. In this study we utilised, a mouse melanoma model, in which the Cdkn2a locus is knocked-out, and HRAS is constitutively active (Cdkn2a<sup>-/-</sup>, Tyr-RAS; from here on referred to as 'control'). This transgenic mouse was crossed to either a Noxa or a Puma knockout mouse resulting in Cdkn2a<sup>-/-</sup>, Tyr-RAS, Noxa<sup>-/-</sup> (referred to as 'Noxa-KO') or Cdkn2a<sup>-/-</sup>, Tyr-RAS, Puma<sup>-/-</sup> (referred to as 'Puma-KO'). PUMA expression was shown to decrease during progression from dysplastic naevus to metastatic melanoma. Previously, we showed that Puma over-expression appeared lethal to melanoma cells and that Noxa over-expression sensitised melanoma cells to temozolamide and other drugs including BH3 mimetic ABT-737. PUMA is a pan-inhibitor of the anti-apoptotic proteins, whereas NOXA specifically inhibits BFL-1 and MCL-1. Therefore, in our model significance of BFL-1 and MCL-1 in melanomagenesis and drug resistance can be ascertained and compared to the other anti-apoptotic proteins (BCL-2, BCL-XL and BCL-w).

Three groups of 30 mice per genotype (control, Puma-KO and Noxa-KO) were compared for tumor latency, penetrance, location and growth. Fifteen mice of each group were UVB irradiated when 3 days old.

Irrespective of UVB irradiation or not, Noxa-KO significantly delayed melanomagenesis (102 days, UVB; 134 days, no UVB) compared to control (90 days, UVB; 88 days, no UVB) and Puma-KO (75 days, UVB; 81 days, no UVB). This resulted in 94% (UVB) and 81% (no UVB) tumor penetrance in Noxa-KO compared to 100% penetrance in controls and Puma-KO. Once tumors were established, Noxa-KO accelerated tumor growth compared to Puma-KO. Interestingly, controls and Puma-KO developed more tumors on the ears than Noxa-KO. Whereas limited tumor numbers on the ear were observed in non-UVB Noxa-KO, none developed in UVB irradiated Noxa-KO mice. Tumor invasion was studied by generating *in vitro* three-dimensional melanoma spheroids from cells isolated from the tumors. In this model spheroids derived from Noxa-KO melanomas showed inhibited spheroid invasion compared to control and Puma-KO spheroids.

Noxa-KO delayed melanomagenesis, decreased tumor penetrance and spheroid invasion but accelerated tumor growth. We speculate that loss of Noxa may be rescued by induction of other BH3-only pro-apoptotic proteins e.g. Puma, which could be responsible for the delayed melanomagenesis and limited spheroid invasion. This suggests that, once tumors are established, Noxa may not be required for melanoma progression.

#### P253 (V22)

##### EphA2 as a target for adenoviral gene transfer or oncolysis of malignant melanoma

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At the stage of metastasis development malignant melanoma is a highly aggressive disease with poor prognosis and no efficient treatment options. A potential new treatment modality, which is currently

under clinical investigation for different cancers, is the use of oncolytic adenoviruses (OncAds). A characteristic of OncAds is their capability to replicate preferentially in tumor cells and not in normal cells. The progeny virus is released and can infect further tumor cells which were not infected after initial virus application. Moreover, OncAds can be 'armed' with therapeutic genes encoding, for example, immunostimulatory cytokines, inducers of apoptosis or prodrug activating enzymes. Safety and tumor specific replication was shown for first generation OncAds in clinical trials.

However, therapeutic efficiency needs to be improved. One reason for the limited efficiency is poor entry into tumor cells. The most commonly used adenovirus is subtype 5 (HAdV-5), which binds to CAR as its natural receptor. CAR is ubiquitously expressed on normal cells but poorly or not expressed in patients tumors. To ensure an efficient and specific uptake into tumor cells, the natural tropism must be ablated and a new specificity towards receptors which are overexpressed in melanomas must be introduced.

We genetically introduced a short 12mer peptide sequence with specificity for the receptor tyrosine kinase EphA2 into the capsid protein fiber and analyzed the capability of this peptide to mediate an EphA2-specific viral cell entry. EphA2 is overexpressed on most types of cancer including malignant melanoma. Here expression level correlates with aggressiveness, a metastatic phenotype and vasculogenic mimicry, making this receptor to a promising target for treatment of advanced melanoma.

EphA2 expression was detected in five of six melanoma cell lines tested by western blot. In particular the aggressive metastatic cell line C8161 expresses high levels of EphA2. Transduction experiments with EphA2-targeted reporter viruses revealed a strong correlation between EphA2 status and transduction efficiency, which was enhanced up to 2 log in comparison to control viruses. Peptide competition experiments demonstrated specificity of the EphA2 interaction. Furthermore, transient EphA2 expression in EphA2 negative cells showed an increase of reporter gene expression up to 2 log in comparison to mock transfection. Next we initiated biodistribution studies in nude mice with subcutaneous tumors or lung metastasis of C8161 cells and quantified reporter gene expression in the tumor/lung in comparison to the liver. Preliminary data show a significant higher tumor/lung to liver ratio for EphA2 targeted viruses in comparison to a non targeted control virus.

To overcome possible obstacles associated with heterogeneous tumors, we currently extend this single targeting strategy towards a double targeting approach. Here, we combine EphA2 targeting with a simultaneous targeting towards further cancer specific receptors by genetically insertion of corresponding peptides into other loops of the fiber protein. In a proof of principle experiment we successfully combined EphA2 targeting with a targeting towards the transferrin receptor or integrins. Transduction experiments *in vitro* revealed that in the absence of EphA2, cell entry is efficiently mediated by the alternative receptors.

Our study establishes a capsid-engineered adenovirus for melanoma-targeted gene transfer and oncology.

## P254 (V29)

### Developing chemotherapeutics which selectively disable the actin cytoskeleton of tumor cells

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The actin cytoskeleton is an important regulator of a variety of cellular functions including cell motility, adhesion, and proliferation, making it an ideal chemotherapeutic target. Despite this promise there are still no anti-actin compounds used in current chemotherapy, primarily due to the inability of existing anti-actin agents to discriminate between the actin cytoskeleton of tumor cells and the actin filaments of the muscle sarcomere. We have previously shown that tropomyosin, an integral component of the actin cytoskeleton, defines functionally distinct populations of actin filaments. We have identified a specific tropomyosin isoform common to all tumor cells tested to date which regulates cell proliferation and have designed a new class of compounds to target this filament population. Anti-tropomyosin (Tm) compounds were selected based on their ability to target the actin cytoskeleton and their efficacy against a panel of neuroblastoma and melanoma cell lines. The lead compound, TR100, was shown to be effective against a panel of tumor cell lines with an average EC<sub>50</sub> of 2–3 M. When tested in 3D melanoma spheroid models, which more accurately mimic the tumour microenvironment, TR100 inhibited melanoma cell growth and motility. This effect translated to a reduction in tumor cell growth *in vivo* in both neuroblastoma and melanoma xenograft models. *In vitro* data using isolated rat cardiomyocytes demonstrated that TR100 had minimal impact on contractile function. *In vivo* data from the drug treated animals also showed no evidence of cardiac damage as measured by blood Troponin I levels and no changes in the intraventricular septum thickness of isolated hearts. These results demonstrate that it is possible to target distinct actin filament populations based on the tropomyosin composition. Next generation anti-Tm compounds with improved efficacy and specificity have now been developed. Preliminary data demonstrate that these compounds exhibit increased selectivity for transformed cells.

Taken together, our findings suggest that the anti-Tm compounds show a significant improvement in the therapeutic window compared to existing anti-actin agents. This novel approach and the development of the new class of anti-Tm compounds may be the key for disabling a long sought after target, the actin cytoskeleton, and may lead to a new class of chemotherapeutics active against a broad range of cancer types.

## P255

### Demethylation of the Brn3a locus causes expression of Brn3a in melanocytes

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The transcription factor Brn3a (POU4F1) is expressed in neural crest cells and influences differentiation and survival of progenitor cells that are destined to become sensory neurons. Similar to neuronal cells, melanocytes develop from the neural crest. We studied neural crest factors in melanoma and observed that Brn3a is highly expressed in human melanoma cells, but not in primary human melanocytes or fibroblasts. Inhibition of Brn3a in melanoma demonstrated that Brn3a is essential for cell proliferation and survival. However, the factors that drive Brn3a expression in melanoma are not known. In differentiated tissue, long-term gene silencing is often maintained by epigenetic mechanisms, e.g., methylation of CpG-rich DNA regions. Analysis of the Brn3a gene locus revealed two large CpG islands within the 5' promoter region. We, therefore, tested whether Brn3a is silenced in melanocytes via promoter methylation. Conversely, loss of DNA methylation may cause re-expression of Brn3a in melanoma cells. When treating melanocytes with the DNA-demethylating reagent 5-aza-2'-deoxycytidine, a 3–6-fold increase in Brn3a expression was observed. Treatment of primary fibroblasts led to a lower increase compared to melanocytes, suggesting that additional factors are involved in Brn3a

expression, which are lineage-specific. Because upregulation of Brn3a in melanocytes by 5-aza-2'-deoxycytidine did not reach the levels observed in melanoma cells, also tumor-specific epigenetic or genetic mechanisms contribute to Brn3a expression in melanoma.

Next, the genetic regulation of Brn3a was examined by analyzing the 5' region of the Brn3a gene. Promoter deletion studies of 5 kb region upstream of the Brn3a translation start site were performed. Luciferase reporter assays revealed that the proximal 1000 bp region is required to promote full promoter activity in melanoma cells, whereas Brn3a-negative fibroblasts showed no reporter activity. Subsequently, transcription factors that putatively bind in this region were identified by *in silico* analysis. In conclusion, the data show that demethylation of the Brn3a gene locus leads to re-expression of Brn3a in melanocytes suggesting that promoter demethylation contributes to Brn3a expression in melanoma cells. In addition, lineage-specific factors as well as other tumor-specific factors are involved in Brn3a expression.

## P256

### Proinflammatory adhesion molecules can be induced on metastatic melanoma vasculature in a xenograft model

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Effective antitumor immune responses require lymphocytic infiltration into melanoma tissue. However, analysis of human metastatic melanoma has shown that tumor blood vessels weakly express leukocyte adhesion receptors thereby impairing entry of cytotoxic lymphocytes. Based on these observations, we aimed to generate a melanoma xenograft mouse model that allows investigation of human melanoma vessels and their modulation *in vivo*. Using this model we wanted to explore whether endothelial activation can induce relevant adhesion molecules on tumor blood vessels for improved lymphocyte extravasation into melanoma tissue.

To test whether adhesion molecules on melanoma vasculature can be induced, we incubated human melanoma metastases with either interferon- $\gamma$ , histamine or TNF- $\alpha$  *in vitro*. In contrast to interferon- $\gamma$  and histamine, TNF- $\alpha$  incubation resulted in fourfold increased expression of ICAM-1 and 72-fold increased expression of E-selectin on tumor vasculature compared to controls detected by qRT-PCR and immunofluorescence staining.

To study vascular adhesion receptor expression and lymphocyte extravasation within human melanoma we characterized tumor blood vessels in a melanoma xenograft mouse model. For this purpose human melanoma metastases were engrafted subcutaneously onto immunodeficient NSG (NOD.Cg-Prkdcscid Il2rgtm1Wjl/Sz) mice. Human blood vessels were preserved inside human melanoma grafts and connected to mouse circulation 2 weeks after transplantation. Simultaneously murine vessels started to infiltrate melanoma tissue. Four weeks after transplantation murine vessels outbalanced human vessels impairing specific studies of human vessels. Tumors showed proliferative activity and histological appearance was similar to melanoma tissue before engraftment. While adhesion molecule expression was moderate on tumor vessels post transplantation intrasclerous injection of TNF- $\alpha$  induced significantly increased expression of ICAM-1 and E-selectin compared to controls.

These findings suggest that subcutaneous transplantation of human metastatic melanoma tissue is well suited to investigate human tumor vasculature in a restricted time frame of 14–28 days after transplantation. Moreover, adhesion molecule expression on tumor blood vessels after transplantation was moderate, but can be significantly increased through intratumoral injection of TNF- $\alpha$ . Ongoing studies examine whether induction of vascular adhesion molecules in grafted melanoma tissue results in increased infiltration of transferred human lymphocytes.

## P257 (V32)

### P-cadherin expression in primary Merkel cell carcinomas is associated with prolonged recurrence-free survival

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Merkel cell carcinomas (MCCs) are uncommon but aggressive skin tumors, associated with advanced age and immunosuppression. Merkel cell polyoma virus (MCV) is believed to be causative for MCC in the majority of cases. As development and progression of cancer can be promoted by changes in the repertoire of cell adhesion proteins, we have previously analyzed homo- and heterotypic cell-cell contacts of normal Merkel cells and MCCs. We observed that in normal human epidermis Merkel cells are interacting with keratinocytes via E- and P-cadherin whereas >90% of MCCs contain N-cadherin and only a smaller proportion E- and P-cadherin, indicating a cadherin switch during carcinogenesis. This prompted us to study the occurrence and prognostic impact of E-, N-, and P-cadherin in a larger series of MCCs. One-hundred forty-eight paraffin-embedded MCCs from 106 patients (72 primary tumours, 17 local recurrences, 22 lymph node metastases, two in transit and 27 distant metastases) were analyzed by double-label immunostaining and immunofluorescence microscopy. In accordance with our previous findings, 92% of all MCC were positive for N-cadherin whereas only 61% or 69% expressed E- or P-cadherin, respectively. Interestingly, P-cadherin was detected significantly more frequently in primary tumors than in lymph node metastases (82% vs 41%,  $P = 0.0002$ ). Moreover, patients with P-cadherin-positive primary tumors were in earlier tumor stages at initial diagnosis as compared to patients with P-cadherin-negative primary tumors ( $P = 0.0054$ ). Both in Kaplan-Meier analysis ( $P = 0.0065$ ) and in Cox proportional-hazards regression analysis adjusting for age, sex, immunosuppression, stage at initial diagnosis and MCV status ( $P = 0.0179$ ), patients with P-cadherin-positive primary MCCs had significantly longer recurrence-free survival than those with P-cadherin-negative primaries (mean: 25.15 vs 11.40 months). However, P-cadherin expression had no influence on tumor-specific and overall survival. This discrepancy might be attributable to the observation that P-cadherin may frequently be restored in distant metastases, 68% of which were P-cadherin-positive. Using real time PCR, MCV DNA was detected in 78% of all MCC, but MCV presence did neither correlate with expression of cadherins nor with recurrence-free, tumor-specific and overall survival. Assuming that MCCs arise from Merkel cells, our results suggest that P-cadherin may be lost during development of primary MCCs and metastatic spread to the lymph nodes but is sometimes re-expressed in distant metastases, in a process that resembles reverting epithelial to mesenchymal transition. Notably, P-cadherin expression in primary MCCs may have favourable prognostic impact, i.e. a prolonged recurrence-free survival.

## P258

### Melanoma cells utilize Thy-1 (CD90) on endothelial cells for metastasis formation

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The adhesion of circulating cells to endothelial cells plays a central role in inflammation as well as in tumour metastasis. Recently, we characterized the human glycoprotein Thy-1 (CD90) as an activation-associated adhesion molecule on human dermal microvascular endothelial cells (EC). Thy-1 on EC mediates both the adhesion of granulocytes via interaction with Mac1 (CD11b/CD18) and the binding of melanoma cells via interaction with  $\alpha\beta3$  (CD51/CD61) *in vitro*. In the present study, we could show that Thy-1 is expressed on EC in human melanoma and metastases, whereas in healthy skin and naevi Thy-1 was not expressed on vessels. VEGF was identified as an inducer of Thy-1 expression on EC. The physiological role of Thy-1 in tumour metastasis was confirmed in a lung metastasis model using Thy-1<sup>-/-</sup> mice. Indeed, in Thy-1<sup>-/-</sup> mice the metastasis to lung was significantly reduced compared to wild type littermate controls. This indicates that tumour cells use Thy-1 on EC for adhesion and transmigration to facilitate their metastatic behaviour.

**P259**  
**An inflammatory microenvironment promotes dedifferentiation of Hgf-Cdk4R24C melanoma cells leading to impaired recognition by gp100-specific CD8+ T-cells**

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Regressive primary human melanomas with dense immune cell infiltrates more frequently contain poorly pigmented tumor cell subpopulations. Recently, it has been suggested that some of these dedifferentiated cells may have acquired a neural crest precursor cell phenotype that would allow progressively growing tumors to evade the surveillance function of T-cells recognizing antigens of the melanocytic lineage. However, experimental evidence that supports this hypothesis and provides insights into the underlying mechanisms is lacking. We experimentally investigated the impact of melanocyte-specific CD8+ T-cells on melanoma development by adoptively transferring TCRtg pmel-1 T-cells recognizing the melanosomal protein gp100 into Hgf-Cdk4R24C mice bearing primary melanomas in the skin. In this model antigen-specific CD8+ T-cells maintain tumor dormancy for an average of 3 months and induce an inflammatory response in the tumor microenvironment that in turn promotes the loss of T-cell effector functions and enables tumor recurrence. These tumors more frequently contain poorly pigmented tumor cell subpopulations expressing strongly decreased levels of gp100. Using the highly pigmented Hgf-Cdk4R24C melanoma cell line HCmel3 established from a primary tumor we could directly demonstrate that dedifferentiated tumor cells derived from inflammatory recurrent tumors escape recognition by gp100-specific pmel-1 T-cells in ELISPOT assays. Interestingly, we found that short-term exposure of HCmel3 melanoma cells to TNF *in vitro* not only stimulated the production of proinflammatory chemokines such as Cxcl2, Cxcl10, and CCL5 but also strongly decreased the protein levels of gp100. This resulted in impaired recognition by gp100-specific pmel-1 T-cells in ELISPOT assays. Prolonged *in vitro* culture of HCmel3 cells in FCS-rich media also leads to a gradual loss of melanocytic differentiation associated with decreased recognition by gp100-specific pmel-1 T-cells. Taken together, our results provide experimental evidence that an inflammatory microenvironment promotes the appearance of dedifferentiated melanoma cells that can escape the surveillance function of melanocyte lineage-specific T-cells. This establishes a novel, previously not described principle of immune escape for primary melanoma. In ongoing experiments we are characterizing the relationship between inflammation, dedifferentiation and the acquisition of a neural crest progenitor phenotype. Furthermore, we are investigating how the activation of proinflammatory signalling pathways converging on NFRB and AP1 affect the control of cellular proliferation and differentiation at the molecular level.

**P260**  
**Phosphorylation of YB-1 induces transcriptional activity and mediates melanoma cell survival and invasion**

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Y-box binding protein 1 (YB-1) is an oncogenic transcription and translation factor and is overexpressed in several types of cancer. Our previous data showed that YB-1 is upregulated and translocated to the nucleus during melanoma progression and that YB-1 is an important transcription factor regulating proliferation, survival, migration, invasion and chemosensitivity of melanoma cells. Here, we show that during melanoma progression *in vivo* YB-1 expression as well as Ser102 phosphorylation as a marker of nuclear activation is increased. We further characterize the mechanisms governing the expression and activity of YB-1 in melanoma cells. We show that the PI3K/AKT and p53 signaling, growth factors and chemotherapeutic agents increase YB-1 promoter activity. However, this is only marginally related to YB-1 protein level. Nuclear translocation and transcriptional activation of YB-1 was reported to be mediated by Ser102 phosphorylation in the nucleic acid binding domain. We demonstrate that the MAPK and PI3K/AKT signaling pathways, both activated in melanoma cells, as well as p53 overexpression increase Ser102 phosphorylation of YB-1, whereas NFB signaling inhibits the phosphorylation. The specific inhibition of Ser102 phosphorylation of YB-1 blocks YB-1 transcriptional activity. On a functional level the inhibition causes reduced melanoma cell invasion and survival whereas cytoplasmic YB-1 negatively regulates RNA translation and inhibits proliferation. We hypothesize that primarily the activation of YB-1 by phosphorylation augments the effects of YB-1 during melanoma progression towards metastasis.

**P261**  
**Regulation of G2-M checkpoint in malignant melanoma**

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The G2-M checkpoint is critical for cell cycle progression and plays a central role for DNA repair before re-entry of cells into cell cycle. Although molecules involved in this process are commonly inactivated in malignant tumours, tumour cells retain key mechanisms of this checkpoint to avoid so-called mitotic catastrophe. Little is known about these mechanisms in malignant melanoma. Here we analysed the individual contributions of different molecules and pathways to G2-M checkpoint control in malignant melanoma. After putting melanoma cells under genotoxic stress by exposure to different chemotherapeutic agents, cells arrested in G2-M, paralleled by an induction of p53/p21, checkpoint kinase 1 (Chk1), WEE1 kinase and inactivation of Cdk1, the major cell cycle kinase for entry into mitosis. Inhibition of p53 and WEE1 by chemical inhibitors reduced this stress-induced G2-M arrest. Thus, both p53/p21 and WEE1/Cdk1 pathways seem to play a role in G2-M arrest in melanoma cells. Moreover, a direct interaction of Cdk1 with p21 was found under stress conditions. Even more important, blockage of the WEE1/Cdk1 pathway led to premature and enhanced induction of p53/p21, which involved common upstream activators in a positive feedback loop. Moreover, downregulation of WEE1 by siRNA induced enhanced expression and activity of p53/p21. Under conditions of WEE1 inhibition, significant levels of apoptosis were induced. Taken together, we identified important and as yet unknown mechanisms of G2-M checkpoint control in malignant melanoma. Under appropriate stress conditions, e.g., by co-treatment with specific chemotherapeutic agents, these may serve as tar-

gets for therapeutic intervention. Interestingly, WEE1 inhibitors are currently tested in clinical trials of other tumour entities.

**P262**  
**Most cells play a protumorigenic role in primary cutaneous lymphoma**

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Primary cutaneous lymphomas (PCLs) are a group of heterogeneous non-HodgkinEUR(TM)s lymphomas that originate in the skin. The interaction of PCL with immune cells of the tumor microenvironment remains poorly understood. Here, we investigated the role of mast cells in the tumor microenvironment of PCL. We found that mast cell numbers were significantly increased in skin biopsies from patients with PCL compared with normal skin. Infiltration of mast cells was particularly prominent in the periphery of PCL, at the invasive tumor front. Interestingly, PCL patients with a progressive course showed higher mast cell counts than stable patients, and mast cell numbers in different stages of cutaneous T-cell lymphoma (CTCL) correlated with malignancy. To investigate functional interactions between PCL and mast cells, we incubated primary PCL cells and PCL cell lines with supernatant of mast cells and observed strongly increased proliferation and production of cytokines upon stimulation with mast cells. Consistently, in a mouse model of PCL, we found significantly decreased tumor growth in mast cell-deficient transgenic mice. Taken together, these experiments show that mast cells play a protumorigenic role in PCL. Our data suggest that tumor-associated mast cells could serve as prognostic marker and therapeutic target in PCL.

**P263**  
**Long-term immune surveillance by gp100-specific pmel-1 T-cells induces an inflammatory tumor microenvironment in primary cutaneous Hgf-Cdk4R24C mouse melanomas**

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Primary human melanomas frequently show signs of regression associated with dense infiltration of antigen-specific CD8+ T-cells. Occasionally, tumors grow progressively and patients die of metastatic disease. According to the cancer immunoeediting theory, tumor progression is due to the emergence of tumor cell variants that escape T-cell recognition. As an alternative explanation, it has been shown that tumor cell-intrinsic changes driving malignant progression can induce an inflammatory microenvironment which promotes local T-cell tolerance. The relative importance of these two mechanisms is controversially debated. Genetically engineered mouse models offer new opportunities to experimentally investigate the role of CD8+ T-cells in melanoma pathogenesis. Here we experimentally studied how primary melanomas in Hgf-Cdk4R24C mice escape the control of adoptively transferred TCRtg pmel-1 CD8+ T-cells recognizing the melanosomal protein gp100. Cohorts of carcinogen-exposed Hgf-Cdk4R24C mice bearing small primary melanomas in the skin received naive pmel-1 T-cells that were activated *in vivo* with the recombinant adenovirus Ad-gp100. This induced tumor regression and dormancy for several weeks after which tumors continued to grow progressively. Repetitive vaccinations boosted circulating levels of pmel-1 T-cells and delayed tumor progression leading to significantly prolonged survival. The majority of tumor cells in recurrent melanomas strongly expressed the target antigen gp100. Further investigations revealed significantly higher numbers of infiltrating myeloid immune cells by immunohistochemistry and flow cytometry as well as increased expression of proinflammatory genes in the tumor microenvironment when compared with melanomas developing in control cohorts of mice that did not receive pmel-1 T-cells. To gain further insights into the underlying mechanisms, we used the highly pigmented Hgf-Cdk4R24C melanoma cell line HCmel3 established from a primary tumor. Macroscopically visible HCmel3 melanoma transplants in the skin of wild-type mice initially regressed following adoptive transfer and *in vivo* activation of pmel-1 T-cells. Most tumors recurred after several weeks of dormancy, largely retained expression of the target antigen gp100 and also showed significantly increased inflammation in the tumor microenvironment. Importantly, serial transplants of recurring HCmel3 melanomas could mostly be controlled by another adoptive transfer of pmel-1 T-cells. In a minority of recurring HCmel3 melanomas we also observed a strongly reduced expression of gp100. Taken together, these results suggest that antigen-specific CD8+ T-cells induce an inflammatory response in the tumor microenvironment that is not due to tumor cell-intrinsic changes. This inflammatory response in turn promotes the loss of T-cell effector functions and enables tumor recurrence. The emergence of tumor cell variants that escape T-cell recognition is a comparatively rare event.

**P264**  
**Specific killing of melanoma cells by infection with an oncolytic measles virus directed to the surface antigen HMWMAA**

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Since there is no curative therapy at hand, innovative treatment methods are desperately needed for advanced melanoma. A novel approach is viral oncolysis, in which viruses are engineered to infect and kill cancer cells in the course of their replication and spread. Ideally, the therapeutic virus only infects tumor cells, but spares healthy tissue. This can be achieved by modifying viral surface molecules to ablate natural virus tropism and redirect the virus specifically to cancer cells. Oncolytic measles viruses (MV) have been shown to be beneficial for several tumor entities in animal models and are currently being evaluated in clinical trials. Due to their membranous envelope, MVs are especially suitable for antibody-mediated targeting. We have now generated the first melanoma-specific MV based on the vaccine strain Edmonston B. The virus specifically recognizes the High Molecular Weight Melanoma-Associated Antigen (HMWMAA, also called MSCP) that has been shown to be widely expressed on the surface of malignant melanoma cells. The affinity-optimized single-chain antibody RAFT3 was fused to the C-terminus of the MV attachment protein hemagglutinin (H), that is simultaneously mutated to ablate binding to its natural receptors.

We showed specificity and biological function of the retargeted H glycoprotein (HaHMWMAA) via fusion assays on HMWMAA-expressing melanoma cells. Transient co-transfection of expression plasmids encoding HaHMWMAA and the MV fusion protein F led to fusion of melanoma cells forming multi-nucleated syncytia. We then generated genetically retargeted viruses (MV-HaHMWMAA) and showed that the modified H glycoprotein HaHMWMAA was incorporated into viral particles at least as efficient as H glycoproteins of analogously retargeted control viruses. In agreement with the fusion assays, targeted infection experiments proved specific entry of MV-HaHMWMAA into antigen-expressing cell lines and low-passage melanoma cells. Broad syncytia formation was indicative of viral spread and significant cytotoxicity

was observed. In all cell lines tested, infection efficacy correlated with HMWMAA surface expression as determined by FACS analysis. Notably, no off-target infection of antigen-negative control cells was detected.

Efficacy and specificity of MV-HaHMWMAA was additionally evaluated by one-step virus growth kinetics. Replication of the retargeted virus in the melanoma cell line Mel888 was comparable to that of an unmodified control virus. Importantly, replication in melanoma cells was 1000-fold stronger than in antigen-negative control cells. With this, the retargeting strategy was successful. Indeed, the selectivity profile was higher than that of an established control MV.

In addition to pure oncolysis by replication, MV offers the possibility to deliver therapeutic transgenes encoding e.g. immunostimulatory or apoptosis-inducing proteins. In this line, we are currently arming MV-HaHMWMAA with the prodrug-converting enzyme cytosine deaminase for melanoma-specific prodrug activation of 5-fluorouracil. This chemovirotherapy approach will further enhance the oncolytic efficacy. The highly melanoma-specific virus developed in this study may become a potent building block of a novel multi-modal therapy for advanced melanoma.

#### P265 sRAGE levels relate to melanoma clinical stages and progression

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Melanoma initiation, growth and progression have been related to microenvironmental factors orchestrating tumor-stroma interaction.

We have recently demonstrated that the receptor for advanced glycation end-products (RAGE) is central for mediating experimental non-melanoma skin tumor formation as well as for experimental chronic inflammation by sustaining positive signaling feed-forward loops regulating specific sets of pro-inflammatory genes such as certain chemokines, COX-2, TNF- $\alpha$  and IL-6. Here, we describe that RAGE activity relates to human melanoma clinical stages and progression and therefore might be central in regulating melanoma growth and development. Markers of RAGE activity include RAGE protein expression, plasma levels of a soluble form of RAGE (sRAGE), phosphorylation of cellular down-stream targets such as transcription factors NF- $\kappa$ B p65, Jun and Stat3 as well as protein expression of RAGE targets/activating ligands such as S100A8/A9, S100B, HMGB1 in human melanoma specimens ( $n = 226$ ). As determined by immunofluorescence on human melanoma tissue sections RAGE protein expression is up-regulated in a stage-dependent manner; by using sRAGE specific ELISA sRAGE levels are significantly down-regulated in the plasma of melanoma patients at stage III compared to patients at stage I and II. sRAGE plasma levels are significantly down regulated in patients at stage IV compared to any other stage. Activity of p65, Jun and Stat3 as well as protein expression of S100A8/A9, S100B, and HMGB1 as determined by a combination of immunofluorescence and ELISA on human melanoma tissue/plasma specimens correlated conversely in a stage-dependent manner. These findings in humans are at least partly resembled in mice by using transplantation melanoma mouse models and transgenic RAGE-deficient mice. Moreover, sRAGE plasma

In conclusion, we provide multiple evidence for a novel role of RAGE signaling in driving melanoma growth and development. Moreover, we have established sRAGE levels in the plasma of melanoma patients as a valuable tool in monitoring disease progression and prognosis.

#### P266 Regulation of the transcription factor c-Jun in malignant melanoma

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**Abstract:** Malignant melanoma is an aggressive tumor derived from melanocytes. Crosstalk between melanocytes and keratinocytes is important in human epidermis. It is known that normal melanocyte phenotype and controlled proliferation of melanocytes is tightly regulated by keratinocytes via E-cadherin. Malignant transformation of melanocytes frequently coincides with loss of E-cadherin expression and the upregulation of N-Cadherin. Recent studies have shown that c-Jun (member of the AP-1 transcription factor family) plays an important role in malignant melanoma and is activated by a novel pathway which is enabled in melanoma beside the Ras/Raf/MEK/ERK (MAPK) and the PI3K/AKT (AKT) signaling pathways. A deregulation of transcriptional activity is often found during tumor development. The constitutive activity of the AP-1 transcription factor family influences the expression of a variety of regulators of cell proliferation, migration and survival, which are significantly involved in melanoma development and metastasis. Furthermore, it is known that epithelial cell adhesion proteins, E-cadherin, play important roles in melanoma development, especially in metastasis.

Recently, we discovered that the loss of E-cadherin leads to dissociation of single tumor cells out of the compact tumor mass, displays the beginning of metastasis and induces c-Jun protein expression. Evidently the mRNA level c-Jun is not affected; hence c-Jun is regulated at post-transcriptional level. Here, we present data that show that the dynamic cytoskeletal network, linked to E-cadherin, is involved in the regulation of the c-Jun protein and transcriptional activity. Moreover, we demonstrate whether there is a direct interaction between tubulin or actin and c-Jun as it is necessary to reveal if c-Jun is stabilized via the cytoskeletal network. In a novel signaling cascade, the loss of E-cadherin activates the transcriptional regulator ETS-1 and consequently leads to the induction of RhoC expression that stabilizes c-Jun in melanoma. The link between RhoC and c-Jun seems to be indirect via the cytoskeleton. We conclude that the loss of E-cadherin mediated cell-adhesion induces c-Jun protein expression in a multistep process, offering several possibilities for therapeutic intervention.

#### P267 Epidermal growth factor influences melanoma lymph node metastasis by affecting lymphangiogenesis

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Soluble factors secreted by malignant tumors are able to influence the development of distant metastases. We aimed to identify the role of such factors in melanoma metastasis using a mouse xenotransplantation model and human patient's material. In our mouse model, a microarray-based screening identified epidermal growth factor (EGF) dependent genes significantly expressed in tumor-negative lymph nodes of tumor bearing mice. In sera from melanoma patients undergoing sentinel-node biopsy, patients with lymph node micrometastases had significantly elevated EGF serum levels. To elucidate a potential role of EGF on lymph node metastasis, EGF-knockdown (EGFkd) in melanoma cells producing high (M24met) and low levels (A375) of EGF. Only in EGF-high producing cells, EGFkd had observable effects. *In vitro*, EGFkd in M24met cells significantly impaired migration but did not affect tumor cell proliferation. *In vivo*, EGFkd significantly reduced lymph node metastasis and lymphangiogenesis in primary tumors. Sprouting of lymphatic but not of blood endothelial cells was inhibited using M24met EGFkd cell supernatants. In xenotransplanted tumors and in human primary melanomas, a direct correlation between EGF/VEGF-C and EGF/Prox-1 mRNA and protein expression levels was found. Taken together, these data indicate that EGF, if present at sufficient levels, influences tumor lymphangiogenesis and may facilitate melanoma metastasis.

#### P268 Indirubin N-glycosides inhibit important intracellular signalling pathways in malignant melanoma

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Despite the recent progress in treatment of metastatic melanoma by targeting of the BRAF/MAPK signaling pathway, there is still a need for new treatment approaches, since relapse rates after BRAF targeting are high and treatment is only successful in patients with a mutated BRAF kinase. In recent years, indirubins and their derivatives have been shown to be highly potent inhibitors of intracellular signalling kinases such as cyclin-dependent kinases CDK2 and CDK4 as well as glycogen synthase kinase  $\beta$  (GSK-3 $\beta$ ). Moreover, they have been shown to be anti-proliferative and pro-apoptotic in a variety of tumour cell lines including melanoma cell lines. In search for their target structures in melanoma cells that might explain their activity, we performed a screen using a phospho-kinase array of more than 45 phospho-proteins, which includes members of all well-known intracellular signalling pathways. We could show that treatment of melanoma cells with different N-glycosylated indirubins led to a significant decrease in phosphorylated Akt, S6 kinase, STAT1 and c-Jun, with phosphorylated c-Jun showing the most significant downregulation. c-Jun is the downstream target of c-Jun N-terminal protein kinases 1/2 (JNK1/2). To further validate these findings, *in vitro* kinase assays were performed with recombinant JNK2, which showed that indirubins were indeed strong inhibitors of JNK2 activity, being even more potent than the positive control inhibitor sorafenib. Thus, indirubins may exert their effects in melanoma cells at least in part through inhibition of JNK signalling. Importantly, human dermal fibroblasts or melanocytes did not significantly respond to indirubin treatment. Together, we identified new intracellular signalling pathways targeted by indirubin derivatives and showed that indirubin derivatives may be interesting substances for new treatment approaches in malignant melanoma.

#### P269 Tumor refractoriness to anti-VEGF therapy is mediated by myeloid cell recruitment in malignant melanoma

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Tumor blood vessels are prime targets for suppressing tumor growth, because they are distinct from normal resting blood vessels and can be selectively destroyed without significantly affecting normal vessels. Angiogenesis inhibitors targeting the VEGF-mediated pro-angiogenic signalling pathways are producing demonstrable clinical benefit for an increasing number of cancer types, but not all cancer patients benefit from such anti-angiogenic therapies. Some, who benefit initially, might develop resistance during the treatment or show some adverse effects. However, angiogenesis is not only dependent on endothelial cell invasion and proliferation, it also requires pericyte coverage of vascular sprouts for stabilization of vascular walls. We were able to show in both experimental settings, MT/ret-transgenic melanoma and human melanoma metastases, taken after adjuvant treatment using bevacizumab, that tumor vessels, resistant to anti-VEGF therapy, are characterized by enhanced vessel diameter and normalization of the vascular bed by coverage of mature pericytes in contrast to partly reduced or lack of pericyte coverage on intratumoral microvessels vessels, sensitive to anti-VEGF therapy.

There is also growing evidence that host stromal-tumor cell interactions play an important role in tumor growth and progression, as stromal cells release a variety of angiogenic factors. Therefore, infiltration by cells of the myeloid lineage might contribute to resistance to anti-angiogenic treatments. Histological analyses of MT/ret-transgenic melanoma, resistant to anti-VEGF therapy, show an enhanced recruitment of CD11b<sup>+</sup> myeloid cells to the vascular wall of tumor-associated blood vessels in contrast to therapy-sensitive tumors. Our findings emphasize that the level of mural cell differentiation and stabilization of the vascular wall but also the recruitment of myeloid cells significantly contribute to the response towards anti-angiogenic therapy in melanoma. This study may be useful to pave the way towards a more rational development of second generation anti-angiogenic combination therapies and provide for the first time a murine model to study this.

#### P270 (V34) Regulation of cell cycle checkpoint kinase Wee1 by miRNA-195 in malignant melanoma

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Wee1 kinase has been described as a major gate keeper at the G2-M cell cycle checkpoint. In a recent study, high levels of Wee1 expression in primary melanomas were associated with poor prognosis in melanoma patients. Here we analyzed expression levels of Wee1 in a series of melanoma cell lines and patient samples using immunoblotting, quantitative real-time PCR (qRT-PCR) and immunohistochemistry. Surprisingly, Wee1 expression was significantly downregulated in melanoma cell lines of high aggressiveness as compared with cell lines of low aggressiveness and in patient samples of metastatic origin as compared to primary melanomas. Thus, metastatic lesions might rely on Wee1 downregulation instead of Wee1 upregulation. Since microRNAs (miRNAs) play an important role in tumor biology and are well-known negative regulators of gene expression, we searched for miRNA candidates that might account for Wee1 downregulation in metastatic melanoma. In different miRNA target databases, miR-195 was found as a top candidate miRNA for targeting of Wee1. Furthermore, our expression analysis revealed an inverse correlation between the expression of Wee1 and miR-195 in melanoma samples. Further experiments showed that transfection of miR-195 indeed reduced mRNA and protein expression of Wee1, and reporter gene analysis confirmed direct targeting of the Wee1 3' untranslated region (3'UTR) by miR-195. We found that overexpression of miR-195 in SK-Mel-28 melanoma cells almost completely abrogated stress-induced G2-M cell cycle arrest. In a rescue experiment, stable over-expression of Wee1 in these cells reversed the miR-195 effect and partially reinstated the stress-induced arrest. This confirms that the miR-195 effects are indeed mediated by direct inhibition of Wee1 in malignant melanoma. Taken together, our study provides significant evidence that Wee1 is directly regulated by miR-195 in malignant melanoma and that Wee1 may be downregulated in metastatic lesions by miR-195 to allow unrestricted growth of tumor cells.

#### P271 Differential effects of casein kinase 1 (CK1) isoforms on proliferation and survival of melanoma cells

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We previously identified CK1 $\alpha$  as a novel tumor suppressor in melanoma and reported that the loss of casein kinase 1 $\alpha$  leads to increased proliferation and invasive growth of melanoma cells by strong acti-

vation of the Wnt/ $\beta$ -catenin signaling pathway (Sinnberg et al. 2010). In this study we analyzed expression and the functional effects of the CK1 isoforms  $\delta$  and  $\epsilon$ . We show that – in contrast to CK1 $\zeta$  – the expression of CK1 $\delta$  and CK1 $\epsilon$  do not significantly change during melanoma progression. Furthermore, downregulation of one CK1 isoform seems not to influence expression of the other CK1 isoforms. Inhibition of the expression and activity of CK1 $\delta$  or CK1 $\epsilon$  by specific inhibitors or siRNAs had no significant effect on the growth and survival of metastatic melanoma cells. Moreover, the over-expression of CK1 $\delta$  or CK1 $\epsilon$  in melanoma cells failed to induce cell death or cell cycle arrest, in contrast to the effects of CK1 $\zeta$ . These data indicate that CK1 $\zeta$  is the dominant active isoform of the casein kinase 1 family, which cannot be replaced by the other CK1 isoforms.

#### P272 Analysis of melanoblasts in cell culture to find previously unknown genes which could be relevant for melanoma

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Melanocytes differentiate from un-differentiated precursors, called melanoblasts, which are derived from neural crest cells during embryogenesis. In turn melanoma originates from melanocytes which are the pigmented cells of our skin.

It is well-known that melanoblast development shares many fundamental molecular processes with melanoma formation, such as self-renewal, proliferation, and migration capacity. Consequently, comparison of expression patterns between melanoblasts, melanocytes, and melanoma is an important issue in cancer research.

Here, under the influence of medium supplemented with Stem Cell factor, Endothelin-3 and Fibroblast Growth Factor-2 melanoblast in culture were generated out of melanocytes.

We then investigated the expression status of genes like E-cadherin, N-cadherin, integrin beta 3, integrin beta 1 and integrin alpha 3 of melanoblasts in cell culture versus melanoma cells and melanocytes, respectively. These genes are known to regulate migration and attachment of melanoma cells and are equally expressed in melanoma cells and melanoblasts. It further seems that these genes were primarily regulated through the de-differentiation status of melanocytes in this *in vitro* system.

Interestingly, we also found genes like SNAIL and MIA (melanoma inhibitory activity) which were differentially regulated between melanoblasts and melanoma. As previously published SNAIL and MIA expression is upregulated in melanoma. In contrast, melanoblasts showed low expression levels of both molecules. These genes were unaffected from (medium dependent) differentiation but could be in fact eminently relevant for early melanoma initiation.

Finally, we are interested to use the *in vitro* system to find the previously unreported key molecule of early melanoma development which is of overriding importance.

#### P273 TGF-beta1 abrogates whereas TNF-alpha enhances resistance to ERK inhibitors in BRAF mutant melanoma cells through Twist1

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Melanomas along with many other cancer types harbor BRAF mutations which constitutively activate the MAPK-ERK1/2 pathway. PLX4032 (vemurafenib) a BRAF inhibitor, showed approximately 80% response during clinical trials, but in turn gave rise to acquired resistance in cancer patients. We found that TGF beta 1 signaling could act as a factor predicting the response for inhibitors of the MAPK-ERK1/2 pathway, as it enhanced the susceptibility of BRAF mutant melanoma cells towards exposure of PD98059 (MEK1 inhibitor) and PLX4032 by almost three times. Inhibition of TGF beta 1 signaling by using an SB431542 (ALK5 inhibitor) almost completely abolished the apoptotic effect of PLX4032 exposure *in vitro*. Over expression of Twist1, an epithelial – mesenchymal transition regulator induced a resistance in BRAF mutant cells to a combination of PLX4032 and TGF beta1. In BRAF mutant cell lines exposure to PLX4032 abrogated the stability of Twist1, whereas exposure to TNF alpha reestablished Twist1 in these cell lines leading to a resistance towards a combined PLX4032 and TGF beta1 exposure. To summarize, our studies suggest that TGF beta 1 with PLX4032 could be a better strategic approach when compared to PLX4032 alone, but the stability of Twist1 inflicted by TNF alpha may contribute to acquired resistance towards inhibitors targeting BRAF - ERK signaling.

#### P274 Highly invasive melanoma cells regulate its VEGF-A expression via a MMP-2/ $\alpha$ = $\beta$ 5- integrin signaling pathway

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Human malignant melanoma is a highly metastatic tumor with poor prognosis and high resistance to treatment. The capacity of tumor cells to form metastases is associated with their ability to interact with the endothelial cell layer and migrate to the surrounding tissue. In this study, we could show that supernatants from high-invasive melanoma cells induce an acute endothelial cell (EC) activation, measured by the release of the pro-inflammatory and pro-thrombotic content of Weibel-Palade bodies (WPB), including P-selectin, Angiopoietin-2 (Ang-2) and von Willebrand factor (VWF), whereas supernatants from low-invasive cells fail to activate ECs directly. Proteome Profile analysis of melanoma supernatants identified vascular endothelial growth factor-A (VEGF-A) as the main mediator of EC activation. Inhibition or knockdown of VEGF-A in melanoma cells as well as the inhibition or knockdown of the VEGF receptor-2 (VEGFR-2) in EC led to a rigorous decrease in acute EC activation. Moreover analysis of supernatants harvested of matrix metalloproteinase-2 (MMP-2) depleted melanoma cells, showed a reduced VEGF-A mRNA content in these cells, and this effect directly correlated with reduced acute EC activation. Further experiments showed that active MMP-2 on the cell surface of melanoma cells regulates VEGF-A mRNA content in melanoma cells via an integrin  $\alpha$  =  $\beta$ 5/phosphoinositide-3-kinase-dependent (PI3K) pathway.

#### P275 The possible role of p16Ink4a in tumor cell growth arrest

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Cell cycle regulation mediated by the p16Ink4a/Rb pathway is frequently impaired in various cancers, including HPV-positive epithelial cancers. In RIP1-Tag2 mice that undergo multistage carcinogenesis and develop  $\beta$  cell cancer, this pathway is incompletely disrupted in pancreatic  $\beta$  cells by the expression of T antigen (Tag) under control of the rat insulin promoter (RIP). We previously showed that Tag-specific Th1 cells enhance the survival of RIP1-Tag2 mice and inhibit tumor progression without causing signs of tissue destruction. Instead, Th1 cells decreased the proliferation rate of the tumor cells *in vivo* by IFN- $\gamma$  and TNF-dependent mechanisms. One possible mechanism that inhibits tumor progression is induction of permanent growth arrest via the p16Ink4a/Rb pathway. Here, we investigated the role of p16Ink4a in arresting tumor cell growth.

We first found *in vivo* that treatment of mice with Tag-Th1 cells dramatically reduced the proliferation of tumor cells as measured by Ki67-staining. Simultaneously, Th1 cells strongly induced p16Ink4a in preneoplastic cancer cells *in vivo*. To test the influence of the two Th1 cytokines IFN- $\gamma$  and TNF on  $\beta$  cancer cells, we isolated tumors from 12 week old RIP1-Tag2 mice. We treated isolated tumor cells with recombinant IFN- $\gamma$  or TNF *in vitro*. Together the two cytokines concentration-dependently reduced proliferation, strongly increased the cell fraction expressing senescence-associated  $\beta$ -galactosidase activity (SA- $\beta$ -Gal), and enhanced the expression of p16Ink4a and Rb. To test the functional relevance, we silenced p16Ink4a by lentiviral infection with shRNA. Downregulation of p16Ink4a caused an increased proliferation of the beta cancer cells thereby demonstrating a link between p16Ink4a and tumor growth arrest. Further experiments with the human rhabdomyosarcoma cell line A204 showed a similar cytokine-induced, p16Ink4a-mediated growth inhibition.

Taken together, these data suggest that immunotherapy of malignant tumors in part relies on cytokine-mediated activation of the p16Ink4a/Rb pathway leading to growth arrest.

#### P276 Merkel cell carcinoma (MCC): mitoses, lymphovascular invasion, expression of Ki-67 and bcl-2 correlate with disease progression.

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Background: MCC is a rare and aggressive skin-tumor. Traditionally, it is classified into three different histopathological types, however, this categorization does not provide any prognostic significance. Aim: The aim of this retrospective study was to investigate whether MCCs might display histological and immunomorphological features of prognostic value. As parameter for progression we used the incidence of local recurrences, lymph node- and/or distant metastases, respectively.

Methods: We investigated the primary tumours of 26 patients by conventional histopathology and immunomorphology and determined (i) type, (ii) size, (iii) number of mitoses, (iv) lymphovascular invasion, (v) proliferation-, and (vi) (anti)-apoptosis-rate of the tumour cells. Lymphatic endothelial cells were identified by staining for podoplanin, proliferation rate was evaluated by labelling with the Ki-67-antibody, anti-apoptosis was determined by the expression of bcl-2. Results were statistically analyzed. Results: A high number of mitoses (mean 8.1 mitoses/HPF;  $n = 26$ , SD = 5.1, min = 1; max = 9; median = 7) and high Ki-67 expression (mean 52%,  $n = 25$ , SD = 28.4%, min = 5%, max = 95%, median = 40%) significantly correlated with lymph node metastases: mitoses  $P = 0.026$ ; Ki-67  $P = 0.008$ . On average 19.6 infiltrated lymphatic vessels per tumour were detected (mean 19.6,  $n = 26$ , SD = 24.4, min = 0; max = 108, median = 14). A higher number of invaded lymphatic capillaries showed a tendency towards a progressive course but no significant correlation. High bcl-2 expression (mean 78%;  $n = 23$ , SD = 30.9%, min = 5%, max = 100%, median 90%) revealed statistical significance as related to local recurrences and a tendency towards lymph node metastases.

Conclusion: Here we show that numbers of mitotic- and proliferating-tumor cells in primary MCCs significantly correlate with a disease progression. In addition we observed a significant correlation of local recurrences with a high bcl-2 expression. Similarly, a tendency was found between the number of lymphatic capillaries invaded by tumor cells and disease progression. We suggest that these peculiar histo- and immunopathological features might be of prognostic value in the primary diagnosis of MCCs.

#### P277 Generation of tumor-specific CD4+, interferon- $\gamma$ -producing T helper lymphocytes for immunotherapy against malignant melanoma

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Immunotherapies such as adoptive T-cell transfer are novel approaches to treat patients with metastatic malignant melanoma. Strategies for adoptive T-cell transfer focused on CD8+ cytotoxic T lymphocytes (CTLs) in the past. But strong CTL responses alone didn't necessarily correlate with sustained anti-tumor protection for the patient. New studies showed predominantly CD4+ T helper (Th1) lymphocytes in the tumor microenvironment, associated with impaired tumor growth and angiogenesis; moreover, tumor growth arrest associated with IFN- $\gamma$  and TNF- $\alpha$ . Thus, Th1 lymphocytes could be more effective for immunotherapies against metastasized malignant melanoma than CTLs. The aim of this study was to design a protocol to generate tumor-specific CD4+, interferon- $\gamma$ -producing T helper (Th1) lymphocytes, to characterize the generated cells and analyze their effector functions on melanoma cells. We primed PBMCs from healthy donors with NY-ESO-1 or MAGE-A1 15-mer overlapping peptide mixes. After 2 weeks of stimulation with IL-2 und IL-7, we enriched IFN- $\gamma$  producing cells with the IFN- $\gamma$  capture technique. Cells were expanded over 2 weeks to analysis. We could generate a cell product with up to  $5 \times 10^9$  cells, containing NY-ESO-1 or MAGE-A1-specific, CD4+ Th1 lymphocytes. After restimulation with the specific tumor-antigen the generated cells produced IFN- $\gamma$  and TNF- $\alpha$ , but not IL-10 or IL-4. These cells proliferated after exposure to the specific tumor-antigen, but not to control-antigen actin as shown by CFSE-staining. In summary, high numbers of tumor-specific CD4+, IFN- $\gamma$ -producing T helper (Th1) lymphocytes were generated for the use as immunotherapy against metastasized malignant melanoma.

#### P278 Caspase-1 and inflammasomes in epithelial skin cancer

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Although chronic inflammation is known to be a promoter of tumor growth and tumor progression, the molecular mechanisms responsible for the tumor-promoting environment are largely elusive. Interleukin-1 $\beta$  (IL-1 $\beta$ ) is one of the key cytokines in innate immunity driving inflammation and is activated by multi-protein-complexes named inflammasomes. In this study, it was our goal to determine the impact of IL-1 signaling on epithelial skin carcinogenesis. We induced epithelial skin cancer using DMBA followed by repetitive TPA treatment in mice deficient for IL-1R or caspase-1, the protease activating IL-1 $\beta$ . The incidence and number of tumors were significantly lower in both IL-1R-deficient mice and caspase-1-deficient mice compared to their wild type littermates. As ASC is the common adapter protein for caspase-1 recruitment in most of the inflammasomes characterized so far, tumor growth was induced in ASC deficient mice. Surprisingly, unlike IL-1R-/- or caspase-1-/- mice, ASC deficient animals did not display altered tumor-numbers compared to wild-type littermates. This discrepancy might be due to the dual function of ASC as a known tumor suppressor in the tumor cells versus ASC being a crucial protein for IL-1 $\beta$ -activation. To confirm our hypothesis, we detected a loss of ASC expression in primary human cutaneous squamous cell carcinomas, while psoriasis lesions as examples for a reactive epidermal proliferation still express ASC in immunohistochemistry. Taken together, our results implicate a role of caspase-1/IL-1 signaling for epithelial tumor-growth and investigate the importance of ASC, with particular focus on its function in the actual tumor cells compared to the tumor-promoting inflammatory microenvironment.

#### P279 Ultraviolet A radiation increases invasiveness of melanoma cell

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Melanoma is a malignant skin tumor deriving from melanocytes, characterised by high morbidity and mortality. While previous studies indicate a causative role for a single high dose of ultraviolet (UV)B

(280–320 nm) radiation in the development of melanoma, the role of UVA (320–400 nm) in the pathogenesis of human melanoma remains unclear. We could previously show, that repetitive exposure of melanoma cell lines to sublethal doses of UVA irradiation increases lactate levels and increases levels of the transketolase-like-1 (TKTL-1) enzyme, which is an important enzyme of the pentose phosphate pathway. These findings reason for an increase of aerobic glycolysis after repetitive UVA exposure, a phenomenon characteristic for many carcinomas, known as the Warburg effect. In order to investigate a potential role of UVA exposure in the modulation of invasiveness, melanoma cells, characterised by different growth patterns (radial, vertical and metastatic) were exposed to sublethal repetitive UVA exposure and invasiveness was assessed by growth in Boyden chambers. In all cell lines the levels of invasiveness increased and this correlated with levels of TKTL-1 expression. Furthermore, UVA exposure in the presence of reactive oxygen quenchers reduced the Warburg effect. Taken together the present data indicate a role for sublethal repetitive UVA in the progression of melanoma cell line towards increased invasiveness which is mediated via the Warburg effect.

#### P280 Improved determination of tumor interstitial fluid pressure in mice xenografts via non-invasive scanning acoustic microscopy

R. Pflanzler<sup>1</sup>, A. Shelke<sup>2</sup>, R. Sader<sup>3</sup>, J. Bererter-Hahn<sup>4</sup> and M. Hofmann<sup>1</sup> <sup>1</sup>Department of Dermatology, Venerology and Allergy, Medical Center of the Goethe-University, Frankfurt am Main, 60590 Frankfurt, Germany; <sup>2</sup>Goethe University Frankfurt am Main, Institute for Cell Biology and Neurosciences, Kinematic Cell Research Group, 60438 Frankfurt, Germany; <sup>3</sup>Department of Oral, Craniomaxillofacial and Facial Plastic Surgery, Medical Center of the Goethe-University, Frankfurt am Main, 60590 Frankfurt, Germany. The chaotically organized vessel network along with an impaired lymphatic drainage in the peripheral layer is a characteristic feature of many solid tumors. It contributes to an often observed elevated tumor interstitial fluid pressure (TIFP) which prevents an efficient uptake of larger, macromolecular anti-cancer drugs - such as monoclonal antibodies (mAb) - in therapy of these types of cancers. High TIFP could also be shown to induce mechanical strain in the tumor cortex providing a trigger factor to cell proliferation. On subcutaneously implanted, vulva-carcinoma derived A431 tumor xenografts in nude mice, pressure values of up to 15 mm Hg could be measured. Two invasive techniques, the wick-in-needle technique and the micropuncture method, are commonly available for these investigations. With scanning acoustic microscopy (SAM) at various frequencies in the range of 15–100 MHz, a novel approach is proposed to overcome the drawbacks of invasive pressure assessment methods. Analysis of amplitude and time-of-flight acoustic signals provide the potential to quantify TIFP. Furthermore, bio-mechanical properties such as tissue attenuation, elasticity and inhomogeneity are more readily accessible. Invasive and non-invasive techniques have been used to assess and calibrate pressure under different treatment regimens on A431 tumors, such as angiogenesis-inducing vascular endothelial growth factor (VEGF-A and VEGF-C) and anti-cancer drugs like Cetuximab-class of antibodies. In addition, making tumor vessel network structures visible via mouse heart perfusion and a maceration preparation process of mouse tumor tissue could provide a helpful tool to support various imaging techniques on tumor microenvironment. Further investigations are undertaken to enhance understanding of tumor architecture and to make non-invasive ultrasound methods available for possible *in-situ* applications in small animals or small, sub-surface areas like subdermal tissues.

#### P281 Metastatic progression of melanoma is driven by Tlr4-dependent inflammatory responses in the tumor microenvironment

T. Bald<sup>1</sup>, J. Landsberg<sup>1</sup>, M. Renn<sup>1</sup>, S. Mikus<sup>1</sup>, A. Limmer<sup>2</sup>, T. Quast<sup>3</sup>, J. Kohnmeyer<sup>1</sup> and T. Tüting<sup>1</sup> <sup>1</sup>Laboratory of Experimental Dermatology, University Hospital Bonn, Bonn, Germany; <sup>2</sup>Institutes of Molecular Medicine and Experimental Immunology, University Hospital Bonn, Bonn, Germany; <sup>3</sup>Life and Medical Sciences Institute Laboratory of Molecular Immunology, University of Bonn, Bonn, Germany. The induction of inflammatory responses in the tumor microenvironment through stimulation of toll receptors (Tlr) represents a double-edged sword in tumor development. It can activate anti-tumor immunity but can also contribute to the survival, proliferation and migration of tumor cells. Here we experimentally investigated how endogenous Tlr4 ligands released in the skin following treatment with the tumor promoter 12-O-Tetradecanoylphorbol-13-acetate (TPA) affect the pathogenesis of cutaneous melanoma in the genetically engineered Hgf-Cdk4R24C mouse model. In our initial investigations we confirmed that two epicutaneous applications of TPA effectively stimulated epidermal hyperplasia, immune cell infiltration and induction of inflammatory genes in the skin of Hgf-Cdk4R24C mice. In subsequent experiments we treated cohorts of Hgf-Cdk4R24C mice with TPA twice weekly for 25 weeks. This induced a diffuse infiltrative expansion of melanoma cells locally in the skin and systemically in draining lymph nodes and lungs. Interestingly, long-term TPA treatment of Hgf-Cdk4R24C mice previously exposed to the carcinogen 7,12-dimethylbenzanthracene (DMBA) selectively increased the infiltrative and metastatic growth of melanoma cells without affecting the development of primary nodular melanomas. Repetitive epicutaneous applications of TPA also promoted the development of spontaneous metastases in lymph nodes and lungs of wild type mice bearing serial transplants of Hgf-Cdk4R24C melanomas in the skin. *In vitro* studies showed that exposure of cultured Hgf-Cdk4R24C melanoma cells to TPA or the Tlr4 ligand LPS directly activated the expression of proinflammatory genes including S100a8 and Cxcl2, stimulated migration in a transwell assay, but did not significantly affect metabolic activity or proliferation. To dissect the relative contribution of Tlr4-dependent effects on tumor cells and on surrounding cells in the microenvironment to the inflammatory response and the increased metastatic spread, we inoculated serial transplants of Hgf-Cdk4R24C melanomas into the skin of Tlr4-deficient mice and treated them with TPA or vehicle. Unexpectedly, the prometastatic effect of TPA on melanoma transplants was completely absent in Tlr4-deficient mice. As a mechanistic explanation, we found that the presence of Tlr4 on recipient mice was required for efficient TPA-dependent induction of inflammatory genes and the expansion of Gr1+ CD11b+ myeloid immune cells that have been shown to promote metastatic dissemination in other experimental models. Taken together, these results provide experimental evidence that Tlr4-dependent inflammatory responses in the microenvironment of primary cutaneous melanomas contribute to the development of metastatic disease.

#### P282 Role for neuropeptide PACAP in immune regulation against malignant melanoma

S. Choi<sup>1</sup>, E. Weihe<sup>2</sup>, M. Herti<sup>1</sup> and A. Bender<sup>1</sup> <sup>1</sup>Dermatology and Allergy, Philipps-University Marburg, 35043 Marburg, Germany; <sup>2</sup>Institute for Anatomy and Cell Biology, Philipps-University Marburg, 35037 Marburg, Germany. Background: The neuropeptides vasoactive intestinal peptide (VIP) and pituitary adenylyl cyclase-activating polypeptide (PACAP), which are structurally related, are known as modulators of the function of inflammatory cells through specific receptors. These are VPAC1 which is constitutively expressed on peripheral T lymphocytes, VPAC2 expressed on activated T lymphocytes, and high affinity receptor for PACAP, PAC1, expressed on antigen presenting cells (APC) like dendritic cells (DC) and macrophages. Several reports show that PACAP mediates potent anti-inflammatory effects through the generation of tolerogenic DCs *in vitro*. In addition, PACAP expression has been demonstrated to be related to the abundance and function of regulatory T cells in animal models. In previous studies, PACAP expression was found in various human malignant tumors, such as colon cancer, prostate cancer, as well as breast cancer. However the presence of PACAP in malignant melanoma and its function on immune surveillance is still unknown.

In this study, we investigated human malignant melanoma for PACAP production and how PACAP may be capable of influencing the immunoregulation in the tumor microenvironment. First, the expression of PACAP was detected in advanced malignant melanoma tissue by immunohistochemistry and in various human melanoma cell lines on mRNA level by PACAP specific RT-PCR as well as protein level with monoclonal antibody by Western blot analysis. Secondly, purified peripheral CD4+ T lymphocytes when stimulated in the presence of neuropeptides *in vitro* may develop a more suppressive phenotype as shown by others. Preliminary data indicate PACAP peptide may differentially influence proliferation and function of human T cell subsets from peripheral blood. In summary: PACAP is produced locally by human malignant melanoma tissue and may therefore augment immunosuppressive function in the tumor microenvironment. This may result in disturbed local cytotoxic T cell responses followed by enhanced tumor growth.

#### P283 The role(s) of the junction protein ZO-1 in malignant melanoma

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We investigated the presence and localization of ZO-1 and other tight junction proteins in tissue sections of MM, dysplastic naevus cell naevi (NZN) and Spitz naevi (SN) as well as several melanoma cell lines by using immunofluorescence microscopy, Western blotting and RNA analysis and looked for consequences of different ZO-1 levels by cytokine arrays, invasion arrays and proliferation assays. Therefore we reduced the expression levels by siRNA mediated knockdown and generated ZO-1 over-expressing cells by transfection with human full length ZO-1. Further we looked for the role of ZO-1 in barrier function by tracer penetration assays and transepithelial resistance measurement.

We observed the presence of ZO-1 in all MM as well as the majority of NZN, SN and melanoma cell lines. This indicates that different ZO-1 expression levels rather than simply its presence may mark invasiveness. In fact, we found a correlation of increased ZO-1 immunoreactivity in invasive areas of MM and Breslow Index as a marker for tumor progression. Invasion assays using melanoma cell lines expressing different levels of ZO-1 further supported this hypothesis. The level of ZO-1 had no influence on proliferation but it had an effect on the release of cytokines from melanoma cells. Despite the presence of other TJ proteins no TJ barrier was observed for ions and larger molecules.

In addition, we observed pronounced alterations of ZO-1 expression in the epidermal tumor microenvironment (TME) of MM compared to dysplastic NZN. While 90% of MM showed a remarkable up-regulation of ZO-1, i.e. expression in all epidermal layers, this was seen in none of the NZN. Spitz naevi showed heterogeneous results. The TME has gained significant interest due to the substantial influence of the interaction of tumor cells with their environment for tumor progression. Looking for putative consequences of presence and absence of ZO-1 in the TME we performed knockdown studies in keratinocytes. They revealed an influence of the presence of ZO-1 on cytokine levels released by the cells, e.g. IL-1 $\beta$  and IL-18, which is likely to result in a downregulation of the immune response. In conclusion, ZO-1 protein expression levels likely correlate with invasiveness in melanoma and the presence of ZO-1 expression in lower layers of the epidermal TME is related to malignancy of the tumor. The latter could be used for diagnostic delineation between melanomas and nevi. In addition, we show that the presence of ZO-1 in melanoma cells and in keratinocytes influences the cytokine milieu and might therefore have consequences for immune response.

#### P284 Morphological alterations in senescent primary human melanocytes

L. J. Mayer, S. Windhövel, L. Völkert, E. Bröcker, M. Goebeler, R. Houben and S. Haferkamp <sup>1</sup>Department of Dermatology, Venerology and Allergy, University of Würzburg, 97080 Würzburg, Germany. Mutagenic agents, UV irradiation or oncogenes put cells under stress and damage their DNA, thereby contributing to the initiation of cancer. However, the organism possesses an intrinsic protection mechanism called stress-induced senescence which counteracts cancer development. This tumor suppressor mechanism is characterized by long-term growth arrest and chromatin-remodelling resulting in the formation of senescence-associated heterochromatin foci. In addition senescent cells show a distinct morphology consisting of cytoplasmic and nuclear enlargement, vacuolisation and an oval to round cell shape. In our current study we used different stress stimuli such as chemotherapeutic agents and the expression of oncogenes to induce senescence in primary human melanocytes and thoroughly analysed the morphological changes associated with the senescence response. Our data show that the senescence phenotype was caused by the rearrangement of actin and tubulin filaments that resulted in the reorganization of the cytoskeleton and the formation of stress fibres. Furthermore, time-lapsed video-microscopy revealed that cellular motility of senescent cells was significantly impaired. Interestingly protein levels of focal adhesion kinase (FAK) and paxillin, both members of the focal adhesion complex family, are increased and both proteins accumulate at focal adhesion plaques in senescent melanocytes. Our data show that senescent melanocytes have a reduced ability to migrate which might be caused by the morphological changes associated with senescence.

#### P285 Sensitive and specific assays for routine serological diagnosis of epidermolysis bullosa acquisita

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anti-laminin 332 mucous membrane pemphigoid (MMP,  $n = 15$ ), pemphigus vulgaris (PV,  $n = 24$ ), and healthy controls ( $n = 254$ ).  
 Results: The cut-off for the ELISA was optimized for accuracy by receiver-operating characteristics (AUC = 0.9952). IgG reactivity against NC1 was detected in 69 of 73 (94.5%) EBA and five control sera (two healthy controls and three BP patients) resulting in a specificity of 98.97%. The IFA showed a sensitivity of 91.8% and specificity of 100%. Reproducibility of the ELISA was demonstrated by an intra-class correlation coefficient of 0.97. Serum levels of anti-NC1 IgG seemed to be correlated with the clinical severity of the disease. IgG subclass analyses by ELISA revealed IgG1, IgG2, IgG3, and IgG4 anti-NC1 reactivity in 83.6%, 85.3%, 37.7%, and 83.6% of EBA sera.  
 Conclusion: Two highly specific and sensitive assays for the detection of serum anti-Col VII autoantibodies were developed to diagnose EBA. Their diagnostic competence was demonstrated in a large cohort of well-characterized EBA sera.

## P286

### Comparison of the antimicrobial effect of PHMB- and silver-containing wound dressings using different *in vitro* test methods

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 Introduction: Wound dressings with antimicrobial agents are increasingly utilized in the management of critical colonized or infected chronic wounds. The dressings' antibacterial activities are mostly evaluated using *in-vitro*-tests. These may differ significantly in their properties and outcome. We have analyzed the antibacterial effect of PHMB- and silver-dressings using *in-vitro*-methods such as the agar diffusion test (ADT), contact tests like JISL1902:2002 or AATCC100, microplate-laser nephelometry (MLN) and luminometric quantification of bacterial ATP (LQB).  
 Materials & Methods: Antibacterial activity of the dressings was tested against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. ADT was performed according to DIN58940-3 with samples of 0.6 cm<sup>2</sup>. For AATCC100 samples sizes of 18 cm<sup>2</sup> and for JISL1902:2002 of 400 mg were used. Dressing extracts were prepared corresponding to DIN10993-12 for MLN and LQB.  
 Results: PHMB- and silver-dressings showed antibacterial activity in all tests. Yet, total effectiveness varied for single methods and properties of the basic dressing materials without active agent (such as alginate, cellulose, or polyurethane). Alginate, cellulose, or polyurethane alone had no effect in ADT. In contrast, alginate showed a strong antibacterial activity in the contact tests (JISL1902:2002, AATCC100) because it is able to sequester bacteria during gel formation. MLN and LQB only determined a bactericidal effect on *S. aureus* and *P. aeruginosa* for the agent-containing dressings.  
 Conclusions: Using *in-vitro*-tests for the evaluation of the antibacterial activity allows quantification and direct comparison of dressings' effectiveness under standard conditions. Various test methods are available that differ in their properties and hence in their outcome, this has to be taken into account when selecting a specific test and interpreting the results.

## P287

### Ingrowth of fibroblasts into large-pored foams during negative pressure wound therapy (NPWT) can be inhibited *in vitro* using a drainage foil

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 Introduction: NPWT has been shown to be clinically effective in the treatment of chronic-stagnating wounds. *In vitro* studies suggest that positive effects of NPWT result from the recruitment of cells to the wound site. It could be shown that the dressings used for NPWT exhibit different effects, cells especially show a significant tendency to grow into large-pored foams\*. *In vivo* this may lead to disruption of newly formed tissue during dressing changes. We have used an *in-vitro*-model for NPWT to investigate if the use of a drainage foil<sup>†</sup> can prevent ingrowth of fibroblasts into a large-pored-foam dressing+.  
 Materials & Methods: Dressing samples\*<sup>+</sup> were placed on fibroblast 3D-cultures. The assembly was positioned in a six-well-plate and sealed with a vacuum-applicator-lid (VAL). VALs were connected to medium supply and vacuum pump. Experiments were carried out at -80 mmHg and -120 mmHg for 48 h. Histology specimens were stained with haematoxylin/eosin and fibroblasts were detected using anti-vimentin-antibodies. Cell viability and ingrowth of cells into samples was determined.  
 Results: Fibroblasts responded to subatmospheric pressure by migrating in direction of the applied vacuum. No difference between cells treated with -80 mmHg and -120 mmHg was observed. Using large-pored foams, cells did not stop at the pellicle edge but continued to migrate into the dressing. Placing drainage foil<sup>†</sup> between collagen pellicle and foam, the ingrowth of cells into the dressing could be inhibited.  
 Conclusions: It could be shown that ingrowth of cells into large-pored foams can be inhibited *in vitro* by application of a drainage foil<sup>†</sup>. *In vivo* this may prevent the disruption of newly formed tissue during dressing changes.

\*V.A.C.GranuFoamDressing/KCI, <sup>†</sup>Suprasorb<sup>®</sup>

<sup>‡</sup>CNP drainage foil/Lohmann & Rauscher, <sup>+</sup>Suprasorb<sup>®</sup> CNP foam/Lohmann & Rauscher.

## P288

### Antifungal effects of cold atmospheric pressure plasma *in vitro*

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 Introduction: Plasma medicine is a promising new tool for clinical practice. So far, cold atmospheric pressure plasmas are mostly used for decontamination and sterilization of implants and heat-sensitive medical products. However, the direct use on the patient is conceivable as more and more about the complex interactions between plasma, micro-organisms and human tissue is understood. The plasma-BLASTER MEF produces an open, potential-free plasma jet. A controlled electrical discharge is ignited between a pencil electrode and a metallic, grounded nozzle. The produced potential-free plasma jet gets directed toward the substrate by gas flow. The thermal load on the substrate is low in comparison to devices which use an arc as a plasma source [1]. We studied the effect of different plasma sources on the treatment efficacy to inhibit yeast growth *in vitro*. *Candida albicans* and *Malassezia pachydermatis* were chosen as model organisms.  
 Materials & Methods: *Candida albicans* and *Malassezia pachydermatis* suspensions were plated onto Sabouraud agar plates in accordance to DIN 58940-3 (susceptibility testing of microbial pathogens to antimicrobial agents). Inoculated plates were incubated for 1 h at 4°C prior to plasma treatment. Plasma treatment was performed using the plasma-BLASTER by TIGRES GmbH with different process gases (air, nitrogen, argon). The plasma jet was fed over the agar plates by meandering. To investigate the antifungal effects the following plasma parameters have been varied: (i) distance from Plasma-Blaster to surface, (ii) electrical power of the Plasma-Blaster, (iii) grid spacing of treatment lines, (iv) number of treatments, and (v) work piece velocity. Afterwards, plates were incubated at 37°C for 24 h (*C. albicans*) and 48 h (*M. pachydermatis*), respectively. Evaluation of treatment efficacy was performed against an untreated control. Results: The generated plasmas had an antimycotic effect that depended on the chosen plasma parameters, in particular on the process gas used, the plasma power and the number of treatments performed. The effect was more pronounced on *Candida albicans* than *Malassezia pachydermatis*.  
 Conclusions: The study showed that cold atmospheric pressure plasmas exhibit antifungal properties *in vitro*. Hence, the selective application of cold plasma for treatment of superficial skin infections such as dermatomycoses could provide a promising alternative or supplementation of the medicinal therapy.  
 Reference: [1] [http://www.tigres.de/pivot/entry.php?id=102&w=pre-treatment\\_stationsbody](http://www.tigres.de/pivot/entry.php?id=102&w=pre-treatment_stationsbody).

## P289

### Comparison of nephelometric and luminometric methods for testing antifungal activity of cyclodextrin-antiseptic-complexes

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 Introduction: The packaging of molecules into cyclodextrins (CD) shows advantages over free active agents. This could for example convey a better solubility and a higher antimicrobial activity. In the present study complexes of  $\alpha$ -,  $\beta$ - and  $\gamma$ -CD with antiseptics such as chlorhexidine diacetate (CHX), iodine and polihexanide (PHMB) were analyzed in respect to antifungal effects on *Candida albicans* and *Malassezia pachydermatis*. Two different *in-vitro*-methods were employed. Microplate-lasernephelometry (MLN) is an optical analytical method and enables a quantitative determination of particle concentrations in solution. Cellular ATP content can be measured using the BacTiter-Glo<sup>™</sup> Microbial Cell Viability Assay (Promega, Mannheim, Germany). The luminescent signal is proportional to the amount of ATP present, which is directly proportional to the number of microbial cells in culture. Subsequently, both methods were compared according to the results.  
 Materials & Methods: Concentration and time dependent effects of CD-antiseptic-complexes on *C. albicans* and *M. pachydermatis* were determined by MLN using the NEPHELOstar Galaxy (BMG LABTECH, Offenburg, Germany). In addition, the antifungal activity was analyzed by chemiluminescent measurement of the cellular ATP content (BacTiter-Glo<sup>™</sup>; Promega, Mannheim, Germany) using the LUMStar Galaxy (BMG LABTECH, Offenburg, Germany).  
 Results: CHX, iodine and PHMB showed already a significant antifungal activity against *C. albicans* and *M. pachydermatis* at low concentrations *in vitro*. CD-antiseptics-complexes also exhibited a significant antifungal activity and achieved a complete inhibition of yeast growth. Only  $\gamma$ -CD-CHX had no effect on *C. albicans*. Furthermore, a drift in antifungal activity in relation with molecule size of CD and antiseptic agent was observed.  
 Conclusions: CD-antiseptic-complexes showed an antifungal activity against *C. albicans* and *M. pachydermatis* *in vitro*. CD exhibit different complexation capacities for the antiseptics tested due to the different molecule size of the agents. According to this, small molecules like CHX and iodine form a better complex with  $\alpha$ -CD.  $\gamma$ -CD shows the highest binding capacity for the large molecule PHMB. Thus, for PHMB the antifungal activity was found to rise from  $\alpha$ - to  $\gamma$ -CD, while for CHX and iodine the  $\alpha$ -CD complex showed the highest antimycotic effects. In addition, it could be demonstrated that both *in-vitro*-methods yield similar results also they differ in the parameter determined, turbidity for MLN and chemiluminescent measurement of cellular ATP content by BacTiter-Glo<sup>™</sup>.

## P290 (V12)

### Enzymatic autoantibody glycan hydrolysis alleviates autoimmunity against type VII collagen

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Autoantibody-mediated diseases comprise a heterogeneous group of disorders in which the pathogenic potential of autoantibodies has been clearly demonstrated. In general, their treatment relies on the long-term use of systemic corticosteroids and other immunosuppressants that are associated with considerable adverse reactions. EndoS, an endoglycosidase derived from *Streptococcus pyogenes*, specifically hydrolyzes the N-linked glycan of native IgG and has previously been shown to modulate the interaction between the Fc portion of autoantibody and Fc $\gamma$  receptors on leukocytes. Here, different models of autoimmunity to type VII collagen, a structural protein of the dermal-epidermal junction (DEJ), were employed to explore the therapeutic potential of EndoS. First, pretreatment of otherwise pathogenic anti-murine type VII collagen (mCOL7) IgG with EndoS significantly reduced split formation at the DEJ in cryosections of murine skin and abrogated clinical disease in mice. Next, the effect of EndoS was also seen when the enzyme was injected into mice after pathogenic anti-mCOL7 IgG had been administered. Finally, to mimic the patient situation even closer, EndoS was applied in mice that had already developed clinical disease after immunization with mCOL7. In all EndoS-treated mice, disease progression was stopped, and in the majority of mice, clinical disease even regressed. Importantly, this effect was seen as early as one week after the first EndoS injection indicating an effect of EndoS on already *in vivo*-bound anti-mCOL7 autoantibodies. Here, for the first time, the therapeutic potential of EndoS in autoantibody-mediated disease was shown by reversing already established clinical disease.

## P291

### Bacterial nanocellulose for antiseptic drugs in the development of active wound dressings

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Great efforts were made in the development of new wound dressings combining different properties for a successful therapy. Promotion of healing, exudate absorption by simultaneous creation of a moist environment, protection against microbial and mechanical attack, as well as an easy handling and good compliance are some characteristics of an ideal wound dressing [1]. Because of its outstanding material properties bacterial nanocellulose has reached great interest for biomedical applications especially in the field of modern wound care. In addition to the already mentioned requirements for a wound dressing BNC shows a broad spectrum of possible variations reaching from shaping during biosynthesis to a controlled delivery of biologically active molecules.  
 In a static cultivation process BNC-hydrogels were synthesized in the form of planar fleeces by *Glucanacetobacter xylinus* in Hestrin-Schramm medium. After treatment with sodium hydroxide and rinsing with deionised water stable BNC-hydrogels with a water content of up to 99% were obtained. Never-dried BNC-fleeces were loaded for up to 48 h with solutions of poly(hexamethylene biguanide) (PHMB), povidone iodine (PVP-iodine) and octenidine to prepare samples for release and biological experiments. By incubation of loaded fleeces in buffer (PVP-iodine, octenidine) or electrolyte solution (PHMB) the release behaviour was investigated over a period of 48 h at 32°C using UV spectrophotometry for quantification of all three antiseptic agents. In a direct challenge test (according to JIS L 1902:2002) *Staphylococcus aureus* was cultivated together with BNC-samples (loaded with PVP-iodine) to examine the antibacterial efficiency of these fleeces. According to DIN EN ISO 10993 extract preparation of BNC/PVP-iodine fleeces was carried out followed by incubation of these extracts with human HaCaT keratinocytes for cytotoxicity experiments. Cell proliferation was quantified using a BCA protein assay as well as a luminometric ATP-luciferase assay. Comparing the uptake of each antiseptic into BNC 10% m/v of loading concentration was absorbed after 48 h, the amount of drug inside BNC-fleeces showed no differences between PHMB, PVP-iodine and octenidine. Analyzing the release behaviour of loaded fleeces in different types of media (depending on the physicochemical characteristics of each drug) a steep initial burst within the first 8 h reaching steady state conditions after 24–30 h was observed. In contrast to native bacterial nanocellulose which showed only slight effects on *Staphylococcus aureus* growth BNC/PVP-iodine fleeces had a deep impact on bacterial growth, resulting in a



reduction of at least 7 log-ranges in the test for antibacterial efficiency. Furthermore, BNC loaded with PVP-iodine did not influence the cell proliferation of HaCaT keratinocytes in cytotoxicity studies. Bacterial Nanocellulose as a biomaterial from renewable sources forms the basis for a new generation of active wound dressings combining drug delivery, biocompatibility and antibacterial efficacy. The authors would like to thank the Thuringian Ministry of Education, Science and Culture (B714-10032) as well as the European Fund for Regional Development for the financial support of this study. Reference: [1] Wiegand C., et al., Wound Repair and Regeneration 2009, 17, 730–738.

#### P292

##### MMP-13 and MMP-14 are dispensable for skin morphogenesis but required for bone metabolism.

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Connective tissue metabolism in skin is characterized by continuous synthesis and degradation of interstitial collagens performed by MMP-14 and MMP-13 the major collagenases synthesized by murine fibroblasts *in vitro*. MMP-14 deficiency *in vivo* results in death of mice by 3 weeks of age and several skeletal abnormalities, while MMP-13 deficiency has no obvious phenotype. *In vivo* we did not detect any abnormality in skin architecture and collagen distribution in both single knockout mice. However, *in vitro*, analysis of collagen expression in MMP-14 deficient fibroblasts showed accumulation of collagen type I as compared to control fibroblasts. Nevertheless, extraction of proteins from neonatal skin of MMP-14 knockout animals did not show any difference in collagen expression when compared to wild type animals. To investigate whether another enzyme, MMP-13, may compensate *in vivo* for lack of MMP-14-derived collagenolytic activity in skin, we generated mice carrying ablation of both genes. Double-deficient mice were born and die within the first three weeks of age thus recapitulating the MMP-14 knockout phenotype. Skin architecture and epidermal differentiation is comparable to control wild type animals. Collagen staining in the dermis seems to be more compacted, however protein extraction did not show any obvious difference. Furthermore, both single and double deficient mice present loss of subcutaneous tissue which is already visible in double mutants at an early time point as compared to MMP-14 single knockout. Interestingly, double MMP-13/MMP-14 deficient mice exhibit altered tooth growth as indicated by the presence of crossed incisors. Furthermore, the bone phenotype observed in the MMP-14 knockout animal is now more prominent being the long bones shorter in MMP-13/MMP-14 deficient animals. Here we show that ablation of both collagenases is compatible with birth and dispensable for collagen metabolism in skin. Double deficiency of both collagenases results in early subcutaneous adipose tissue loss and altered skeleton and tooth development.

#### P293

##### Detection of hazardous polycyclic aromatic hydrocarbons (PAH) in black tattooed human skin and lymph nodes

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Millions of people have at least one tattoo. In the process of tattooing, the tattooists inject high amounts of pigment suspension in the dermis along with numerous unknown ingredients. Many of the tattoos consist only of black inks, which are predominantly composed of soot products like carbon black or polycyclic aromatic hydrocarbons (PAH).

In the present study, we aimed to investigate whether and to which extent PAH are still present in skin or lymph nodes months and years after tattooing.

A recently established method was applied that allows quantitative extraction of PAH from tissue. 14 black tattooed human skin specimen and related lymph nodes, which were obtained from forensic medicine, were investigated.

A variety of 20 PAH and phenol were extracted from 14 skin and lymph node specimens, respectively, and quantified by HPLC – DAD monitoring. In 12 of the 14 tattoos and in nine corresponding lymph nodes we were able to identify and subsequently to quantify the concentration of PAH. The mean PAH concentration in the tattooed skin was 6.57 g/cm<sup>2</sup> and 14.96 g/g related lymph nodes.

Our results provide clear evidence that PAH are present in skin and lymph nodes of tattooed individuals. PAH are known to be carcinogenic and/or mutagenic. In addition they can produce reactive oxygen species (ROS) when exposed to UV light, which can damage human tissue. These results are a first basis to estimate a possible risk of tattooing that is related to PAH.

#### P294

##### Trypsinase therapy counteracts lethal effects of snake venom: a new treatment for snakebite

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Currently, the primary treatment for poisonous snake envenomation is species-specific antibody serum raised in large host animals. Previously we have shown, using genetically modified mouse models, that mast cells (MC) and more specifically MC proteases, are essential for resistance to non-lethal doses of some animal venoms. To test whether MC proteases may be used as a new form of therapy for snake envenomation, we performed survival assays both in a zebrafish embryo model and in a mouse model. Six different clinically relevant snake venoms were incubated with purified human trypsinase, chymase, or CPA and then added at lethal concentrations to microwells containing single zebrafish embryos at 50 h post fertilization. Lethality was significantly decreased, or delayed, in embryos receiving venom pretreated with the trypsinase, but not chymase, CPA, or PBS. Similarly, trypsinase was also protective when administered, instead, therapeutically up to 10 min following venom exposure. These results were validated using the traditional method of antivenom testing in mice. Wild type mice were injected intraperitoneally with venom pretreated with human trypsinase, or with venom followed at intervals by therapeutic trypsinase injection. Survival and change in subcutaneous temperature were monitored. Our results point to human mast cell trypsinase as a potential basis for development of a novel, non-species-specific antidote to snake bite.

#### P295

##### Influences of regulatory beta1 integrin antibodies on epithelial hair follicle progenitor cell activation in an novel ECM model assay

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Integrins control important biological processes like cell growth, differentiation and polarity. In the human epithelial stem cell (eSC) niche this integrin mediated signalling is fundamental for controlling cell-cell- and cell-matrix-interactions of adult eSCs and so the extracellular matrix (ECM) of the stem cell niche is thought to be important for regulating stem cell fate.

Our aim was to explore if there is a direct influence in regulating epithelial hair follicle (HF) progenitor cell functions through the beta1 integrin receptor via adding the receptor activating 12G10(+) and

inhibiting mab13(-) antibodies as we could demonstrate previously their positive and negative influences on human HF elongation and keratinocyte proliferation.

Therefore we developed a novel ECM model assay mimicking parts of the eSC niche which optimizes HF epithelial cell proliferation and differentiation. Disperse pretreated HFs were embedded into a mixture of MatrigelTM and collagen I (ratio 1:1) in KSFM (keratinocyte serum-free medium) and cultured over 4 days in the presence or absence of the two different beta1 integrin antibodies in comparison to our standard culturing method in supplemented Williams E medium. We analyzed HF cell outgrowth by measuring the whole area around the HF and the three largest outgrowth points every second day. Cultured HFs were cryosectioned and the immunoreactivity pattern of endogenous K15 protein, CD200, Ki-67 (proliferation) and TUNEL (apoptosis) were quantified.

Embedding in MatrigelTM/collagen I supports epithelial cell outgrowth of disperse pretreated HFs compared to the standardized culture conditions. The activating antibody 12G10(+) as well as the inhibiting antibody mab13(-) decreased outgrowth in the ECM composed of MatrigelTM/collagen I whereas the untreated but ECM embedded control HFs showed a similar enhanced outgrowth of bulge and bulb located epithelial progenitors. These preliminary data suggest that there is a supporting effect of the ECM model for epithelial cell proliferation and inhibition of apoptosis alone. By additional use of the inhibitory and the stimulatory beta1 integrin antibodies we were further able to functionally distinguish between epithelial progenitors of the bulge region and cells of the bulb as we could show that the inhibitory antibody mab13(-) enhanced the proliferation of the bulge located progenitors whereas the stimulatory 12G10(+) antibody enhanced the proliferation of only bulb located epithelial progenitors (transient amplifying cells and their progeny).

#### P296

##### Mobile Erfassung des DLQI für die medizinische Versorgung und Forschung in der Dermatologie

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Einleitung/Hintergrund: Lebensqualität und Krankheitsempfinden werden von den Patienten und deren behandelnden Ärzten oft abweichend beurteilt, daher ist eine Erhebung dieser Daten durch die Patienten sinnvoll. Patienten können während der Wartezeit vor der Behandlung Fragebögen auf Papier ausfüllen. Bei Lebensqualitätsbögen wie dem DLQI werden die entsprechenden Scores später per Hand ausgerechnet und zu Forschungszwecken anschließend von Dokumentationsassistenten in Forschungsdatenbanken übertragen. Dieses Verfahren ist ressourcenintensiv und wird deshalb außerhalb von klinischen Studien selten angewandt. Um Lebensqualitätsparameter in der Behandlung mit zu berücksichtigen, ist eine effiziente Methode notwendig, die Daten direkt vom Patienten elektronisch erfassen zu lassen und im Klinikinformationssystem (KIS) zur Weiterverwertung bereit zu stellen.

Material und Methoden: Folgende Patientenfragebögen wurden im Kompetenzzentrum Pruritus der Hautklinik des Universitätsklinikums Münster getestet: zur Erfassung der Lebensqualität der Dermatology Life Quality Index (DLQI), zu Angst und Depression der Hospital Anxiety and Depression Scale (HADS) und zu Behandlungszielen der Patient Benefit Index (PBI prä und post).

Diese wurden mit den Tools unseres lokalen KIS ORBIS® implementiert und zusätzlich über eine selbstentwickelte Webapplikation bereitgestellt. Mit mobilen Geräten, z.B. Apples iPad, lässt sich die Webapplikation aufrufen, um die Bögen von dem Patienten ausfüllen zu lassen.

Ergebnisse: Kompetenzzentrum Pruritus werden alle Fragebögen von jedem der ca. 200 im Monat behandelten Patienten elektronisch auf dem iPad ausgefüllt. Die überwiegend älteren Patienten (Durchschnitt 60 Jahre) kommen sehr gut mit der Bedienung zurecht. Die vormals vom medizinischen Personal durchgeführte manuelle Übertragung der Daten in das KIS sowie in Forschungsdatenbanken und die Prüfung auf Vollständigkeit entfallen, da dies nun automatisch geschieht.

Diskussion/Schlussfolgerungen: Mit dem hier vorgestellten Verfahren lassen sich Informationen zur Lebensqualität von Patienten ohne Mehraufwand erfassen. Die erfassten Daten stehen den behandelnden Ärzten unmittelbar für die Behandlung und zur weiteren Kommunikation (z.B. innerhalb eines Arztbriefes) zur Verfügung. Für wissenschaftliche Fragestellungen lassen sich die Daten über das gesamte Patientenkollektiv hinweg, gemeinsam mit anderen relevanten Parametern, auswerten.

Schlüsselwörter: Lebensqualität, Patient Reported Outcomes, Mobile Erfassung, Klinikinformationssystem.

#### P297

##### Wound healing described by an alternative to the Kaplan–Meier curve

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Background: Demonstration of the effectiveness of medications and therapeutic measures is becoming ever more important due to the constantly rising costs in all areas of health care. Therefore, it makes sense to convert empirical data into mathematical functions. By mathematical evaluation it is possible to determine numeric parameters that characterize the analysed group. Such parameters are helpful in comparing different therapeutic methods or in evaluating survival probabilities on a qualitative basis by measurable values of mathematical parameters. Our developed model is based on physiological evidence.

Methods: Clinical studies are the method of choice for improving treatment measures. In most cases, qualitative findings are statistically assured which, however, requires relatively large test groups. For a mathematical description of a survival curve, we take up the idea of Gompertz and adapted it with following preconditions:

1 The prognosis for the course of disease is different for all patients within the group. Hence, those with good healing or bad survival prognosis are the first who will leave the group.

2 Changes in the size of the group over time will be proportional to the size of the group. This implies that in two groups of equal size and the same preconditions, the same number of events will occur over time.

3 The temporal sequence of events is group-specific and reflects the different effects of the disease or the treatment on the observed group

Results: With the aim of achieving quantitative results, numerous approaches to apply mathematical equations to biological processes were evaluated within this study. According to the radioactive decay, the decrease of group size can be described by the differential equation  $\frac{dN(t)}{dt} = -v(t) \cdot N(t)$ . The pre-condition a) correlates to the equation  $v(t) = w \cdot e^{-\alpha t}$  and we receive the solution:  $N(t) = N_0 \cdot e^{-w(e^{-\alpha t} - 1)}$ .  $N(t)$  are those persons who stay within the group. Those who have left the group are dead or e.g. healed:  $N_{healed} = N_0 - N(t)$ .

Parameters  $w$  and  $\alpha$  are calculated by 'best fitting'. In case of  $t \rightarrow \infty$ , the proportion  $\frac{N(t)}{N_0} = e^{-w}$  of the patient group will survive. Looking at the time during which 50% of the events observed took place  $N(t_{50\%}) = 0.5 \cdot N_{max}$  we can define a further parameter for the group, which we will call  $t_{50\%}$ . Furthermore,  $w$  and  $\alpha$  will be determinants for the recruitment and the post-observation period of the patients. 'Censored patients' are defined as those patients who drop out of sight without leaving a data trail. If the group size changes during time by such instances of censored patients, the number of events in this group, e.g. deaths or healing, will need to be weighted with an adjusting factor (precondition b) that takes into consideration the patients lost up to that point. To the surviving patients we should, therefore, apply the equation:  $N(t) = f(t) \cdot N_0 = f(t) \cdot g \cdot [N_0 - L(t)] \Rightarrow g = \frac{N(t)}{N_0 - L(t)}$ .  $L(t)$  is the total number of all lost patients at the time point  $t$ . All further events after  $t$  are multiplied with the factor  $g$ , e.g.  $g = 2$  if group size is diminished to 50% by censored patients. By this, the initial group size is virtually preserved.

Conclusion: We managed to provide a physiological basis for these variables and thus make the formula applicable for routine clinical application.

Parameters  $w$  and  $\alpha$  provide important guidance when we compare the effectiveness of two different therapeutic measures. The compared groups and their therapy are defined by its pair of parameters  $w$

and  $\alpha$ . This pair of parameters allows us to determine the maximum value of the observed events for  $t \rightarrow \infty$ , as well as the period within which 50% of these events occur. This mathematic procedure is easy, clinically based and more precisely as Kaplan–Meier estimation, so we think that it has the potential to replace Kaplan–Meier curve.

### P298 Impact of age on epidermal barrier function and skin microbiota: comparison of elderly and middle aged adults

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Prevalence of xerosis increases with aging in skin type I-II and can lead to red, cracked, fissured and itchy skin, a condition called xerotic eczema. Xerosis is described as a dysfunction of the epidermal barrier. Aged epidermal barrier integrity and recovery is reduced compared to young skin, shown in humans and mice. The impaired aged epidermal barrier function seems to be linked to an increased stratum corneum pH in aged skin. This study was performed to verify these results in very old skin type I-II and further compare skin microbiota in skin type I-II elderly and middle aged adults. First, we compared skin surface pH of 43 middle aged adults (age range 31–50, mean 40.1 7.4 SD) and 43 elderly (80–97, 85.8 4.8). Second, 20 middle aged adults (31–50, 40.6 7.6) and 20 elderly (80–97, 87.0 5.4) were compared. Measurements of skin hydration and transepidermal water loss (TEWL) were performed. Parallel, dynamic studies to investigate barrier integrity (no. of tape strippings required to increase TEWL by threefold), barrier cohesion (protein amount removed per tape) and after 24 h barrier recovery were designed. Moreover, the 'wash and scrub' method was used to sample skin flora, presented as colony forming units (CFU) per cm<sup>2</sup> skin. Microorganisms were identified by morphology, gram staining and standardized biochemical tests.

A significant ( $P = 0.002$ ) higher skin surface pH on the volar forearm was observed in the elderly (5.70 0.54) compared to the middle aged adults (5.15 0.39). Skin hydration ( $P = 0.000$ ) and TEWL ( $P = 0.000$ ) was significant lower in aged skin (skin hydration 29.6 4.2 AU; TEWL 4.63 1.77 g/m<sup>2</sup>/h) compared to the younger counterparts (43.0 5.6 AU; 9.51 2.43 g/m<sup>2</sup>/h). The dynamic studies show a reduced barrier integrity ( $P = 0.000$ ) and cohesion ( $P = 0.058$ ) in the elderly. Further, barrier recovery 24 h after perturbation by tape stripping was significant ( $P = 0.020$ ) delayed in aged skin. In the young 328 (median) CFU per cm<sup>2</sup> skin were isolated in comparison to 167 CFU per cm<sup>2</sup> skin in the elderly, whereas no significance ( $P = 0.715$ ) was calculated for this difference. In the young 16 subjects (80%) were observed with a mixed resident flora (mixed growth: more than three resident germs) compared to six elderly (30%).

Because of the increased skin surface pH in elderly, which leads to reduced epidermal barrier function, we point out, that maintenance of the physiological skin pH is essential. Specialized skin care to normalize skin pH in elderly may lead to improved epidermal barrier function, stabilized resident flora and reduced skin problems, like xerotic eczema.

### P299 Effect of pH 4.0 skin care on epidermal barrier function and skin microbiota: a randomized, controlled and double blind long-term study in a nursing home

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The normal stratum corneum (SC) pH regulates epidermal barrier function and skin microbiota. While the physiological SC pH is just below 5, in aged skin it is elevated up to 6 leading to a reduced barrier integrity/cohesion and delayed barrier recovery. The resident microflora is reduced in aged skin and dissociation of resident bacteria from skin surface is enhanced under alkaline pH conditions. We addressed the question whether pH4 skin care shows positive clinical, biophysical and microbial effects in aged skin.

Therefore, we performed a randomized, controlled and double blind long-term study. Elderly people from a local nursing home were randomly separated in two groups: group A ( $n = 12$ ; age range 80–95; mean 88.1 4.9 SD) and group B ( $n = 8$ ; 80–97; 85.4 5.9). Over 7 weeks both groups used habitual the same test products (cream, lotion, syndet), but with a different pH value (group A: pH 4.0 and group B: pH 6.0). All measurements were taken before (baseline) and after treatment. 'Dry skin' scoring, skin surface pH, skin hydration and transepidermal water loss (TEWL) were performed according to the guidelines. Barrier integrity (no. of tape strippings required to increase TEWL by threefold), barrier cohesion (protein amount removed per tape) and barrier recovery after 24 h were evaluated. To document the impact of the test products on microbiota, we used the 'wash and scrub' method for sampling skin flora, presented as colony forming units (CFU) per cm<sup>2</sup> skin. Microorganisms were identified by morphology, gram staining and standardized biochemical tests.

After 7 weeks of consistent skin care, skin dryness was reduced without significant differences between the groups. Barrier integrity shows a significant ( $P = 0.007$ ) improvement of group A compared to baseline, insignificant changes of group B ( $P = 0.672$ ) were observed. A significant ( $P = 0.025$ ) decline in barrier cohesion was observed in group B, whereas in group A barrier cohesion remained stable ( $P = 0.814$ ). Barrier recovery 24 h after perturbation increased significantly in group A ( $P = 0.004$ ) compared to baseline. Barrier recovery in group B was not significant, comparing baseline to after treatment data ( $P = 0.327$ ). Moreover, skin surface pH correlated negatively with the number of tape strippings required to increase the TEWL by threefold ( $P = 0.037$ ) and positively with protein amount removed per tape ( $P = 0.040$ ). Skin flora evaluation showed a significant increase in CFU per cm<sup>2</sup> skin in group A ( $P = 0.016$ ) and B ( $P = 0.017$ ). After treatment group A shows more subjects (100%) with a mixed resident flora (mixed growth: more than three resident germs) compared to group B (88%). The reduced epidermal barrier function in elderly is linked to an increased skin surface pH. Long-term treatment with pH 4.0 leave-on and rinse-off products results in a significant improvement of barrier integrity, cohesion and recovery compared to subjects, who used the same products with a given pH of 6.0. Effects of the 7-week treatment on skin dryness and resident flora are demonstrated, whereas without significant differences between the two groups.

We postulate that long-term skin care with pH 4.0 products improves epidermal barrier function in elderly. Moreover, positive effects on skin dryness and skin microbiota seem to be applicable.

### P300 In vitro analysis of hemostatic properties and hemocompatibility of collagen and/or ORC-containing wound dressings

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Introduction: Physiological wound healing is a highly regulated process, which can be divided in the three stages of hemostasis, inflammation, and repair. Hemostasis with fibrin formation contributes to

the formation of a protective wound scab. This facilitates the following steps by providing a matrix within which cell migration and angiogenesis can take place. Wound dressings consisting of oxidized regenerated cellulose or collagen are often used in treatment of surgical wounds as well as chronic wound care. These biomaterials offer interesting properties such as being absorbable and possessing hemostatic effects. A comprehensive *in vitro* study was performed to compare the hemostatic properties of wound dressings consisting of collagen and/or oxidized regenerated cellulose.

Materials & Methods: Wound dressings containing ORC (oxidized regenerated cellulose, Tabotamp, Johnson & Johnson), bovine collagen type I (Suprasorb C, Lohmann & Rauscher), or both (Promogran, Systagenix) have been tested. Influence of the materials on the generation of thrombin in human plasma was assessed using the Thrombin Generation Assay (Technothrombin TGA, Technoclone GmbH). Effect on prothrombin time (PT) and activated partial thromboplastin time (aPTT) was determined with the coagulation analyzer MCI (Greiner Biochemica GmbH). Impact on clotting of whole blood was analyzed by measurement of the blood clotting index (BCI). Furthermore, the wound dressings were tested for their hemocompatibility, assessing their hemolytic effect and their potential to activate the release of PMN elastase by granulocytes.

Results: All biomaterials tested were found to overall enhance coagulation. However, they yielded different results in the various *in vitro* tests used. None of the materials affected PT and aPTT. Only bovine collagen achieved a significant shortening of the time to thrombin generation in the thrombin generation assay. The most pronounced effect on the blood clotting index was observed for collagen + ORC. Furthermore, none of the materials led to a distinct release of PMN elastase from granulocytes. However, a slight hemolytic effect of ORC and collagen + ORC was detected.

Conclusions: The use of *in vitro* techniques enables the direct comparison of the hemostatic properties of wound dressings under standard conditions. Diverse biomaterials have different effects on hemostasis. Collagen only shortened the thrombin generation in the cascade of blood coagulation. Bovine collagen showed the highest hemocompatibility *in vitro*. Hence, products consisting only of collagen might be superior to the combination product of collagen and ORC.

### P301 Determination of antimicrobial properties of ceramic surfaces with perfluoroctyltriethoxysilane coating

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Introduction: Antimicrobial coatings are commonly used in household, food industry and hospital. New coating systems benefit from chemical vapor deposition under atmospheric-pressure plasma. In this study, the antimicrobial effect of a 1H,1H,2H,2H-perfluoroctyltriethoxysilane finish on a ceramic surface was analyzed. Furthermore, the influence of different process managements during precursor vapor deposition was determined.

Materials & Methods: Atmospheric-pressure plasma (300W) was used for chemical precursor (hexamethyldisiloxane) deposition on ceramic surfaces. Plasma conditions were modified with an accessory plasma-activation (400W) or the addition of isopropyl alcohol and isopropyl alcohol : water (1:1), respectively. Precursor activated ceramic surfaces were finished with a 1H,1H,2H,2H-perfluoroctyltriethoxysilane coating. These ceramic samples were tested according to the ISO 22196 for their antibacterial activity. In brief, samples of 50 × 50 mm were inoculated with *Staphylococcus aureus* and *Klebsiella pneumoniae*, respectively. Ceramics without coating were used as control. Samples were incubated for 24 h at 37°C under aerobic conditions. Subsequently, the samples were washed and serial dilutions were plated on agar plates. Colonies were counted after 24 h at 37°C.

Results: Ceramic surfaces with a perfluoroctyltriethoxysilane coating after precursor surface activation at 300W exhibited a strong antimicrobial activity against *Klebsiella pneumoniae* but had no effect on *Staphylococcus aureus*. Additional plasma activation (400W) reduced the antimicrobial effect. Application of isopropyl alcohol or isopropyl alcohol:water (1:1) during plasma surface coating with the precursor improved antimicrobial activity against both *Staphylococcus aureus* and *Klebsiella pneumoniae*. Conclusions: In this study, ceramics with a 1H,1H,2H,2H-perfluoroctyltriethoxysilane finish have been tested according to the ISO 22196. It was shown, that such a coating exhibits antimicrobial activity against *Staphylococcus aureus* and *Klebsiella pneumoniae*. However, antimicrobial properties depended on the process management during precursor deposition. Application of isopropyl alcohol (with or without water) has an advantageous effect on the antimicrobial activity. In conclusion, 1H,1H,2H,2H-perfluoroctyltriethoxysilane has the potential for a stable antimicrobial coating of ceramic surfaces.

### P302 Hemocompatibility of polyamide-6 foil equipped with a zinc-complexed hyperbranched polymer

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Introduction: Hemocompatibility is an important feature for materials in contact with human blood. Materials with advanced hemocompatibility have great potential for intracorporeal and extracorporeal applications. Blood response to dendrimer-equipped foil with zinc additive was determined using different *in vitro* assays according to the DIN norm EN ISO 10933-4 (2002, 2006). The study included the analysis of the effects on intrinsic and extrinsic activation pathways in whole blood as well as plasma. Dendrimer-equipped polyamide foil with zinc additive was used as an example of a multifunctional material with high cell compatibility combined with antimicrobial properties.

Materials & Methods: The polyamide foil tested was equipped with a dendritic polymer and zinc additive (DF-Zn). For reference a polyamide foil (RF) without the additives was used for comparison. Samples of the two foils were obtained using punch biopsies. The time-dependent thrombin generation in normal plasma was determined via cleavage of a fluorogenic substrate by the serine protease thrombin using the Thrombin Generation Assay (Technothrombin TGA, Technoclone GmbH). The fluorescent signal was measured with the SPECTROstar Omega (BMG LABTECH GmbH). Prothrombin time (PT) and activated partial thromboplastin time (aPTT) in plasma were measured in the coagulation analyzer MCI (Greiner Biochemica GmbH). Blood coagulation was analyzed by incubation of the samples with citrate blood. The free erythrocytes, not captured in a fibrin clot, were lysed with Aqua dest. And the hemoglobin content in the supernatant was measured at 540 nm with the SPECTROstar Omega.

Results: Both, DF-Zn and RF accelerated thrombin generation in normal plasma in comparison to the control. Furthermore, the time to the start of the thrombin generation (lag-time) and the time till the maximal thrombin activity was reached (time-to-peak) was significantly shortened. In whole blood DF-Zn and RF also increased clot formation compared to the control. No effect on prothrombin time (PT) and activated partial thromboplastin time (aPTT) was observed *in vitro*. No differences were observed between DF-Zn and RF.

Conclusions: It could be shown that a polyamide foil equipped with dendritic polymer and zinc additive is not completely hemocompatible. Hence, intracorporeal usage could be problematic *in vivo*. However, acceleration of blood coagulation is advantageous in wound management.

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