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Allergy

P001

Epidermal PAR2 overexpression causes atopic dermatitis-like skin disease: neuro-epidermal communication

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Protease-activated receptor-2 (PAR2) activation has been implicated in the pathophysiology of atopic dermatitis, Netherton syndrome, pruritus, as well as impaired skin barrier regulation. With the aim to study the effects of epidermal PAR-2 function on skin inflammation and itch, we generated a mouse that overexpresses PAR2 in keratinocytes only (KC-PAR2OE). Although KC-PAR2OE newborns display no overt abnormalities, they spontaneously develop dry skin, severe pruritus, and subsequently eczematous skin lesions after several weeks. Analysis of barrier function and immune response in lesional KC-PAR2OE mice revealed the hallmarks of atopic dermatitis-like skin lesions including acanthosis, parakeratosis, significant downregulation of filaggrin and other epidermal structure proteins, a mast cell- and T cell-driven inflammatory infiltrate. Of note, and in close correlation to patients with atopic dermatitis, repeated topical application of house dust mite (HDM) allergens onto KC-PAR2OE mice induced earlier and more severe lesions and pruritus in these mice (as determined by increased skin lesion score, scratching bouts, TEWL, total IgE). Our electrophysiological, morphological and molecular studies show that KCPAR2OE mice have an increased density of unmyelinated nerve fibers, increased NGF and endothelin expression levels in the skin, which may explain our findings of higher susceptibility of KC-PAR2OE mice to pruritogens and the development of spontaneously increased pruritus. In sum, our results suggest that certain proteases and KC-PAR2 are critically involved in the pathophysiology of pruritus and atopic dermatitis. KC-derived PAR2 seems to be an important link in neuro-epidermal communication with the keratinocyte-protease-PAR2 system as a forefront of sensory signaling and neuro-immune communication in inflammatory skin diseases.

P002 (O01/02)

Regulatory T cells induced by epicutaneous and oral low zone tolerance prevent the development of a Th1/Th17-mediated colitis

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The TNBS-induced colitis is a mice model which mimics Crohn's disease in humans. Crohn's disease is characterized by an inflammation affecting the entire gastrointestinal tract and each intestinal layer. Considering the pathophysiological similarities of TNBS-induced colitis to Crohn's disease it is a suitable model to investigate immune mechanisms. The hallmark of TNBS-induced colitis is a persistent inflammation of the gut, which is mediated by CD4+ Th1/Th17 T cells. Previously, we demonstrated that epicutaneous and oral applications of subimmunogenic amounts of allergens (e.g. TNCB) result in low zone tolerance (LZT), which inhibits the development of an allergic skin inflammation (contact hypersensitivity reaction) in mice. In the current study, we have analyzed the impact of orally and epicutaneously-induced LZT on the outcome of the TNBS-colitis and the underlying immune mechanisms.

Notably, we found that repeated oral as well as epicutaneous applications of low doses of the hapten TNCB or TNBS, respectively, abolished the clinical symptoms of the colitis, resulting in a significantly reduced inflammation of the gut. These data were evaluated *in vivo* by use of mini-endoscopy to assess a panel of inflammatory parameter (vascularity, granularity, translucency of the gut wall, fibrin, consistency of stool) and by histology (infiltration of inflammatory cells, vessel density, colon wall thickness). Analysis of the underlying immune processes revealed a significantly diminished hapten-specific T cell-proliferation and reduced Th1/Th17-cytokine production (IFN-γ, IL-2, IL-17) after both routes of tolerization, thereby confirming the clinical data and indicating an inhibition of TNBS-induced colitis by LZT. Furthermore, we put into question the role of CD4+CD25+FOXP3+ regulatory T cells (Tregs) in LZT modulation of colitis. Here, for Treg depletion either wild-type BL/6J mice were treated with anti-CD25 mAb or DEREG mice with diphtheria toxin prior to LZT induction. In the absence of CD4+CD25+ Tregs, LZT failed to abolish the Th1/Th17- mediated colitis (increased colitis score, vigorous T cell proliferation), indicating a pivotal role for Tregs in colitis prevention by LZT. In addition, LZT induced colitis prevention was an allergen-specific mechanism as demonstrated by use of a second unrelated contact allergen (e.g. DNFB) for tolerization.

Our data demonstrates that LZT inhibits a CD4+ Th1/Th17-mediated colitis independent of the site of tolerance induction (oral and epicutaneous) in an allergenspecific manner by activation of CD4+CD25+FOXP3+ Tregs.

P003 (O04/01)

Specific TLR activation mediates innate immune signal triggered co-factor dependent anaphylaxis

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Classically, anaphylaxis is induced by cross linking of IgE bound to FcεRI on mast cells (MC) and basophils. Alternatively, cross linking of FcγRs by allergen-specific IgG antibodies can also initiate anaphylaxis. However, the clinical relevance of this observation in humans is still unclear. In contrast, the clinical significance of co factors triggering anaphylaxis is broadly accepted. This is best documented for wheat dependent exercise induced anaphylaxis. Other well documented cofactors are alcohol consumption or infections. To study how infections trigger anaphylaxis, we sensitized mice with Ovalbumine (OVA) and prior to low dose OVAchallenge, pretreated them with pathogen associated molecular patterns (PAMPs) to mimic infection. Our results clearly show that pretreatment with Peptidoglycan (PGN), Pam2cys and also CpG triggers full-blown anaphylaxis as measured by significantly decreased core body temperature, correlating significant reduction in systolic blood pressure and increased serum histamine levels. Experiments using either passively or actively sensitized basophil depleted and mast-cell-deficient Kitw-sh/w-sh mice showed that PAMP driven anaphylaxis is not only basophil dependent and MC independent but also independent from IgE but dependent on IgG. In mice deficient in the pattern recognition receptors (PRR) TLR2 or NOD2, the latter sensing the PGN fragment MDP, pretreatment with PGN still triggers anaphylaxis. However, PAMP dependent anaphylaxis after pretreatment with PGN is strongly impaired, although not absent, in mice double deficient in TLR2 and NOD2. In contrast, PAMP triggered anaphylaxis is completely absent in TLR2−/− mice after pretreatment with Pam2cys or in TLR9−/− mice after pretreatment with CpG, respectively. Thus, our results for the first time show a relevance of IgG and basophil dependent anaphylaxis in a clinically relevant setting. In addition, the modulating effect of PAMPs on the onset of anaphylaxis is mediated by cellular activation via specific TLRs. Those results can be of major clinical importance for the diagnosis and management in patients with anaphylaxis and may help to develop new therapeutic strategies.

P004

Atopic dermatitis and attention deficit hyperactivity disorder: altered granule transport mechanisms pave the road to atopy

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Epidemiology suggests atopic dermatitis (AD) to be an independent risk factor for attention deficit hyperactivity disorder (ADHD) and vice versa. We recently demonstrated a significant increase of parameters typical for AD pathophysiology in ADHD children treated with methylphenidate (MP, Ritalin) as compared to healthy controls (HC). These preliminary data were now expanded. In addition, we investigated the putative influence of MP-treatment on these parameters.

Serum levels of IgE, eosinophilic cationic protein (ECP) and tryptase (Pharmacia- CAP-system), eosinophils (%) and the portion of CD8hi+ cytotoxic T cells (CTL) positive for lytic perforin-containing granules before and after stimulation with iomomycin/PMA (flow cytometry) as well as the dermographism were determined in 21 ADHD children treated with MP after diagnosis was confirmed in a day-care clinical setting over several weeks, 12 matched AD children diagnosed according to the criteria of Rajka and Hanifin and 9 healthy control individuals w/o any atopic/psychiatric background. In addition, a subgroup of MP-treated ADHD children ($n = 14$) were investigated before and after a therapeutic break of 42 ± 5 days.

3/33 children suffered from AD and ADHS and were excluded from further analysis. All other ADHD children did not show any history for nor any clinical evidence of atopy. ADHD and AD children showed significantly higher (i) IgE serum level (185 ± 305 and 375 ± 745; HC: 33 ± 51 kU/l), (ii) eosinophils (6.4 ± 8% and 8.7 ± 6%; HC: 1.9 ± 1%) and (iii) ECP level (27 ± 26 and 44 ± 26; HC: 12 ± 7 μg/l). In >90% of ADHD children a white dermographism (mediated by noradrenalin-granule release) was demonstrable. CTL of AD- and ADHD children released their perforin-containing granules significantly faster as CTL of HC. During the MP treatment break, the following significant changes were observed: percentage of perforinpos lymphocytes and serum tryptase levels rose; ionomycin/PMA stimulated perforin release from CTL was slower. The latter two parameters correlated positively. Changes in IgE and ECP levels after the break correlated positively as well.

Our data demonstrate an unexpected tight connection between AD and ADHD on the pathophysiological level and, thus, support epidemiological findings. Both entities showed a facilitated and accelerated release of storage granules (noradrenalin, perforin). Since the perforin-granule system is involved in control of IgE production and noradrenaline supports T-type2 reactions, abnormalities described may contribute in these patients to their increased disposition for atopy. The finding of altered granule transport mechanisms in ADHD opens new views on ADHD pathophysiology. Surprisingly, MP treatment altered granule based mechanisms (tryptase, perforin). One may speculate that part of MP's beneficial effect is mediated by influencing granule transport mechanisms in the central nervous system as well.

P005

House dust mite-specific T cells display a Th2, Th17, and Th2/Th17 phenotype in atopic dermatitis

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Background: In atopic dermatitis (AD), Th2-polarized T cells represent a key factor in allergic inflammation and it has been demonstrated that allergen-specific Th2 cells are reduced after successful specific immunotherapy. Furthermore, the Th17 and Th22 subsets seem to play distinct roles in allergic diseases and in the acute phase of AD. MHC multimer technology has been applied to investigate the specific T cell response in allergic rhinitis and asthma patients during the last years, while less data is available regarding AD.

Aim: This study aimed to describe the phenotype of house dust mite allergen Der p1- and Der p 2-specific T cells in the circulation of AD patients applying MHC tetramers directly *ex-vivo*.

Methods: Der p 1 and Der p 2-specific $CD4^+$ T cells bound by MHC class II tetramers were isolated from the circulation of HLA-matched, HDMsensitized patients with mild to severe AD ($n = 17$). Surface expression of Th1, Th2, and Th17 markers (IL-18-R, CRTh2, CCR6) as well as T cell differentiation markers (CD45RA, CD45R0, CD27) were assessed on a single cell level applying the imaging technique ChipCytometry. The respective cytokines (IFN-γ, IL-4, IL-17, IL-22) were assessed by ELISA in cell culture supernatants from T cell lines ($n = 26$) which were generated in presence of either Der p 1 or Der p 2.

Results: We detected Der p-specific T cells of the Th2, Th17, and Th2/ Th17 subtypes and demonstrated the production of the cytokines IL-4, IL-17, and IL-22 in response to the two major house dust mite allergens Der p 1 and Der p 2. Surface differentiation markers indicate a role of these T cell subsets in the inflammation. Patients with detectable $tetramer^+$ T cells suffered from significantly more severe AD than those without.

Conclusion: Our experiments demonstrate that the T cell response to Der p 1- and Der p 2-allergens is complex since not being restricted to the classical Th2 phenotype. Interestingly, we observed similar cytokine responses to Der p 1 and Der p 2. Applying our experimental methods, we were able to identify a Th2/Th17 T cell phenotype in the circulation of sensitized AD patients, which has been described in allergic rhinitis and asthma recently.

P006 (O06/05)

Three-year follow-up of regulatory T cells during wasp venom immunotherapy

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Background: During wasp venom immunotherapy (VIT) induction of allergen-specific regulatory T cells (Treg) is believed to play a pivotal role in promoting long lasting immune tolerance. We have reported that Treg are specifically activated within the first month of VIT followed by a decline to baseline levels after 6 months (J Allergy Clin Immunol, 2011;127:495–501). Furthermore, we could demonstrate oligoclonal expansion of activated Treg bearing the T cell receptor (TCR) Vβ2 and Vβ5.1 chains (J Allergy Clin Immunol, 2012;130:994–996).

Objective: We investigated 10 patients over a time period of 3 years VIT for the peripheral trafficking and functional capacity of CD4+CD25+CD45RO+ memory Treg, CD4+CD25−CD45RO+ memory T cells as well as CD4+CD25+CD45RO− naïve Treg and CD4+CD25−CD45RO− naïve T cells.

Patients and methods: Treg and conventional T cells of freshly isolated peripheral blood mononuclear cells (PBMC) of 10 wasp venom allergic patients were analyzed longitudinally (i.e. before VIT, after 1, 6 and 36 months of VIT) by flow cytometry for surface expression of Vβ2, Vβ5.1, Vβ3, Vβ8, Vβ13.1, Vβ17 as well as lymph node seeking CCR7 and integrin CD62L, respectively. In addition, wasp venom specific proliferation and cytokine secretion of PBMC were monitored.

Results: In concert with an enhanced activation of Treg after 1 month VIT as analysed by an increased CD45RO expression, an induction of the co-expression of the lymph node homing receptors CCR7/CD62L on Treg was noticed. In parallel, relative Treg numbers in the peripheral blood declined, strongly suggesting a pronounced recirculation of activated Treg into secondary lymphoid organs. Interestingly, relative Treg numbers as well as the activation status normalized to baseline levels after 6 months and 3 years, respectively, indicating that an enhanced Treg recirculation capacity into secondary lymphoid organs may be critical in the early phase of VIT. This is supported by the abolishment of the wasp venomspecific proliferation after 1 month of VIT (stimulation index (SI) 0.8), followed by a pronounced induction of proliferation after 6 months of VIT (SI 4.8) and almost equal stimulation indices after 3 years as compared to the initiation of therapy (SI 2.5 vs 2.9). Abolition of wasp venom-specific proliferation was accompanied with a markedly increase in IL-10 secretion after 1 month, while wasp venom-specific IFN-γ production was readily detected after 6 and 36 months of VIT.

Over a time period of 3 years VIT sustained the oligoclonal expansion of Vβ2+ and Vβ5+ Treg while the expression of Vβ3, Vβ8, Vβ13.1 and Vβ17 chains remained constant.

Conclusions: The increased Treg homing capacities after 1 month of VIT returned to baseline levels after 3 years strongly suggesting that the action of Treg is critical in the early phase of VIT. In contrast, VIT promotes a sustained level of oligoclonal expanded Vβ2+ and Vβ5+ allergen-specific Treg populations over 3 years. Therefore, the functional role of Treg during VIT may change over time from early suppression of wasp-venom specific proliferation to fine-tuned maintenance of tolerance.

P007

Hapten-induced extracellular ATP is degraded by Treg, impairing egress of effector T cells from lymph nodes and reducing contact hypersensitivity reactions

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ATP is released in the skin upon application of contact sensitizers. It acts as a danger signals by activating inflammasomes and other proinflammatory pathways in T-cells and dendritic cells. This action is mandatory for sensitization. In a murine model of TNCB-induced contact hypersensitivity (CHS) we injected regulatory T-cells (Treg) before sensitization and noted increased size and cellularity of the skin draining lymph nodes (LN). Of note, the ear swelling reaction following hapten challenge was abrogated too, indicating absent sensitization. To dissect the underlying mechanisms, we analyzed the LN 2–3 h after Treg injection and sensitization, and found enhanced numbers of CD62L+CD4+ T cells in the dLN. In contrast, without injection of Treg, hapten-application resulted in downregulation of CD62L expression in T cells. As for the mechanism of CD62L downregulation, we found that ATP released into the extracellular space at the site of hapten application is inducing the sheddase ADAM17. ADAM17 removes CD62L from the surface of effector T cells enabling the cells to leave the LN and to migrate into distant tissues. However, Treg express the ectonucleotidases CD39 and CD73 that degrade ATP into adenosine. Thus, after injection of Treg extracellular ATP is removed from the tissue and shedding of CD62L in effector T cells is abrogated, impairing their migration to tissues sites. These results were confirmed by experiments using Treg from CD39 deficient animals. Injection these Treg failed to prevent CD62L shedding *in vivo* and were unable to block the sensitization phase. In summary these data indicate that the regulation of ATP turnover by Treg and other cells in skin and LN is an important regulator for immune responses.

P008

Nonsteroidal anti-inflammatory drug (NSAID) hypersensitivity – possible association with elevated serum tryptase and tolerance of COX-2-inhibitors

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NSAIDs are a frequent cause for hypersensitivity reactions resulting in skin, respiratory or severe non-allergic anaphylaxis and even death. Elevated serum tryptase is detected in patients with manifest or occult mastocytosis, a disease that was confirmed to be associated with a higher risk of anaphylaxis. Certain drugs, amongst them NSAIDs, have been described to induce immediate type reactions in such patients, which is frequently discussed, however, only supported by case reports. Furthermore, selective COX-2-inhibitors were reported to be well tolerated by patients with NSAID hypersensitivity, although to date this has not been confirmed and there is no clear recommendation for the treatment of these patients with COX-2- inhibitors. Single-blinded, placebo-controlled drug provocation testing is the gold standard to evaluate possible NSAID hypersensitivity and cross reactions between different drugs.

We generated a data base including all patients that were worked up for NSAID hypersensitivity and received drug-provocation testing in our allergy clinic from 2004 to 2014. Data was analyzed for specific hypersensitivity reactions, basal serum tryptase level, positive skin reactions and tolerance of COX-2-inhibitors. In order to detect patients with immediate and delayed type allergic reactions skin prick tests (SPT) or patch tests (PT) were performed.

We evaluated drug skin tests and 1256 drug challenge tests in 441 patients (37% men, 63% women, mean age 47.6). In our 441 patients we observed 25 positive skin test reactions (5.7%), with 8 positive PT (32%) and 17 positive SPT (68%). Most frequently skin reactions occurred for metamizol (4 PT, 8 SPT). Skin tests were rarely positive for acetylsalicylic acid (ASA) (1 PT), acetaminophen (1 SPT), celecoxib (2 PT) and diclofenac (1 PT, 2 SPT). Upon oral challenge, in total 18.0% showed hypersensitivity reactions to the exposed substance. The highest percentage of reactions in exposed individuals occurred with ASA (18.6%), followed by ibuprofen (6.6%), diclofenac (3.45%) and acetaminophen (3.25%). Although frequently exposed ($n = 324$ challenges) COX-2-inhibitors (celecoxib, rofecoxib, valdecoxib, etoricoxib) induced reactions in only 0.62% of challenges. 83% ($n = 50$) of the patients with a proven ASA hypersensitivity were exposed to celecoxib and acetaminophen. All of them tolerated celecoxib and 98% tolerated acetaminophen. Only 1 patient cross-reacted to ASA and acetaminophen. Serum tryptase levels were elevated (>11.4 μg/l) in 19 patients, but only 1 of these patients had a positive reaction to ASA during challenge. In comparison to a published normal population that showed elevated serum tryptase in 4.3%, levels were not significantly higher in our group of patients with suspected NSAID hypersensitivity (4.6%). Of the 83 patients with exposure proven NSAID hypersensitivity only 1 patient (1.2%) showed elevated serum tryptase.

Concluding we found that selective COX-2-inhibitors and acetaminophen are well tolerated by the majority of patients showing hypersensitivity to other NSAIDs. Skin testing is an additional diagnostic approach if a reaction to metamizol is reported in the history. Serum tryptase levels are not elevated in patients with suspected or confirmed NSAID hypersensitivity.

P009

Analysis of basophilic activation and T cell responses by allergens encapsulated into degradable PEG-nanocarriers

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Polymer nanoparticles are used to protect therapeutic proteins or drugs from degradation, obtaining higher local concentrations and enabling targeted transport. Encapsulation of allergens inside polymeric nanoparticles could also help to avoid antibody-mediated side effects occurring during allergen-specific immunotherapy. We have synthesized a novel type of difunctional, water soluble poly(ethylene glycol) dimethacrylate macromonomer with acetal-sites that degrade at acidic pH. The allergen and the macromonomer were entrapped inside liposomes as templates that were produced by dual asymmetric centrifugation. Radical polymerization of the methacrylate groups inside the liposomes generated PEG-nanocarriers. The allergen-loaded nanocarriers were approximately 150–200 nm in size and showed low polydispersity indices. It could be demonstrated in in-vitro studies that immature dendritic cells (DC) internalize these protein-loaded, non-toxic PEG-nanocarriers. Viability and surface marker expression of DC was not affected by allergen-loaded nanoparticles. Furthermore, nanoparticles themselves did not lead to DC maturation. To investigate the targeted delivery of allergens into the antigen-presenting pathway, T cell proliferation was investigated. DC treated with allergen-loaded nanoparticles induced an allergen-specific proliferation reaching about 50% of the proliferation intensity observed for T cells stimulated with allergen-treated DC alone. Allergen is shielded by the nanocarrier, which was analyzed via allergen detection by IgE-loaded basophilic leucocytes and subsequent leukotriene release. These data indicate that allergen-loaded nanoparticles are capable of inducing specific immune responses necessary for specific immunotherapy. Furthermore, this finding encourages functionalization of nanoparticles with targeting molecules like mannose for increased allergen uptake and presentation by DC.

P010

Chronic urticaria registry (CURE) – the first international disease registry for chronic urticaria

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Background: Chronic urticaria (CU) is a highly frequent allergological and dermatological disorder. Many CU patients are affected for years and exhibit a severe impairment of their quality of life. As of now, the epidemiology, duration, course, response to treatment and underlying causes of CU are still ill defined. While a registry would be an appropriate tool to assess these features, this was, until recently, not available. Therefore, the Chronic Urticaria Registry (CURE) was set up as an academia-driven, open-ended registry for all subforms of CU. CURE is investigator-initiated and hosted by the non for profit organization Urticaria Network e.V. (UNEV) with support by the GA^{2}LEN, EAACI and EADV Task Forces for CU. CURE partners with physicians, scientists, patient organizations, payers, health authorities and industry.

Objectives: CURE aims to collect quality, real-life data on CU patient characteristics, the course of disease, underlying causes, comorbidities, treatment responses, quality of life impairment and health care costs.

Study design: The registry setup is divided in several steps. In a first step an International Steering Committee (ISC) for CURE was convened to specify the most important open questions in CU and to translate these questions into appropriate CURE items. In addition, its purpose is to decide on the cooperation with and the acceptance of CURE partners, to discuss and suggest data explorations of the CURE data base and arising publications, and to decide on the future addition or deletion of CURE items. In a second step, the CURE questionnaires for a basic entry and follow-up entries were generated, reviewed by the ISC, and will be transformed into a webbased, online registry. In a third step, CURE will be launched in Germany, Spain, France, Italy and the UK and all urticaria-treating physicians in these countries will be invited to enter patient data. The entering physicians will keep access to the data they entered and may suggest data analyses to the ISC in case more than 10 patient data sets were entered. Later, CURE will be expanded to a global registry. The data of CURE will be analysed in regular intervals and its results will be published.

Conclusions: CURE is the first disease registry for CU of all subforms. It is academiadriven and set up by a joined, multinational effort of urticaria specialists to obtain data that are needed to better understand CU and to improve CU patient care.

P011

Anaphylaxis: discrepancy in guideline adherence between acute management and medication at discharge

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Background: Anaphylaxis is a life-threatening condition that is often poorly recognized and treated. Reliable epidemiological data on incidence is sparse and emergency departments have the largest experience in its management.

Objective: In this study, the fast encounter of the emergency specialist was investigated together with adherence to the current WAO-guidelines in management of anaphylaxis.

Methods: Data on anaphylactic patients was collected over a period of 4 years (2009–2012). During this period emergency physicians were encouraged to complete a questionnaire for adult patients with anaphylaxis presenting in the emergency room. 212 368 patients visited the emergency department of the Sint-Pieters hospital, Brussels and 100 cases were included in the study. Inclusion criteria to administer the questionnaire were based on the Sampson criteria of gradation and definition of anaphylaxis (JACI, 2006). To prevent over-diagnosis of anaphylaxis, grade I anaphylaxis cases,

according to the classification of Ring (The Lancet, 1977), were not included in our database. All data were analyzed using a Microsoft Excel database. Collected data elements included age, blood pressure, pulse, past medical history, symptoms, possible etiology of anaphylaxis, tryptase values, emergency management and recommendations at discharge.
Results: 0.05% of all emergency visits in adults presented with anaphylaxis. In both women and men, dyspnea and urticaria were the most frequently noted symptoms. More men displayed larynx and pharynx oedema, while women had digestive complaints. Only 28% received medical help within 30 min of onset and 20% had their first medical contact within 31–90 min.
81% of cases presented with grade 4 or 5 anaphylaxis. As age increased, severity of anaphylaxis increased.
Treating physicians noted that 49% of cases had a possible etiology of food, 44% medications and 3% hymenoptera venom. In 4% specialists indicate other causes. Women anamnestically indicated more allergy in their previous history. 48% of patients reported a food-related anaphylaxis in their previous history.
67% of all patients were treated with adrenaline, 85% received antihistamines, 89% were given methylprednisolone. 11% of grade 5 category had resuscitation, 78% received adrenaline, 78% received antihistamines and 89% were treated with methylprednisolone.
46% of all patients were discharged directly from the emergency room. 87% of those patients received recommendations for further treatment or follow-up including 67% corticosteroids, 83% antihistamines, 9% Adrenaline IM (Epipen®), 74% were instructed to consult an allergist.
Conclusion: Our urban population showed the same characteristics, concerning symptoms and frequent elicitors, as previous cohorts, confirming that age over 50 is a risk factor for more severe anaphylaxis.
The majority of patients was treated according to the WAO-guidelines regarding the acute management of anaphylaxis with use of IM Adrenaline, while only a minority received the recommended Adrenaline IM (Epipen®) at discharge. However, 72% of all patients received medical help at least 30 min after appearance of first symptoms, underlining the necessity of the prescription of Adrenaline auto-injectors.
This data shows that current guidelines on acute management of anaphylaxis were followed in this emergency department. However recommendations at discharge were in most patients insufficient, regarding the prescription of adrenaline autoinjectors. Furthermore the majority of patients were advised to consult an allergist: the collaboration between the emergency care and the allergist might improve diagnosis and follow-up.

P012

Interaction of brain derived neurotrophic factor (BDNF) with the cholinergic system of the skin under stress

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Secretion of BDNF in the central nervous system and in peripheral tissues has been shown to be regulated by oxidative stress as well as psychoemotional stress. Recent studies indicate that this process is tightly regulated by the expression of acetylcholine (ACh) and muscarinic ACh receptors (mAChR). Through the mediation of the miR-376b-5p, BDNF-mAChR M3 interaction plays a potentially protective role in Alzheimer disease development and cardiac ischemia, while it is negative for the development of asthma. In skin mAChR M3 is prominently expressed in mast cells. Hence, the interaction with BDNF may have a vital role in the skin's cholinergic system. However, such an interaction is unknown in the skin and carries in itself a great therapeutic potential in the treatment of atopic dermatitis. Using a mouse model for atopic dermatitis-like allergic dermatitis (AlD, inflammatory stress) and a mouse model for noise exposure (psychosocial stress), treatment with BDNF neutralizing antibodies partially abolished the stress-induced barrier dysfunction. In organ cultures full thickness skin biopsies (*ex-vivo*), transfection of mir-376b-5p down regulated BDNF expression. Using this model of the back skin biopsies from C57BL/6 Wt or nAChRα7 KO mice, subjected to H2O2 induced stress, were analyzed for mRNA expression of the cholinergic markers such as choline acetyltransferase (ChAT), vesicular acetylcholine transporter (VAChT), Secreted Ly-6/uPAR-related protein 1 (SLURP1) and BDNF by RT-PCR. H2O2 stress down regulated the mRNA expression of mAChR M3 and BDNF in Wt mice. Muscarine (a Pan-muscarinic agonist) upregulated the mRNA expression of BDNF in Wt mouse skin indicating a potential regulation of BDNF by mAChR M3. It is clear from these results that there is an interaction between BDNF and mAChR M3 in the murine skin under stress. Further immunohistochemistry and biochemical analysis as well as ongoing experiments in mAChR M3 KO skin will shed more light in to this fascinating interaction between BDNF and mAChR M3.

P013

Long-term outcome after discontinuation of hymenoptera venom-specific immunotherapy

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Background: Allergen-specific immunotherapy (ASIT) is very effective in patients suffering from IgE-mediated allergy against hymenoptera venom (HV). Induction of regulatory T(reg) cells, loss of HV-specific Th2 cells and synthesis of allergenblocking IgG antibodies are discussed as potential mechanisms responsible for the establishment of allergen tolerance. However, only a few clinical studies have investigated the clinical course of HV-allergic patients after discontinuing ASIT, and post-ASIT analysis of cellular and humoral alterations in these patients are virtually missing.
Material and methods: HV-allergic patients ($n = 50$) treated with ASIT for 3–5 years were evaluated up to 11 years after finishing ASIT. Analysis included medical history, i.e. repeated bee or wasp stings and their clinical outcome, skin tests with HV and evaluation of peripheral blood immune parameters. HV-specific IFN-γ-, IL-5- or IL-10-secreting T cells, representing Th1, Th2 and type-1-Treg (Tr1) cells, respectively, were quantified by ELISPOT assay, CD4+CD25+CD127low Treg cells were characterized by flow cytometry, and allergen-specific IgE, IgG and IgG4 antibodies were determined by ImmunoCAP. The *in vitro*-capacity to block allergen- IgE complex formation was measured by the recently established cell-free HVELIFAB (enzyme-linked immunosorbent facilitated antigen binding) assay.
Results: Post-ASIT analysis of HV-specific T cells showed increasing numbers of Th2 but reduced frequencies of Th1 and Tr1 cells compared to control individuals, while CD4+CD25+CD127low Treg cells were unaltered. HV-specific IgE antibody concentrations were not affected. However, IgG4 antibody levels started to decrease after cessation of ASIT, which was associated by a decline of patients' serum blocking capacity. Almost 30% of the patients (14/50) experienced a re-sting, which was well tolerated by 8 but resulted in anaphylactic reactions in 6 patients. Interestingly, HV-specific IgG4 antibody concentrations did not differ substantially between the two groups.
Conclusion: Our results suggest that both T cellular and humoral alterations induced by ASIT are not maintained for prolonged time frames in HV-allergic patients after stopping treatment. However, it remains to be determined which parameters are indicative for long-term protection against repeated exposure to HV.

P014

Allergic diseases do affect the true elderly

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Background: Data on allergic sensitisation and prevalence of allergic diseases in the elderly are scarce. As cross-sectional studies performed in mid-age patients (>45 years of age) hint at a decrease in IgE with age, it was hypothesized that true *de novo* sensitization does not occur in the elderly. Observations on food allergy in patients >70 years have demonstrated cross-reactive sensitisations as well as *de novo* sensitization and food allergy following loss of stomach acidity. We aimed at describing the prevalence of sensitisation and allergic disease in the aged and addressed *de novo* sensitization in advanced age by assessing sensitisation to the plant ambrosia (ragweed) which is a neophyte to our catchment area spreading for the past ten years.
Method: We recruited 896 patients in three age strata (60–69; 70–79; 80–89 years)at seven test sites in south west Germany, having never resided in regions with ambrosia habitats (e.g. US, Canada, Hungary, Northern Italy). Patients underwent physical examination, questionnaire, skin prick testing (SPT), sIgE measurements, and appr. 100 patients also provocation testing.
Results: Data on birch, mugwort, ambrosia and house dust mite sensitization will be presented. We found that sensitization to the allergens tested can be frequently found in the aged, but tends to decrease with age. This was demonstrated for indoor as well as outdoor allergens (house dust mite SPTpositive: 60–69 years: 26%; 70–79 years: 22%; 80–89 years: 15%; birch pollen SPT positive: 60–69 years: 28%; 70–79 years: 20%; 80–89 years: 15%). Most subjects sensitized to ambrosia were also sensitized to mugwort, exhibiting cross-reactivity, but 2.8% of the aged tested were sensitized to ambrosia only. This was interpreted as *de novo* sensitization. In contrast to sensitization against house dust mite allergens for which atopy was a risk factor, only 10% of ambrosia only sensitized patients were atopic according to the Hanifin and Rajka criteria. While RCA was the most frequent manifestation of allergic disease, with 24%, asthma and eczema did occur in just 8% of the sensitized. When age at manifestation was assessed by questionnaire, asthma, like eczema was remembered to have manifested by most affected before age 20, and only in 11% past age 60 (eczema 17%), while RCA manifested mostly in adulthood (50% 20–60 years; 8% >60 years; 42% <20 years).
Conclusion: Allergic diseases do affect the true elderly population. New sensitisations and new manifestations of disease do occur past age 60. Even though the burden of disease and the prevalence of sensitisations decrease during age, the projected development of age distribution for the next decades will necessitate services directed at therapy for these patients.
In a very small subgroup of patients true sensitisation to the neophytic antigen ambrosia/ragweed is observed. As this allergen is just emerging in Germany, this may be due to new sensitization at an age beyond 60 years of age.
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P015

Lipid mediators from pollen inhibit the innate antiviral defense

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Background: Asthmatics and allergic rhinitis patients are more susceptible to respiratory infections and viral exacerbations than healthy individuals.
Aim of the study: To elucidate whether pollen exposure itself can compromise the innate antiviral response of airway epithelium.
Methods: Primary human nasal epithelial cells were stimulated with ligands of TLR3, RIG-I and MDA-5 in the absence or presence of aqueous pollen extracts (APE). Human 3D bronchial epithelial cultures were infected with rhinovirus in the absence or presence APE. Culture supernatants were analysed for pro-inflammatory cytokines and type III interferons. IRF-3 and -7 were detected in nuclear lysates by Western Blotting. *In vivo* effects of intranasal pollen exposure were assessed in a murine respiratory syncytial virus (RSV) model.
Results: APE dose-dependently inhibited the TLR-3-, RIG-1/MDA-5 and rhinovirus induced production of type III interferons. APE and candidate compounds, 9-,13-HODE/9-,13- HOTE, block nuclear translocation of pIRF-3 and -7. In the murine RSV model, intranasal exposure to APE directly after RSV infection resulted in increased weight loss.
Conclusion: Lipids released from pollen can inhibit antiviral type III interferons and proinflammatory cytokines. This might indicate a compromised response against respiratory viruses during episodes of pollen exposure.

P016

Low dose omalizumab therapy inhibits IgE production *ex vivo* using PBMCs

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Omalizumab, an anti-human IgE antibody, improves symptoms of allergic asthma, allergic rhinoconjunctivitis and chronic spontaneous urticaria. During therapy, serum levels of free circulating IgE drop down to about 10% of the original level. The objective of this research was to identify and/or quantify the effect of inhibition of peripheral IgE production.
Therefore, 8 patients with chronic urticaria refractory to level III of the international EAACI/WAO/EDC/GA2LEN 2014 therapeutic guidelines and 6 patients with chronic but acute exacerbated atopic dermatitis and hyper-IgE-syndrome (>2000 kU/l total serum IgE) were recruited. Systemic therapy was changed to cetirizin. After 4 weeks, low dose omalizumab therapy (150 mg s.c./month) was initiated, peripheral blood was obtained every 4 weeks for 16 weeks. To estimate IgE production initiated *in vivo*, ficoll-isolated peripheral blood mononuclear cells were incubated in RPMI containing 10% fetal calf serum at 37°C for 8 days. Cell free supernatants were harvested and kept at −80°C until IgE was determined (Pharmacia CAP system, low level method). Results are expressed as relative IgE levels (maximal IgE level produced by an individual was set for 100%). In parallel, numbers of peripheral CD20+ B lymphocytes, percentage of perforin-granule contaning cytotoxic T cells (CTL, a known IgE regulating cell type) and the surface expression of FcεRI on HLADR+ CD123+ basophils were quantified in a FACS-CANTO flow cytometer. 6 healthy volunteers served as an untreated control cohort.
4 weeks after the first dose of omalizumab, 4 individuals had reduced their IgE production *ex vivo* down to <50%. In 4 other patients, IgE production dropped down more slowly. In contrast, in 6 individuals IgE production was upregulated firstly. In all of them, IgE levels produced *ex vivo* dropped down again 4 weeks after the third omalizumab injection. At this time point, reduction of IgE production within the cohort reached statistical significance ($P < 0.05$). The percentage of CD20+ lymphocytes as well as the portion of perforin+ CD8+ CTL were not altered significantly during omalizumab therapy. Surface expression of FceRI on basophils as estimated by mean fluorescence intensity was reduced significantly starting 4 weeks after the first dose of omalizumab in 11/14

patients. However, in the remaining three individuals, surface expression of FcεRI was upregulated over several weeks but dropped to low levels after the fourth dose.
We report here for the first time that Omalizumab is capable of inhibiting IgE production in humans. Removal of a protein from peripheral blood, e.g. of an autoantibody by immuno absorption, can lead to its subsequent (over)-production. IgE upregulation in 6/14 patients during the first weeks of treatment might represent such a phenomenon. However, repeated application of omalizumab suppressed IgE production to low levels in all patients. So far, from all cell types putatively involved in IgE production, only basophils seem to be altered during omalizumab therapy. Mechanisms how omalizumab achieves inhibition of IgE production remain to be elucidated.

Cellular Biology

P017

Cockayne syndrome and disturbed protein homeostasis

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Aging can successfully be studied by exploring premature aging diseases that exhibit an 'accelerated' aging phenotype with defined genetic mutations. Deciphering the mechanisms that drive the 'accelerated' aging process will help to better understand the driving forces of aging in general.
An interesting proponent of premature aging diseases is Cockayne syndrome, a severe developmental and degenerative disorder that comprises skin atrophy, neurological degeneration with deafness and mental retardation, retinaatrophy and severe growth retardation that can even lead to early childhood death. It can be caused by recessive mutations in five different genes with mutations of the Cockayne syndrome proteins A (CSA) and B (CSB) are causing the majority of the cases. The Cockayne syndrome proteins are all involved in the repair of UV-lesions in DNA by the nucleotide excision repair pathway, thus explaining the hypersensitivity to UV light, typical for this disease, however, mutations in CSA and CSB can also cause the mild cutaneous hypersensitivity syndrome UV-sensitive syndrome (UVs) with normal development and live expectancy. Cells of these patients also show hypersensitivity to UV-light but, in contrast to Cockayne syndrome cells are not hypersensitive to oxidative stress. Thus, a problem to cope with oxidative stress may cause premature aging and neurodegeneration in Cockayne syndrome.
We previously showed that all CS proteins play a role in the key step of ribosomal biogenesis, transcription of the rRNA by RNA polymerase I. Ribosomal biogenesis, growth and protein synthesis are severely impaired by mutations in CS proteins. Starting from these observations we hypothesised that a failure in protein homeostasis may qualify as a common denominator in Cockayne syndrome causing both premature aging and neurodegeneration.
The analysis of cells from severely affected CSB patients compared with cells from mildly affected UVs patients revealed that Cockayne syndrome cells have a markedly reduced number of ribosomes and a decrease in protein synthesis per ribosome. Of note, CS cells show reduced translational fidelity and an increase in protein oxidation. Additionally, cells from CS patients exhibit significantly higher protein turnover compared to control cells. Taken together, cells from Cockayne syndrome patients but not from UVs patients have a reduced number of ribosomes that display quantitative and qualitative disturbances in protein synthesis and – possibly due to oxidation – an elevated protein turnover. Hence, a previously unreported severe imbalance in proteostasis may contribute to or even cause premature aging and neurodegeneration.

P018

Platelet-derived growth factors induce the expression of the antimicrobial peptide human beta-defensin-2 in primary keratinocytes

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Background: Platelet-derived Growth factors (PRGF) is a thrombocyte concentrate's lysate containing a variety of chemokines, cytokines and growth factors. *In vitro* it is supposed to stimulate cell proliferation and tissue regeneration, to modify cell and tissue differentiation and to support angiogenesis. *In vivo* thrombocyte concentrate's lysates are recently used to support healing of chronic or complicated infected wounds. Human beta-defensin-2 (hBD-2) is an anti-microbial peptide expressed in normal human keratinocytes exhibiting potent antimicrobial activity against Gramnegative bacteria that often cause severe clinical wound healing problems. The aim of this study was to analyze the influence of PRGF on hBD-2 gene and protein expression in human primary keratinocytes.
Material and methods: We isolated PRGF from freshly donated human thrombocyte concentrates and used it for *in vitro* stimulation of human primary keratinocytes. Subsequently, total RNA was isolated and reverse transcribed in cDNA which served as template in a realtime- PCR to analyze gene expression of hBD-2 and various cytokines. In addition, ELISA was used to investigate hBD-2 protein release in culture supernatants of keratinocytes treated with PRGF.
Results: We observed a concentration-dependent significant induction of hBD-2 gene expression in PRGF-treated keratinocytes. In concordance with the gene expression data ELISA analyses of cell culture supernatants revealed that PRGF induced the secretion of hBD-2 in keratinocytes. Induction of hBD-2 was time-dependent with highest levels after 24–72 h.
Since PRGF is known to contain various growth factors we used an antibody (cetuximab) directed against the epidermal growth factor receptor (EGFR) which significantly blocked the induction of hBD-2 by PRGF.
An analysis of cytokine expression in keratinocytes stimulated with PRGF revealed a high induction of IL-6 already after 4 h. To evaluate the potential influence of IL-6 on the observed PRGF-mediated hBD-2 induction we used an antibody (tocilizumab) directed against the IL-6 receptor. Tocilizumab significantly reduced hBD-2 induction in keratinocytes stimulated with PRGF.
Discussion: We demonstrated that PRGF stimulation of primary keratinocytes caused a statistically significant increase of hBD-2 gene and protein expression in a concentration – and time-dependent manner. These effects were partially mediated by the EGF- and IL-6 receptor. Our results suggest that the induction of hBD-2 by PRGF may contribute to the observed beneficial effects of thrombocyte concentrate's lysates in the treatment of chronic or infected wounds in-vivo.

P019

Epithelial transdifferentiation of adipocyte-derived stromal cells (ADSC) – comparison of different medium compositions

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Adult stem cells derived from adipose tissue hold great promise for regenerative medicine. By using specific medium supplements, ADSCs can be transdifferentiated *in vitro* into various lineages including osteocytes, chondrocytes, myocytes, endothelial cells, adipocytes or epithelial cells. At first, ADSCs were isolated from abdominal subcutaneous fat tissue and propagated in serumfree medium. Cells were characterized by stemness-associated antigen markers (CD31−, CD45−, CD34+, CD54+, CD90+, CD105+, CD166+, HLA−ABC+, HLA−DR−). The present study is dedicated to compare and optimize culture conditions allowing transdifferentiation into epithelial cells. As read-out serve morphological changes and the expression of pan-cytokeratin as detected by immunocytochemistry and FACS analysis. From a plethora of different parameters tested, particularly all-trans retinoic acid (ATRA) triggered successful transdifferentiation, as indicated by epitheloid cell shapes and the presence of approximately 20% cytokeratin-positive cells. Ongoing experiments test if these results can be further improved. Our findings might help to define condition promoting epithelisation of non-healing ulcers.

P020

Oxidative stress induced IGF-1 resistance in fibroblasts through concomitant activation of the key phosphatases PTP1B and PTEN

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The insulin/IGF-1 signalling pathway is evolutionary conserved in many organism including mammals. The regulation of this pathway is critically involved in determining longevity, metabolism, tissue homeostasis as well as cancer progression. This regulation depends on the delicate balance between activating kinases and suppressing phosphatases at different steps of insulin/IGF-1 downstream signalling. In this report, we demonstrated the novel finding that accumulation of superoxide anion radicals (O2o-) in the mitochondria resulted in a significant activation of two key phosphatases, PTP1B and PTEN, eventually dampening the IGF-1 induced signalling cascade via dephosphorylation of specific tyrosine residues of the IGF-1 receptor (IGF-1Rβ chain) and phosphatidylinositol 3,4,5-trisphosphate (PIP3), respectively. Dephosphorylation of these key mediators of insulin/IGF-1 pathway resulted in reduced activation of PI3 kinase, ribosomal S6 kinase and AKT. Specific inhibition of PTP1B and PTEN either by small molecule inhibitors or by shRNAs significantly attenuated the O2o-induced IGF-1 resistance, which further proved the specific involvement of PTP1B and PTEN. The dephosphorylation of IGF-1Rβ chain and subsequent inactivation of downstream effectors are supposed to supress cell growth by minimising the biosynthesis of translational components and components of the extracellular matrix. In fact, we found that the O2o-mediated IGF-1 resistance resulted in decreased proliferation of murine dermal fibroblasts and significantly reduced mRNA levels of α1 (I), α1 (III), and α2 (I) collagen chains, molecular hallmarks of skin aging. These data are of high clinical relevance as the accumulation of superoxide anion radicals is found to be increased in replicative senescent human fibroblasts. Moreover, skin sections from old human individuals showed higher oxidative damage compared with young individuals. In addition, the IGF-1 signalling pathway was found to be down-regulated in the skin of elderly individuals. Collectively, these findings suggest that O2o-, PTP1B and PTEN represent promising targets for drug development to prevent and treat skin aging and age-related disorders driven by persistent insulin/IGF-1 resistance.

P021

In-silico knock-out predictions identify NEMO as a possible target to prevent the onset of the senescence associated secretory phenotype

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Cells are subjected to continual stresses from exogenous and endogenous sources. These events can cause a number of responses, ranging from complete recovery to malfunction and ultimately cell death. Alternatively some cells can undergo the transition into permanent cell-cycle arrest, to protect from putative cellular and tissue homeostasis disturbing damage. This state called cellular senescence seems to be a fundamental mechanism of aging, and development. Additionally, cellular senescence can be accompanied by the senescence associated secretory phenotype that causes chronic inflammation and paracrine reinforcement of senescence through the release of proinflammatory cytokines by senescent cells. However, the master regulators of these processes are still unknown.
Here we present a core gene regulatory network of the development and maintenance of senescence and the senescence associated secretory phenotype incorporating published gene expression and interaction data of different signaling pathways like IL-1, IL-6, p53 and NF-B. Using computational Boolean network models, we simulate stable states of these complex pathway interactions between p53/p16 steered senescence, IL-1/IL-6 driven inflammatory activity and the emergence and retention of the senescence associated secretory phenotype through NF-B and its targets.
The modeled simulations correspond to published and expected data on cellular senescence and the senescence associated secretory phenotype. This Boolean network reconstructs the different steps of p53/p16 mediated cell cycle arrest and the arising senescence associated secretory phenotype and therefore enables the highlighting of key players in these processes. Furthermore we can predict different in-silico knock-outs that prevent key SASP-players, like IL-1 and IL-6, from getting activated upon cell cycle arrest. In a first screening we found different gene knock-outs and knock-out combinations that prevent the activation of IL-6 and IL-8 signaling, two factors that are responsible for the spreading and retaining of the SASP. In this way we could single out the NFκB Essential Modifier (NEMO) as a target. Under the assumption of DNA damage, a NEMO-knockout was enough to prevent the activation of IL-6 and IL-8 in-silico. Additionally, an in-silico NEMOknockout in combination with a MEK1/2-knockout could not only prevent the activation, but it was also able to terminate a pre-existing activity of IL-6 and IL-8.
Consequently, this gives us the power to create *in vitro* and *in vivo* models that help to better understand the dynamics of the SASP and can be used to broaden our understanding of highly important aging mechanisms. Ultimately, this may help to give rise to future targets for therapy to, at least, facilitate the burdens of aging.

P022 (O01/01)

MSCs sense TGF-β1 deficiency and via adaptive up-regulation of miR-21 enhance TGF-β1 release restoring impaired wound healing in a murine LAD1 model

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Mutations in the gene encoding CD18, the common β-chain of the β2 integrin family, result in severely disturbed wound healing with increased propensity for life-threatening systemic infections in human patients with leukocyte adhesion deficiency-1 (LAD1). Previously, we generated a CD18-deficient (CD18−/−) mouse strain, which similar to LAD1 patients revealed a delay in wound healing due to a lack of neutrophil engulfment by macrophages which subsequently cannot release sufficient TGF-β1. By contrast to PBS injection, adipose tissue derived mesenchymal stem cells (AT-MSCs) injected around full-thickness wounds significantly accelerated wound healing and restored TGF-β1 concentrations to that of wild type mice at all wound stages in CD18−/− mice. Of note, the adaptive TGF-β1 release by MSCs led to augmented α-SMA+ myofibroblast differentiation from wound resident fibroblasts eventually restoring wound contraction and collagen deposition. These beneficial effects were almost completely abrogated when wounds were injected with TGF-β1 silenced AT-MSCs. In order to delineate the mechanism underlying MSC sensing of their wound environment, MSCs were exposed to increasing TGF-β1 concentrations *in vitro* in the presence of S35 labelled methionine/cysteine. Low TGF-β1 concentrations representative for the microenvironment in CD18−/− wounds induced TGF-β1 release from AT-MSCs, whereas high concentrations of exogenous TGF-β1

suppressed *de novo* TGF-β1 production by MSCs as measured by newly synthesized S35 labelled TGF-β1. This adaptive response was mediated via microRNA-21, which suppress the translation of its target Smad7, the negative regulator of TGF-β1 signaling *in vitro* and in wounds *in vivo*. In fact, increase in microRNA-21 reduced Smad7 and enhanced TGF-β1 synthesis when MSCs were exposed to low TGF-β1 concentrations. This regulatory adaptive loop was disrupted if MSCs were silenced with micrRNA-21 antagomirs or were lentivirally transduced with vectors with enhanced Smad7 expression. Also antibodies against subunits of the TFG-β1 receptor or the receptor dependent ALK kinase responsible for relaying TGF-β1 signals intracellularly abrogate the adaptive TGF-β1 release. These data indicate that the TGF-β1 receptor itself and downstream signaling effectors constitute the sensing and the adaptive response mechanism qualifying MSCs as an 'adaptive drugstore' which depending on microenvironmental demands at the wound site substitute for TGF-β1 deficiency. Hence, local delivery of AT-MSCs represents a promising strategy to improve impaired healing in LAD1 patients. Our results are of particular clinical relevance as decreased TGF-β1 also constitute a major hallmark in the widely occurring chronic venous leg and diabetic foot ulcers in humans.

P023

Towards further characterization of ABCB5+ mesenchymal stem cells in the ageing skin

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The ATP-binding cassette transporter ABCB5 was originally found to be expressed on epidermal CD133+ malignant melanoma initiating stem cells and in several other malignancies, responsible for increased resistance against a wide spectrum of chemotherapeutic drugs. We describe a novel population of dermal ABCB5+ multipotent stromal cells (MSCs) with a surface marker expression profile similar to conventional MSCs and functional properties distinct from dermal fibroblasts. To address possible functions of ABCB5+ MSCs in their tissue of origin in the context of inflamm-aging, we analysed their capacity to suppress inflammation. Interestingly, we found that ABCB5+ MSCs could suppress M1 macrophage activation *in vitro* by suppression of NO, TNF-alpha and IL-12 release while no M1 macrophage suppression was observed when ABCB5- dermal fibroblasts were *in vitro* co-cultured with activated macrophages. *In situ*, a highly significant age-dependent decrease in the number of ABCB5+ MSCs from 3.2% of total dermal cells in skin of young individuals to only 1.6% of total dermal cells in the skin of individuals above 70 years of age was found by immunostaining. Double staining for NG2, an established pericyte marker and ABCB5 showed that ABCB5+ MSCs constitute a population independent of pericytes. Interestingly, double staining for ABCB5 and nestin, a marker of hair follicle connective tissue sheath and dermal papilla stem cells termed Skin-derived Precursors (SKPs), revealed two independent cell populations. To exclude an epidermal origin of the observed dermal ABCB5+ MSCs, we performed double staining of ABCB5 with MelanA, a marker indicative of melanocyte origin, CD133, a marker indicative of cancer stem cells and Lgr5, a marker of epidermal stem cells in the outer root sheath of hair follicles. Notably, we did not find staining of ABCB5 with the above mentioned markers, further strengthening the view that ABCB5+ MSCs are of dermal origin and distinct from ABCB5+ CD133+ malignant melanoma initiating cells. Furthermore, ABCB5+ dermal MSCs co-express SSEA4, a marker that was recently found to enrich for dermal MSCs. Double staining for ABCB5 and endothelial cell marker CD31 showed ABCB5+ MSCs as a population distinct from endothelia, residing in a perivascular and interfollicular localisation. Notably, an age-dependent change in niche preference of ABCB5+ MSCs was observed: while in the skin of young individuals, 75% of ABCB5+ MSCs reside in close association to endothelia, in the skin of old individuals only 10% of ABCB5+ MSCs were found in a perivascular localization. Interestingly, in parallel the number of dermal cells expressing the extracellular matrix protein osteopontin was found to be significantly decreased in the skin of old compared to young individuals. These data indicate that ABCB5+ MSCs are a novel stem cell population in the skin important in tissue homeostasis especially in the context of persistent inflammation and are furthermore implicated in skin ageing.

P024

Biocompatibility of aminocelluloses in monolayer keratinocyte cultures and three-dimensional skin models

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Introduction: Aminocelluloses (AC) are chemical modified biomacromolecules. They are produced by introduction of amino groups at the C6-position of cellulose by nucleophilic substitution. We have already shown that functionalized celluloses are antimicrobial active. This reveals a broad spectrum of medical applications. For this biocompatibility tests are essential. Therefore, we tested the effects of AC on human keratinocytes in monolayer culture as well as in a 3d human skin model. Here, the tested aminocelluloses were functionalized with ethylendiamine (EDA) or triaminotriethylamine (TAEA) and differed in the degree of substitution of tosyl groups bonded to cellulose backbone (DS 0.3–0.56).
Methods: The effects on human HaCaT keratinocyte's cell viability and proliferation were analysed by measuring cellular ATP and protein content. Cytotoxicity was detected by photometric LDH measurement and cell culture supernatants were collected for quantitative measurement of IL-8 and IL-6. The biocompatibility of all ACs was also tested using a 3d model of human skin consisting of a dermis and epidermis. In addition, cell viability and cytotoxicity were determined with PrestoBlue, WST-1 and by LDH measurement. Expression rates of IL-6, IL-8, TNF-α and IL-1α were examined with qRT-PCR.
Results: In this study AC with EDA [LC50: 1.38 mg/ml (DS 0.55) and 2.76 mg/ml (DS 0.45)] functionalization demonstrated a better cell compatibility in keratinocyte cultures than TAEA-AC [LC50: 0.026 mg/ml (DS 0.56) and 0.037 mg/ml (DS 0.35)]. A similar trend was observed in the 3d skin models. Here, only the highest concentration of TAEA-AC with DS 0.56 (10 mg/ml) was cytotoxic.
Conclusions: From the results obtained, it can be concluded that the DS is crucial for the biocompatibility of AC *in vitro*. Here, it was observed that lower DS convey a better cell compatibility. This finding is crucial for the functionalization of celluloses with antimicrobial activity for medical applications.

P025

Aminocellulose-coating of glass slides improves keratinocyte attachment and proliferation *in vitro*

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Introduction: Cultivation of keratinocytes is challenging and a substrate that favours their growth and adhesion would overcome associated problems. Most modification methods employ immobilization of adhesion molecules onto surfaces, commonly substances of the extracellular matrix such as collagen, fibronectin, vitronectin or laminin. However, this needs tedious crosslinking processes with very low yield. Hence, alternatives are of great interest. Here, we have tested attachment and proliferation of keratinocytes on aminocellulose-coated glass slides. Aminocelluloses (AC) are aminodeoxy derivates of cellulose synthesized from p-toluenesulfonicacid esters of cellulose (tosyl-celluloses) by a nucleophilic displacement reaction (SN) with amines such as ethylenediamine (EDA-celluose) or tetraethylendiamine (TAEAcellulose). Their self-assembling layers can be used to modify surface properties such as hydrophilicity and charge.
Methods: Aqueous solutions of the aminocelluloses (0.01–0.00025%) were used to coat glass slides. HaCaT cells were seeded onto these AC-coated glass slides and incubated for 10–48 h at 37°C in a humidified atmosphere containing 5% CO_2. Cell attachment and cell viability was determined by chemiluminescent measurement of the cellular ATP content (ATPlite, Perkin Elmer) using the LUMIstar Galaxy (BMG LABTECH).
Results: Glass slides were easily coated with EDA- and TAEA-cellulose in aqueous solution without any organic solvent being involved, yielding an even layer of these aminocelluloses on the glass. AC-functionalization increased cell attachment and proliferation compared to untreated glass slides *in vitro*. Concentration and time dependent effects of EDA- and TAEA-cellulose could be observed and an effective concentration range for both aminocelluloses could be identified.
Conclusions: Cells grow more easily on positively charged surfaces and both, EDAand TAEA-cellulose can be used in a simple surface functionalization technique to obtain hydrophilic coatings that increase keratinocyte adhesion and proliferation. Hence, it can be concluded that the self-assembling layers of these aminocelluloses can be used to also modify surface properties of other materials for biomedical utilizations, e.g. implants where a rapid cover with cells is needed.

P026

Evaluation of antimicrobial efficacy and cell compatibility of cold atmospheric pressure plasma *in vitro*

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Objective: 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. This study investigates the antimicrobial efficacy of physical plasmas on micro-organisms causing skin infections, such as Staphylococcus aureus, Pseudomonas aeruginosa, Candida albicans, and Malassezia pachydermatis and compares it to its cell compatibility in dependence on the process gas and the electrical power used for plasma generation.
Method: Micro-organisms were plated onto MH2 agar plates in accordance to DIN 58940-3. Inoculated plates were incubated for 1 h at 4°C prior to plasma treatment. 2-weeks-old 3d-skin models, comprised of a dermal fibroblast collagen matrix with an epidermal keratinocyte layer on top, were used for cell compatibility testing. Plasma treatment was performed using the plasma-BLASTER (TIGRES GmbH) with either air or nitrogen as process gases at increasing electrical power. The following plasma parameters have been kept constant: distance from Plasma- Blaster to surface, grid spacing of treatment lines, number of treatments, and work piece velocity. After treatment, MH2 plates were incubated at 37°C for 24 h under aerobic conditions. Evaluation of antimicrobial efficacy was performed against an untreated control. 3d-skin models were investigated for cell viability by determination of metabolically active cells (Alamar Blue assay) and effects on morphology by histology and immunochemistry.
Results: The generated plasmas exhibited a significant antimicrobial efficacy depending on the process gas used and the plasma power. Nitrogen as process gas conveyed a stronger antimicrobial activity compared to air. Antimicrobial effects further increased with rising power as did the cytotoxicity of the plasma determined in the 3d-skin model assays. A balance between cytotoxic influence and antimicrobial activity has to be achieved at moderate power using either air or nitrogen as process gas to ensure treatment safety as well as treatment efficacy.
Conclusions: The study showed that cold atmospheric pressure plasmas exhibit antimicrobial properties *in vitro*. Moreover, cell compatible plasma parameter sets could be identified. Hence, the selective application of cold plasma for treatment of wound infections as well as other superficial skin infections such as dermatomycoses could provide a promising alternative or supplementation of the medicinal therapy.

P027

Regulation of von Willebrand factor (VWF)-mediated cutaneous inflammation

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Von Willebrand factor (VWF), a well-known key player in hemostasis, is increasingly recognized as a pro-inflammatory protein. Previously, we demonstrated that VWF is an important regulator of neutrophil-mediated cutaneous inflammation.
Here, we show that VWF plays a crucial role in the regulation of T cell-mediated inflammation of the skin. 1-fluor-2,4-dinitrobenzol (DNFB)-induced contact hypersensitivity (CHS) was performed either in mice treated with VWF-blocking antibodies or in VWF−/− mice. We observed a significant VWF-dependent reduction of the cutaneous inflammatory response as measured by ear swelling, edema formation of DNFB-challenged back skin or by histological analysis. In all studied animals – WT, anti-VWF-treated and VWF−/− mice – gene expression of pro-inflammatory markers such as tumor necrosis factor α (TNFα), interleukin-1ß (IL-1ß) or interferon γ (IFNγ) was significantly increased in DNFB-challenged skin related to non-challenged control skin. In contrast, mRNA levels of the anti-inflammatory marker interleukin-10 (IL-10) were significantly increased only in DNFB-challenged skin of anti-VWFtreated and VWF−/− mice. Thus, in the presence of VWF protein anti-inflammatory mediators like IL-10 are suppressed during T cell-mediated cutaneous inflammation. Further investigations are required to clarify how VWF supports the inflammatory response during both neutrophil-mediated inflammation [immune complex-mediated vasculitis (ICV) and irritative contact dermatitis (ICD)] and also T cell-mediated contact hypersensitivity (DNFB-induced CHS).
Moreover, we here study the role of ADAMTS-13 (a disintegrin and metalloproteinase with a thrombospondin type I motif-13) for VWF-mediated cutaneous inflammation. In the circulation, VWF multimers are strictly controlled by ADAMTS-13 which constitutively cleaves ultralarge VWF strings into smaller, less adhesive multimers. Recent studies have shown that ADAMTS-13 has protective effects against ischemic brain damage and reduces VWF-mediated acute cerebral inflammation following stroke. However, an involvement of ADAMTS-13 in cutaneous VWF-mediated inflammation is yet unknown. First experiments reveal a role of ADAMTS-13 for vascular permeability in the skin. Histamine- and ICV-induced vascular leakage was significantly increased in ADAMTS-13−/− mice compared to WT control mice. In addition, VWF plasma levels were significantly higher in inflamed ADAMTS-13−/− mice compared to inflamed WT mice. Ongoing research will investigate the putative regulatory role of ADAMTS 13 for VWF-mediated cutaneous inflammation in more detail.
In conclusion, targeting VWF provides an innovative therapeutic anti-inflammatory approach for treatment of diverse cutaneous inflammatory diseases and might be implemented by therapeutic substitution of the VWF cleaving protease ADAMTS-13.

P028

Cytokine mediated induction of mTORC1 signaling prevents proper differentiation of keratinocytes and contributes to the pathogenesis of psoriasis

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Although biologics directed against cytokines, show promising results in the therapy of psoriasis, a comprehensive understanding of signaling mechanisms contributing to the pathogenesis is still missing.

We could previously show that the PI3K/Akt pathway coordinates the equilibrium between proliferation and differentiation in keratinocytes and is deregulated in psoriasis. Downstream of Akt the mTOR (mechanistic target of rapamycin) cascade is a major integrator of different signals and plays a key role in cell growth and homeostasis. We found that the mTOR kinase as well as components of the active mTOR1 complex (mTORC1) are hyperactivated in psoriatic lesions. At the same time mediators of mTORC1 signaling, the ribosomal protein S6 and 4E-BP1 are strongly phosphorylated.

In vitro psoriatic cytokines such as TNF-a, IL-1b and IL-17A are able to strongly induce the Akt/mTOR pathway. As a consequence of this activation, PI3-K/Akt activity mainly drives proliferation, while mTOR only partially mediates proliferative responses as blockade of mTOR signaling only has a minor effect on cellular proliferation. In contrast, under healthy conditions, mTOR signaling is turned off as soon as keratinocytes start to differentiate. This is supported by the finding that isolated keratinocyte stem cells (KSCs) show strong activation of Akt and mTOR. In contrast transient amplifying cells generated from KSC do hardly display any activity of the Akt/mTOR pathway, but undergo terminal differentiation.

When simulating the psoriatic inflammation by treating keratinocytes chronically with IL-1b or TNF-a and thereby keeping the Akt/mTOR signaling activity at a constant high level, proper differentiation is blocked. Conversely, regular differentiation can be restored under these conditions if mTOR signaling is blocked through siRNA mediated knockdown of components of mTORC1.

In summary, our data suggest that cytokine induced activation of the Akt/mTOR cascade contributes to the induction and/or maintenance of the psoriatic phenotype through the induction of proliferation and blockade of proper differentiation, thus pointing towards mTOR as novel target for anti-psoriatic therapies.

P029

Establishment of keratinocyte cell lines from human hair follicles

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Primary keratinocytes (KC) have a finite cellular lifespan *in vitro*. Although KC cell lines are widely used in dermatological research, investigations on keratinocyte differentiation are limited due to dysfunctions of their differentiation program. Therefore, the establishment of KC cell lines that are still able to execute the normal KC-differentiation program would be of added value for studying many aspects of KC biology *in vitro*. In the present study autonomously growing KC cell lines derived from human hair follicles and, as a control, interfollicular epidermis were established and characterized.

KC isolated from hair follicles and interfollicular epidermis were immortalized by ectopic expression of SV40 early region and hTERT. Both, the isolated primary KC as well as the generated KC cell lines differentiated into a stratified epidermis with an identifiable stratum granulosum and stratum corneum in our organotypic skin model, showing normal K1, K5, K10, K17, involucrin, filaggrin and caspase 14 expression. The epidermal diffusion barrier function was intact in organotypic skin cultures of both hair and skin-derived KC cell lines. Mechanistically we found that SV40 large T antigen and p53 expression was only detectable in the basal layer of the *in vitro* reconstructed epidermis. Furthermore, inhibition of DNA methylation circumvented SV40 large T antigen shutdown in the suprabasal layers and led to incomplete KCdifferentiation.

Our study demonstrates that immortalization of primary KC by overexpression of SV40 early region and hTERT generates cell lines which are able to fully differentiate in an organotypic skin model. Since hair follicles – as source for KC – can be obtained by minimally invasive procedures, our approach will allow the generation of cell lines from patients with skin diseases and therefore represents an advantageous new tool in the search for underlying pathogenic processes.

P030

Anti-inflammatory properties of a bacterial endopeptidase *in vivo* and *in vitro*

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O-sialoglycoprotein endopeptidase (OSGEP) is a neutral metalloprotease that can be purified from culture supernatant of the bovine lung pathogen Mannheimia Haemolytica. OSGEP specifically cleaves O-sialoglycoproteins and has become a well-established cutting tool in studying receptor-ligand interactions. In the present project, we investigate the anti-inflammatory properties of OSGEP *in vitro* and *in vivo*.

Therefore, we used two cutaneous inflammation models, immune complexmediated vasculitis (ICV) and irritative contact dermatitis (ICD) and compared edema formation, as well as leukocyte infiltration at the sites of inflammation, in mice injected either with OSGEP or vehicle. We found a significant decrease in both, edema formation (64% in ICV, $P \leq 0.001$; 68.4% in ICD, $P \leq 0.001$) and leukocyte infiltration (74.9% in ICV, $P \leq 0.001$; 75.8% in ICD, $P \leq 0.001$) in the OSGEP treated mice. We then tested physiological functions of leukocytes upon OSGEP treatment. Vitality assays did not reveal any cytotoxic effect of OSGEP on peripheral blood cells. FACS analysis of bone marrow-derived neutrophils (BMC) revealed no decrease of Ca^{2+} influx, indicating no impaired receptor induced activation ability after OSGEP treatment. In adhesion assays on ligand coated surfaces, we observed a significant reduction in integrin mediated adhesion of OSGEP treated BMC compared to vehicle control treated cells ($P \leq 0.01$). Also, OSGEP treated BMC did not reveal altered migratory capacity in transmigration assays through permeable membranes, compared to control treated BMC. However, measuring the amount of BMC migrated towards chemotactic factors through a stimulated layer of endothelial cells, showed less migration upon OSGEP treatment. Interestingly, the reduced migration was not only observed when OSGEP treatment was performed on BMC, but also by treating the endothelial cell layer prior to migration experiments, using non treated BMC. This finding indicates that OSGEP exerts its effect by modifying the neutrophil-endothelial interaction. It is matter of ongoing research in how far OSGEP can be used as a therapeutic tool in cutaneous inflammatory disease.

P031 (O02/05)

Ceramide synthase 4 is involved in the regulation of hair follicle stem cell homeostasis

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Ceramides are central components of mammalian membranes and key players in different intracellular signaling pathways. Ceramide production is dependent on ceramide synthases (CerS) the family of which consists of 6 members (CerS1-6). Ceramides are crucial for skin barrier function but little is known on their role in the formation and maintenance of epidermal appendages and whether stem cell populations that control epidermal regeneration depend on specific ceramide species.

Our investigation shows that ceramide synthase 4 (CerS4) is highly expressed in adult murine epidermis where it is localized in the interfollicular epidermis and in the bulge and junctional zone of the hair follicle. To examine the functional significance of CerS4 in ceramide production and epidermal homeostasis, we generated CerS4 knockout mice. Inactivation of CerS4 induced precocious activation of hair follicle bulge stem cells. This was manifested in a loss of label retaining cells and a continuous anagen-like growth state of hair follicles after the second catagen and ultimately led to an almost complete depletion of bulge stem cells. At the second catagen to telogen transition a reduction in BMP target gene mRNA expression was identified, indicating a decrease in BMP signaling in CerS4-deficient epidermis. This may explain the inability of hair follicle stem cells to properly enter telogen. Further the reduction in BMP activity likely promoted enhanced Wnt target gene mRNA expression in CerS4-deficient mice. Our data reveal an essential role of CerS4- directed epidermal ceramide composition in the control of hair follicle stem and progenitor cell activation and dynamics potentially through the regulation of BMP and Wnt signaling. Thus our data suggest a novel means of hair follicle stem cell activation, which is of interest for understanding the regulation of adult stem cell populations.

P032

Tight junction proteins: new players in pathogenesis of chronic wounds

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Besides their important role in barrier formation, tight junction (TJ) proteins are known to be involved in proliferation and differentiation. These processes are essential for normal wound healing and impaired in chronic wounds. Therefore we investigated the TJ proteins Occludin (Ocln), Claudin-1 (Cldn-1) and ZO-1 in porcine and human *ex-vivo* wound healing models as well as in tissue samples of acute and chronic human wounds by immunohistological stainings.

We observed striking differences in the localization of Ocln, Cldn-1 and ZO-1 between normal healing wounds and chronic wounds, with the latter ones often showing an at least partial loss of all three proteins at the wound margins while there was an increased expression of ZO-1 and Ocln behind the wound margins.

To elucidate the putative role of the loss of Ocln, Cldn-1 and ZO-1 in wound healing, we knocked-down these proteins in primary human keratinocytes by using two different siRNAs each and subjected these cells to proliferation (BrdU), scratch wound, adhesion, differentiation and cytokine release assays.

Knock-down of Claudin-1 expression resulted in significantly impaired scratch wound healing, where both migration and proliferation seem to be affected. This argues for an important role of the loss of this protein in wound healing impairment in chronic wounds. Putative signalling pathways have been investigated.

For Ocln, downregulation resulted in increased wound healing in scratch assays which was unexpected, because it is downregulated in chronic wounds. However, after subjecting the cells to mechanical stress – which is normally present in wounds – this improvement of wound healing was abolished. This argues for a role of the loss of Ocln in chronic wounds associated with mechanical stress and therefore cell adhesion. In line with this hypothesis, we observed reduced cell-cell and cell-matrix adhesion in Ocln knock-down cells. Further, also differentiation was altered, while proliferation was unchanged.

ZO-1 knock-down had only a slight impact on cell scratch healing and no influence on proliferation, but resulted in an increase of IL-1β release, a cytokine with elevated levels in chronic wounds. Thus, ZO-1 might be involved in pathogenesis of chronic wounds via contributing to these elevated levels.

In conclusion, our results show that there is a difference between expression and localization of Ocln, Cldn-1 and ZO-1 between normal wound healing and chronic wounds and that all three proteins are likely involved in the pathogenesis of chronic wounds, however, by executing different effects. This argues for TJ structure independent effects and highlights the functions of individual TJ proteins in the epidermis.

P033

Differential responses of human melanoma cells to c-Rel down-regulation

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Since melanoma cells are prone to develop therapy resistance mechanisms, novel therapeutic targets may improve the treatment of melanoma patients. Such potential target structures include subunits of the central transcription factor NFKappaB (nuclear factor 'kappa-light-chain-enhancer' of activated B-cells). We have focused on the c-Rel subunit of NF-KappaB, because we have recently shown increased c-Rel expression in squamous cell carcinomas and identified a role on cell cycle progression during G2/M phase as well as on apoptosis in human keratinocytes. Thus, c-Rel exerts anti-apoptotic and pro-oncogenic activities in human keratinocytes, two features that have not been reported together for any other NF-KappaB subunit. Here, we analyzed seven human melanoma cell lines and we found constitutive c-Rel expression in all of them. When we interfered with c- Rel functions by transfection of four of these melanoma lines with six different small interfering RNA (siRNA) constructs, we identified one construct that consistently achieved down-regulation of c-Rel by approximately 60%, while the other constructs were markedly less effective. Of note, melanoma lines showed reduced cell growth after c-Rel knock-down compared to control siRNA transfected cells. Furthermore, a significant increase of apoptosis was induced in three cell lines (LOX, MV3 and Sk- Mel-23) originating from metastatic melanomas, but not in A375 cells, which were derived from a primary tumor. Regarding cell cycle phase distribution and mitotic cell number, c-Rel down-regulation provoked a significant shift toward cells in G2/M phase together with an induction of the mitotic cell portion in the LOX, MV3 and Sk- Mel-23 cell lines. Altogether, our data suggest an anti-apoptotic and pro-proliferative role for c-Rel in some human melanoma cells.

P034

Desmoglein 3-specific autoantibodies recognizing a membrane-proximal epitope induce loss of keratinocyte adhesion

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Pemphigus vulgaris (PV) is an organ-specific potentially life-threatening human autoimmune disease characterized by intraepidermal loss of keratinocyte adhesion resulting in flaccid blisters and erosions. There is ample experimental evidence that IgG autoantibodies (autoAb) against the desmosomal cadherins desmoglein 3 (Dsg3) and Dsg1 lead to acantholysis in PV. The precise molecular mechanisms that finally induce loss of keratinocyte adhesion upon autoAb binding, including the activation of signalling pathways in keratinocytes, induction of apoptosis and depletion of non-desmosomal desmogleins, are still controversially discussed. Furthermore, PV patients demonstrate autoAb reactivities against various epitopes of the Dsg3 ectodoamin. AutoAb targeting epitopes that are located in the NH2- terminal region of the Dsg3 ectodomain which is critical for the transinteraction of these adhesion molecules have been shown to be pathogenic. The aim of this study was to characterize pathogenic effects of a monoclonal mouse IgG antibody against the membrane-proximal domain (extracellular domain 5, EC5) of human Dsg3. The Dsg3-EC5-specific monoclonal

antibody 2G4, was affinity-purified from supernatans of B cell hybridomas that were generated in Dsg3-immunized mice. The epitope specificity of 2G4 was verified by using recombinant single Dsg3-EC-domain constructs in ELISA and immunoblot assays, respectively. Functional *in vitro* studies such as the keratinocyte dissociation assay showed that 2G4 is capable of inducing loss of epidermal keratinocyte adhesion. Moreover, applying the *ex vivo* skin biopsy model, 2G4 induced split formation at the suprabasilar level of the epidermis in human skin, correlating with binding of the antibody to the cell surface of keratinocytes as demonstrated by immunofluorescence. Furthermore, single molecule atomic force microscopy revealed that this EC5-reactive antibody impaired Dsg3 interaction suggesting that COOH-terminally located epitopes interfere with Dsg3-mediated keratinocyte adhesion. P38 MAPK (mitogen-activated protein kinase) as a representative signal transduction pathway that has been described to be activated after binding of PV autoAb was investigated upon incubation of human keratinocytes with 2G4. 2G4 lead to phosphorylation of p38 MAPK which was comparable with the effect of the well characterised Dsg3-EC1-specific monoclonal antibody Ak23. P38 MAPK inhibition experiments are being performed to further study the dependence of 2G4-mediated loss of keratinocyte adhesion on the activation of this signalling pathway. Other possible 2G4-induced mechanisms, such as induction of apoptosis in epidermal keratinocytes, are being investigated as well. Finally, synergistic effects of 2G4 with other Dsg3-reactive autoAb will be analysed. In summary, our results suggest that an autoAb directed against the membraneproximal EC5 domain of the Dsg3 protein clearly exhibits pathogenic activity. These findings expand the current concept on pathogenic autoAb that primarily react with NH2-located epitopes in PV and they provide new aspects for a more comprehensive understanding of the underlying mechanisms leading to blister formation in this autoimmune disorder.

P035

Proteome analysis of primary human skin mast cells

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Dermal mast cells are primarily defined by their contribution to allergic and inflammatory skin diseases such as urticaria and atopic dermatitis. However, their function in skin homeostasis is still not completely elucidated. Recently, proteomics has evolved as a powerful method to identify the protein signature of cells, leading to a better understanding of their biological roles.

We enriched mast cells from human abdominal skin by magnetic separation with a purity of 98% as evaluated by toluidine blue and tryptase staining. Mast cell proteins were extracted and separated using SDS-PAGE, silver-stained and gel slices were digested with trypsin. Individual peptides were separated with UHPLC and analyzed by mass spectrometry. Data were analyzed using Proteome discoverer 1.4 and filtered allowing only peptides with a FDR less than 0.01. The identified 3147 proteins were classified and further analyzed with the database for annotation, visualization and integrated discovery (DAVID). Key mast cell proteins – e.g. stem cell factorreceptor, chymase, tryptase and prostaglandin synthases – were found among the highly enriched proteins. Moreover, the existing proteome data from the mast cell line LAD2 on exocytosis related proteins was confirmed and extended. We identified several proteins that were more abundant in mast cells as compared to other skin cells including CD26 (dipeptidylpeptidase IV), an enzyme widely expressed on immune cells, but not yet described for dermal mast cells. CD26 is involved in inflammatory processes as activator of vasoactive peptides, growth factors, cytokines and chemokines and was found to be increased in the skin of patients with mast cellinvolving diseases, e.g. atopic dermatitis. In follow-up experiments we were able to confirm the expression of CD26 on isolated mast cells as well as on skin mast cells *in situ*.

With proteome analysis we identified several proteins uniquely or principally expressed in human dermal mast cells. Detailed analyses of CD26 and other proteins will allow us to get more insights into mast cell biology and their role in health and disease.

P036

NADH dehydrogenase subunit 2 mutation leads to delayed fibroblast ageing

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Mitochondrial dysfunction of parts of mitochondrial respiratory chain is suggested to contribute to organismal ageing. The largest and first complex of the respiratory chain NADH dehydrogenase contains 45 subunits, 7 of them are encoded by mitochondrial (mt)DNA. Mutations in one of the seven mitochondrial subunits were suggested to play a role in diverse diseases and ageing. To analyze the influence of mutations in mitochondrial genes of the respiratory chain in the context of skin ageing conplastic mouse strains were generated. In the present study, we analyzed isolated skin fibroblasts of the mouse strain C57BL/6J-mtALR/LTJ with a single nucleotide exchange (nt4738A) in the NADH dehydrogenase subunit 2 gene (Nd2-mutant) in complex I. Skin fibroblasts of mice of different age (3 and 12 month) were analyzed at different time points for expression and secretion of age-related markers under untreated conditions or cellular stresses like doxorubicin.

The skin fibroblasts of Nd2-mutant mice showed decreased ROS production, enhanced ATP levels and an enhanced proliferation rate compared with the control strain C57BL/6J-mtAKR/J, in 3 and 12-month-old mice. Furthermore, the mutation in Nd2-mutant fibroblasts led to higher complex I activity as compared to control mice. β-galactosidase activity, another important senescence marker is significantly reduced in Nd2-mutant mouse fibroblasts. Furthermore, the cytokine levels of IL-6 and IL-8 in fibroblasts of Nd2-mutant mice increased 4 and 8 days after doxorubicin treatment, but the basal secretion level of these cytokines was lower compared with the control strain. Immunoblots showed that expression of the age-related marker H3K9me3 was delayed in Nd2-mutant mice after doxorubicin treatment. Pathway analysis revealed that the MAPK pathway is strongly involved in mediation of reduced senescence effects of Nd2 mutant fibroblasts.

These results demonstrate an obvious reduction in senescence features in fibroblasts of mutant mice as compared with control mice. The investigation of life span is still ongoing. However, in humans it has been shown, that a single nucleotide polymorphism (C5178A) in the NADH dehydrogenase subunit 2 gene is associated with longevity in a Japanese population. Taken together, in the present report we identified a mitochondrial gene polymorphism that could be age-protective in mice and humans.

P037

Transcriptome analysis of FcεRI-stimulated mast cells reveals Syk-independent regulation of gene expression

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εMast cells (MC) play an essential role in the initiation and progression of immunological diseases like allergy and asthma. Cross-linking of the high affinity receptor for IgE (FceRI) through IgE-allergen complexes results in the rapid release of preformed inflammatory mediators from granules – like proteases and histamine – as well as *de novo* synthesized lipids and a broad range of cyto- and chemokines. The spleen tyrosine kinase (Syk) is a key player in IgE/FcεRI-mediated activation of MC and is therefore a potential therapeutic target for MC-driven diseases such as asthma, and allergy. Here we set out to explore if Syk is an essential regulator of the proinflammatory phenotype of MC. Using the Cre-LoxP system, deletion of the Syk gene led to an abrogation of effector responses to antigen stimulation such as Ca^{2+} waves, degranulation or selected cyto- and chemokine release in MC. Unexpectedly, however, MC still displayed FceRI-dependent transcriptional activity. By using next generation sequencing technology (RNAseq, Illumina), for whole transcriptome analysis of IgE-antigen stimulated Syk deficient MCs, we preliminarily identified genes that are, compared to stimulated wt cells, either evenly regulated, oppositely regulated or solely regulated in the absence of Syk. Suprisingly, some of the genes whose expression was markedly increased, in Syk deficient cells, are strong promoters of inflammation. Moreover, costimulation of Syk deficient MCs with IgE/Antigen in the presence of substance P or LPS was sufficient to trigger cytokine release or degranulation respectively in an IgE-dependent manner *in vitro* and *in vivo*. These findings demonstrate the use of Syk-independent signaling pathway(s) downstream of FceRI and provide a new view of the key regulatory and/or signalling networks that are Syk-dependent and Syk-independent. Importantly, this work also continues to delineate the signals required for FcεRI-dependent regulation of MC gene expression.

P038

Impact of different spa waters on inflammation parameters in human keratinocytes

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The treatment of different skin conditions with spa waters has a long tradition going back to at least the late Hellenism. Interestingly, independent scientific examinations studying the effect of spa waters are scarce. In the present *in vitro* study it was tested whether culture media supplemented with different spa waters have impact on physiological parameters in human skin keratinocytes. It was found that two popular thermal spring waters (La Roche-Posay, Avne) suppressed proliferation and also cell damage. Moreover, these waters reversed the induction of IL-6, measured by ELISA and promoter transactivation, and the formation of reactive oxygen species after UVB stimulation. Of note, two natural mineral waters (Heppinger, Adelholzener) distributed as drinking waters have also some effect on the above mentioned parameters but to a lesser extent. In sum, our results show that spa waters and particularly those derived from thermal springs reduce parameters involved in inflammation. It seems likely that trace elements such as selenium and zinc are critical for the observed effects.

P039 (O05/03)

The necroptosis-sensitizing effect of TRAF2 knockdown upon TRAIL stimulation is a genuine TRAIL signalling effect and can be mimicked by TWEAK

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The relevance and functions of the adaptor protein TRAF2 and the TRAF2- associated E3-ligases cIAP1 and cIAP2 for signal transduction of the death receptor TNF receptor-1 (TNFR1) and CD95 are well established. Whether TRAF2 also plays a role in signalling by death receptors TNF-related apoptosis inducing ligand (TRAIL)- receptor-1 (TRAILR1) and TRAILR2 is poorly understood. The TNF-related weak inducer of apoptosis (TWEAK) is known to recruit cytosolic TRAF2 to the TWEAKreceptor Fn14, thereby reducing the cytosolic pool of TRAF2/cIAP complexes.

As we have recently shown, TRAF2 inhibits TRAIL-mediated necroptosis and apoptosis *in vitro*. Interference with the activity of the TRAF2/cIAP complex can trigger the induction of endogenous TNF and subsequent TNFR activation. To exclude that the observed sensitization towards TRAIL-triggered cell death in TRAF2-depleted cells was an indirect effect of TNF, we repeated our experiments in the presence of TNFR2-Fc (Etanercept, Enbrel®), which inhibits TNF-alpha and LT-alpha, or the antagonistic anti-TNF-alpha antibody Adalimumab (Humira®). Effects of TRAIL stimulation under necroptotic conditions were not altered by coincubation with TNFR2-Fc or anti-TNF-alpha, respectively. Inhibition of classical NF-kappaB signalling, which plays a major role in TNF induction but also induces survival proteins, with the IKK2 inhibitor TPCA-1 did also not affect the cell deathenhancing effects of TRAF2 depletion. The cell death-sensitizing properties of TRAF2 knockdown observed in TRAIL-stimulated cells thus reflect a genuine effect of TRAIL-related death signalling rather than an indirect TNF effect.

In the context of TNFR1 signalling, priming of cells with the Fn14 ligand TWEAK leads to a strong enhancement of TNF-induced caspase-8 activation and apoptosis. We thus analysed the effects of Fn14 stimulation by soluble TWEAK on TRAILinduced cell death. In another setting we depleted IAPs by incubating the cells with the IAP antagonist BV6. TWEAK priming and BV6 treatment specifically sensitized for TRAIL-induced necroptosis and in this respect mimic a major effect of TRAF2 depletion. Thus, the latter may act partly by reducing the capacity of cIAP1/2 to target RIP1.

Demonstrating that TRAF2 knockdown, TWEAK priming and BV6 treatment result in enhanced TRAIL-induced necroptosis in the presence of a caspase inhibitor, we provide several lines of independent evidence for an inhibitory role of TRAF2/ cIAP complexes in TRAIL-induced necroptotic signalling. To identify conditions that sensitize relative resistant tumour cells towards TRAIL-induced cell death remains an important goal in oncology and mechanisms that inhibit necroptosis are critical for maintenance of tissue homeostasis. TWEAK might serve as a physiological imitator of the necroptotis-sensitizing effect observed with TRAF2 knockdown or with the IAP antagonist BV6.

P040

Interplay of histone H2A deubiquitinase 2A-DUB/Mysm1 with p53-mediated anti-apoptotic and anti-proliferative programs in skin development and homeostasis

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Developmental processes depend on collaboration of sequence-specific transcription factors with histone-modifying enzymes for timely activation and silencing of lineagespecific genes. To characterize the function of histone H2A deubiquitinase 2A-DUB/ Mysm1 in the skin, we systematically analyzed expression and potential interactions of this epigenetic regulator during development using Mysm1-deficient and p53−/− Mysm1−/− double-deficient mice and skin-derived cell lines. In context with a belly spot-and-tail phenotype and hematopoietic anomalies, skin of newborn and young adult Mysm1−/− mice was atrophic with reduced thickness of epidermis, dermis and subcutis compared to wild-type littermates. Diminished cell numbers and atrophy resulted from reduced proliferation of skin progenitor populations and increased fractions of apoptotic cells in Mysm1-deficient epidermis and hair follicles. In support of a role of Mysm1 in the DNA-damage response, increases in DNA-damage marker γH2AX were detectable. In addition, skin pigmentation and melanocyte migration to the hair

follicles were analyzed in embryonic and early postnatal development of Mysm1−/− mice. Western Blot and IF analyses of Mysm1−/− mouse skin and other tissues confirmed our hypothesis that levels of tumor suppressor p53, a major regulator of apoptosis and cell cycle inhibition, were significantly elevated in part via a p19Arf-dependent mechanism whereas IGF-I levels were not consistently decreased. In p53−/−Mysm1−/− double-deficient (DKO) mice, a significant rescue of skin atrophy and delayed pigmentation was observed, substantiating the involvement of the p53 pathway in the skin defects caused by Mysm1-deficiency. Partial recovery of the alterations in skin lacking Mysm1 occurred in p19Arf−/−Mysm1−/− double-mutant mice. Moreover, preliminary screening experiments indicate additional functions of Mysm1 in the skin in response to tissue damage in wound healing, inflammation and UVirradiation models. In context with our finding that other defects of Mysm1-deficient mice were ameliorated by p53 deletion, this investigation uncovers a novel role for histone H2A deubiquitinase 2A-DUB/Mysm1 in suppression of anti-apoptotic and anti-proliferative programs mediated by the p19Arf/p53 axis during development with potential impact on susceptibility to DNA-damage and transformation.

P041

Involvement of BCL-XL in regulation of mast cell survival

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Mast cells exert beneficial as well as detrimental functions in host defense and various diseases, but the regulation of mast cell homeostasis is only partially understood. BCL-XL belongs to the group of anti-apoptotic BCL-2 proteins and is indispensable for embryogenesis. Furthermore, BCL-XL has been reported to be essential for mast cell development from mouse embryonic stem cells.

In order to explore the role of BCL-XL in mast cells, we generated mast cellspecific Bcl-x knockout mice by crossing Bcl-xfl/fl mice to the Mcpt5Cre strain, which expresses Cre recombinase in connective tissue type mast cells. For *in vitro* experiments, Bcl-xfl/fl mice were also bred with the Mx1Cre strain, enabling deletion of the Bcl-x gene upon injection of polyinosinic-polycytidylic acid (poly I:C). Upon deletion of Bcl-x, bone marrow was isolated to culture bone marrow-derived mast cells (BMMC).

Mcpt5Cre/Bcl-xfl/fl mice were viable, fertile and showed no apparent phenotype. Interestingly, we observed significantly reduced numbers of mast cells in peritoneal cavity, tongue and heart, but not in back skin, ear, mesentery and stomach in Mcpt5Cre/Bcl-xfl/fl mice compared to Bcl-xfl/fl controls. However, these reductions were not sufficient to affect IgE-mediated passive systemic anaphylaxis. BMMC developed from Mx1Cre/Bcl-xfl/fl mice and showed unaltered viability, apoptosis and mast cell counts after 4 weeks in culture. Western blot analysis revealed absence of BCL-XL and increased expression of the anti-apoptotic protein MCL-1 in Mx1Cre/ Bcl-xfl/fl BMMC, indicating that it may compensate for the loss of BCL-XL. While apoptosis upon deprivation of growth factors was comparable between Mx1Cre/Bclxfl/ fl and Bcl-xfl/fl BMMC, Mx1Cre/Bcl-xfl/fl BMMC showed significantly enhanced susceptibility towards the antineoplastic agents actinomycin D and etoposide. Also, apoptosis in response to TRAIL in combination with actinomycin D was increased in Mx1Cre/ Bcl-xfl/fl BMMC compared to Bcl-xfl/fl controls.

Collectively, our findings demonstrate that BCL-XL is dispensable for development of mast cells, but crucially participates in the regulation of mast cell survival.

P042

Nrf2 activation in keratinocytes enhances wound closure and barrier reestablishment

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Nrf2 is a cytoprotective transcription factor with a crucial function in ROS and electrophile detoxification. Therefore, Nrf2 is an attractive target to enhance cytoprotection and to prevent cancer development. We recently showed that genetic and pharmacological activation of Nrf2 in keratinocytes protects from UVBinduced apoptosis due to enhanced ROS detoxification. On the other hand, strong Nrf2 activation results in barrier defects, acanthosis, and hyperkeratosis due to upregulation of small proline rich proteins (Sprr2) and secretory leukocyte protease inhibitor (Slpi).

We now investigated the consequences of genetic Nrf2 activation on cutaneous wound healing using mice expressing a constitutively active Nrf2 (caNrf2) mutant in keratinocytes. caNrf2 transgenic mice exhibited an increase in the length and area of the wound epithelium, resulting in a marked increase in wound closure at 5 days post wounding. We identified enhanced migration and proliferation of wound keratinocytes as the underlying mechanism. This is most likely due to Nrf2-mediated detoxification of ROS, which is known to inhibit these processes.

Surprisingly, barrier re-establishment was accelerated during early wound healing of caNrf2 transgenic mice. Sprr2 or other cornified envelope proteins were not upregulated during the early wound healing phase, however, increased levels of Slpi and more pronounced hyperkeratosis were observed. This most likely enhanced barrier functionality of the wound epithelium.

Thus, Nrf2 activation in keratinocytes enhanced wound healing, presumably due to increased ROS detoxification combined with accelerated epidermal barrier re-establishment. Future experiments will reveal whether Nrf2 activation also accelerates closure when wound healing is disturbed. This could be of interest for the treatment of chronic, non-healing wounds.

P043

Erk5 inhibits endothelial migration via KLF2-dependent downregulation of PAK1

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The MEK5/Erk5 pathway mediates beneficial effects of laminar flow, a major physiological factor preventing vascular dysfunction. Forced Erk5 activation induces a protective phenotype in endothelial cells that is associated with a dramatically decreased migration capacity of those cells. Transcriptional profiling identified the Krüppel-like transcription factors KLF2 and KLF4 as central mediators of Erk5-dependent gene expression. However, their downstream role regarding migration is unclear and relevant secondary effectors remain elusive. Here, we further investigated the mechanism underlying Erk5-dependent migration arrest in endothelial cells.

Method and results: Our experiments reveal KLF2-dependent loss of the pro-migratory Rac/Cdc42 mediator PAK1 as important mechanism of Erk5-induced migration inhibition. We show that endothelial Erk5 activation by expression of a constitutively active MEK5 mutant, by statin treatment or by application of laminar shear stress strongly decreased PAK1 mRNA and protein expression. Knockdown of KLF2 but not of KLF4 prevented Erk5-mediated PAK1 mRNA inhibition revealing KLF2 as novel PAK1 repressor in endothelial cells. Importantly, both PAK1 re-expression and KLF2 knockdown restored the migration capacity of Erk5-activated endothelial cells underscoring their functional relevance downstream of Erk5.

Conclusion: Our data provide first evidence for existence of a previously unknown Erk5/KLF2/ PAK1 axis, which may limit undesired cell migration in unperturbed endothelium and lower its sensitivity for migratory cues that promote vascular diseases.

P044

Expression of the focal adhesion protein kindlin-1 as protective factor against UV-B induced apoptosis

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Kindlin-1 is an epithelial-specific protein, mainly expressed in the basal keratinocytes of the epidermis. The protein contains a four point one ezrin radixin and moesin (FERM) as well as a pleckstrin homology (PH) domain, and acts as a focal adhesion adaptor protein, linking the actin cytoskeleton to membrane-bound integrin receptor molecules and activating the integrin beta1 subunit. Recently, kindlin-1 was shown to play a role in in cell cycle progression and stem cell proliferation. Mutations in the gene FERMT1, encoding for the protein kindlin-1, result in Kindler syndrome (KS), a recessively inherited skin blistering disorder and distinct type of epidermolysis bullosa (EB). KS manifests first with skin blistering and photosensitivity, followed by progressive poikiloderma, skin atrophy and mucosal involvement. Here, we used immortalized keratinocytes from KS patients (KSK) to show the impact of disturbed focal adhesion (FA) formation on UV-B sensitivity and to explore the underlying molecular mechanisms *in vitro*.

Compared to normal human keratinocytes (NHK), the amount of mature FA (demonstrated by immunofluorescence staining of vinculin and talin) is significantly reduced in KSK, probably due to the lack of kindlin-1 as a recruiter for the assembly of FA proteins. After medium-dosage UV-B irradiation (60 mJ/cm^2), apoptosis is significantly increased in KSK (~2.5 fold, $P < 0.05$) compared to NHK (TUNEL assay). Likewise, UV-B irradiation causes a significant increase in p38 phosphorylation (Pp38, ~2 fold, $P < 0.05$) and IL-6 mRNA expression (IL-6, ~2 fold, $P < 0.05$) in KSK. Inhibition of p38 phosphorylation with the specific inhibitor SB203580 is sufficient to reduce UV-B induced apoptosis and IL-6 expression in KSK significantly, identifying the p38 MAPK pathway as a central target to modulate UV-B induced apoptosis in our cellular model of UV-B sensitivity.

Another factor, being significantly increased in KSK compared to NHK after UV-B irradiation is TNF-alpha (~4 fold mRNA levels, $P < 0.05$). Experiments with blocking of p38 phosphorylation or TNF-alpha receptor binding indicate that TNF-alpha is not involved in the p38 MAPK pathway in our model, neither upstream nor downstream. Therefore TNFemerges as a second key regulator in the pathomechanism of photosensitivity in KS, as blocking of TNF-alpha signalling significantly reduces UV-B induced apoptosis.

Furthermore, we show that low levels of kindlin-1 expression in KSK significantly reduced UV-B induced apoptosis and increased formation of FA, whereas overexpression of kindlin-2 had no effect. These data indicate a crucial role for the FA protein kindlin-1 in the protection against apoptosis after UV-B irradiation in human epidermis.

P045

The NADPH oxidase isoform Nox4 controls TGF-beta1-mediated activation of human dermal fibroblasts – a promising new target for the treatment of systemic sclerosis?

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The pathogenesis of systemic sclerosis (SSc) is still incompletely understood. Transforming growth factor-β1 (TGF-beta1)-mediated activation of fibroblasts plays a central role in this disease and oxidative stress has long been implicated in the development of tissue fibrosis. However, the precise molecular interplay between TGF-beta1 and oxidative stress generating intracellular enzymes remains largely unexplored in human dermal fibroblasts (HDFs). We showed that the NADPH oxidase isoform Nox4 is the exclusively expressed Nox isoform in neonatal and adult HDFs as well as in HDFs from SSc patients. Expression of the Nox4 adaptor proteins p22phox and Poldip2 which regulate Nox4 enzyme activity and stability was also detected in these cells. Stimulation of normal HDFs with TGF-beta1 resulted in a time- and dose-dependent induction of Nox4 mRNA and protein. This effect of TGF-beta1 was mediated by transcriptional induction and associated with increased NADPH activity in membrane fractions of HDFs. Moreover, TGF-beta1- induced Nox4 expression in HDFs was dependent on functional SMAD signalling as shown by preincubation with the SMAD3 inhibitor SIS3. Immunofluorescence analysis with laser confocal microscopy studies further revealed that Nox4 localizes to the endoplasmic reticulum as demonstrated by double staining with protein disulfide isomerase. Importantly, pharmacological inhibition of NADPH oxidase activity by the Nox inhibitor – diphenyleneiodonium- or Nox4-specific knock-down not only suppressed TGF-beta1-mediated expression of collagen type I COL(I) but also induction of both alpha-smooth muscle actin and fibronectin 1 in normal HDFs. Likewise, alpha-melanocyte-stimulating hormone, a neuropeptide with antioxidative and antifibrogenic effects, suppressed TGF-beta1-mediated expression of Nox4 in HDFs. At the moment we are performing *in vivo* experiments with different pharmacological Nox inhibitors in mice with experimentally induced skin fibrosis to extend the above *in vitro* findings and to facilitate the translation of our results from bench to bedside.

P046

Water filtered infrared A influences wound healing associated cytokines and cell division

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The sun emission can be divided into three radiation categories: ultraviolet radiation, visible light and infrared radiation (IR). Whereas IRB (1400–3000 nm) and IRC (3000 nm–1 mm) are absorbed by the water molecules in the atmosphere IRA (700–1400 nm) reaches the surface. Artificial IRA radiation sources recreating the atmospheric filtering process by water-filtering the emitted IR radiation are described to beneficially influence e.g. wound healing and temperature homeostasis *in vivo*. Aim of our study was to characterize the effects of water filtered infrared A (wIRA) on primary dermal and epidermal cells and on organotypic tissue cultured skin equivalents.

Primary fibroblasts or keratinocytes were seeded in monolayer cultures. After adherence they divided into four treatment groups. One group was irradiated with 154 mW/cm^2 wIRA for 2 hrs, one group was irradiated with 154mW/cm^2 wIRA for 2 h and 150 mJ/cm^2 UVB, one group was irradiated with 150 mJ/cm^2 UVB and one group was left untreated. 24 h after the respective treatment the cytokine profile of the cell free supernatants were analysed. Furthermore wound situations were induced in keratinocyte (HaCaT and primary kerartinocyte) and fibroblast monolayers. After wound induction the control group was left untreated whereas the wIRA group was irradiated for 20 min or 2 h with

154 mW/cm^2 wIRA. Wound closure was monitored for 30 h with a light microscopic live cell imaging system and the cytokine profiles of the cell free supernatants were analysed.
We could show that wound healing associated cytokines e.g. GM-CSF, MCP-1, TIMP-1 were - induced after wIRA treatment whereas interleukin 29 and TNF-α were reduced. For the wound situation we observed that wound closure was achieved at an earlier time point due to wIRA irradiation. In accordance increased cell division could be documented in the wound area after wIRA irradiation.
The herein presented results are a first step to understand the *in vivo* observed wIRA dependent improved wound healing.

P047

HDAC inhibitors decreases lymphangiogenesis by inducing apoptosis and cell cycle arrest via p53/p21-dependent pathways

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Question: Lymphangiogenesis is a crucial step in the progression of cancer. Formation of new lymphatic vessels provides an additional route for tumor cells to metastasize. Therefore, inhibiting lymphangiogenesis represents an interesting target in cancer therapy. Recent evidence suggests that histone deacetylase inhibitors (HDACi) may mediate part of their antitumor effects by interfering with angiogenesis. We therefore examined the potential impact of three different HDACi, trichostatin A (TSA), sodium butyrate (NaB) and valproic acid (VPA) on cell proliferation in primary human lymphatic endothelial cells (LEC).
Methods: We hypothesized that HDACi might have anti-lymphangiogenic qualities. To prove this assumption, we performed cytotoxicity-, ELISA-, lymphangiogenesis- and cell cycle FACS-assays as well as Western blot and mRNA analysis with primary human lymphendothelial cells (LEC) during the treatment with HDACi.
Results: HDACi inhibited cell proliferation in a concentration-dependent manner. We found that TSA induced G0/G1 arrest in LEC. Cell cycle arrest was accompanied by upregulation of p53 and p21. Moreover, we found that p21 mRNA was significantly upregulated by TSA, while the protein and mRNA half-life remains largely unaffected. The promoter activity of p21 was enhanced by TSA indicating a transcriptional mechanism. Subsequent EMSA analyses showed increased constitutive Sp1/3- dependent DNA binding in response to HDAC inhibition. We demonstrated that p53 was required for TSA induced p21 expression. Interestingly, siRNA-mediated p21 depletion reduced the antiproliferative effects of TSA in LEC. In addition, TSA induced apoptosis by cytochrome c release, activating Caspase-9/-7 and down-regulating the anti-apoptotic proteins cIAP-1/-2. In further analysis, we could demonstrate an inhibition of the formation of capillary like structures by TSA treatment.
Conclusion: In conclusion, we demonstrate that HDACi have distinct anti-lymphangiogenic effects by activating the intrinsic apoptotic pathway and cell cycle arrest via p53/p21- dependent pathways.

P048

Resveratrol impairs lymphangiogenesis through G0/G1 cell cycle arrest and apoptosis

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Question: There is growing evidence that lymphatic vessels are linked to immune regulation, atherosclerosis, or metabolic diseases. In addition, the lymphatic vessels provide a route for tumor cells to metastasize. Therefore, influencing lymphangiogenesis is an interesting target in various pathological conditions. Recent studies suggest that Resveratrol, a natural phenol and phytoalexin found in the skin of red grapes, may mediate part of their antitumor effects by interfering with angiogenesis. We therefore examined the potential impact of Resveratrol on cell proliferation in primary human lymphatic endothelial cells.
Methods: Human lymphatic endothelial cells (LEC) were cultured *in vitro* and treated with or without Resveratrol. Effects of Resveratrol on proliferation, cell cycle progress and apoptosis were analyzed mainly by BrdU-Assay, flow cytometry, Caspase-3/7 activity assay as well as Western blot analysis. *In vitro* angiogenesis was investigated using the Matrigel tube formation assay.
Results: Reveratrol inhibited cell proliferation in a concentration-dependent manner. We found that Resveratrol induced G0/G1 cell cycle arrest in LEC. Cell cycle arrest was accompanied by up-regulation of p53 and p21. In addition, Reveratrol induced apoptosis by activating Caspase-3/7 and cleavage of poly ADP-ribose polymerase (PARP) in LEC. Furthermore, we could demonstrate an inhibition of the formation of lymphatic capillary like structures by Resveratrol treatment.
Conclusion: In conclusion, our results provide for the first time clear evidence, that Resveratrol has distinct anti-lymphangiogenic effects mainly by cell cycle arrest as well as apoptosis.

P049

Unraveling the role of aPKCλ in the regulation of oriented cell division and stem cell homeostasis in the mammalian epidermis

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The atypical protein kinase C (aPKC) is a major regulator of polarity processes throughout all species. Best described are these functions in lower organisms as Drosophila and *C. elegans*, here, aPKC is essential for the regulation of division orientation and thereby the cell fate decisions by coupling the polarized distribution of cell fate determinants to the orientation of the mitotic spindle. Despite the conserved function of aPKC in the mammalian system, the role of aPKC in the regulation of oriented cell division has been very controversial and appears to be tissue specific. To unravel aPKC function in the mammalian epidermis, we generated an epidermis specific knockout of aPKCλ (aPKCλepi−/−), which was characterized by an overall increase in differentiation promoting asymmetric cell divisions and increased stem cell activation maybe mediated by increased Wnt/β-catenin signaling. Moreover, these changes in the ratio of symmetric (SCD) to asymmetric (ACD) cell divisions led to the gradual exhaustion of the hair follicle stem cell populations, cell fate changes and furthermore a premature ageing phenotype. These findings indicate that aPKCλ couples division orientation and cell fate decisions in the mammalian epidermis. However the molecular mechanism of how aPKCλ regulates division orientation and cell fate remains still unclear. Therefore, to further elucidate this molecular mechanism we generated a transgenic mouse model expressing a membrane-targeted form of aPKCλ (aPKCλCAAX), to analyze the regulation of self-renewal and differentiation in aPKCλ-deficient and 'constitutive active' background. Interestingly, expression of aPKCλCAAX induced a reversed phenotype regarding the aPKCλepi−/− mice, characterized by an expansion of the bulge stem cell compartment and reduced stem cell activation maybe due to impaired Wnt/β-catenin signaling. These results indicate that aPKCλ might directly regulate hair follicle stem cell activation, division orientation and concomitant cell fate decisions and present the polarity kinase as an essential player in stem cell homeostasis in the mammalian epidermis. Currently we are examining whether these newly identified aPKC functions promote or inhibit nonmelanoma skin cancer using two stage skin cancer protocols on aPKCλepi−/− and aPKCλCAAX mice.

P050

Targeted deletion of suprabasal keratins K2 and K10 causes upregulation of K1 and K16

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Keratins K1, K2 and K10 are the main components of the cytoskeleton in keratinocytes of the suprabasal layers of the epidermis. In the mouse K1 is absent from skin regions of the ears, soles and the tail where K2 is expressed. K1 and K2 heterodimerize with K10 to form intermediate filaments. To investigate the biological effects of genetic disturbances of the keratinocyte cytoskeleton, we have generated and characterized mice in which both K2 and K10 are inactivated by targeted gene deletions. Mice deficient of both K2 and K10 were viable but developed hyperkeratotic epidermis on their ears and soles. Protein extraction, electrophoresis and nano-HPLC ESI-MS/MS showed that K2 and K10 were the most abundant proteins in corneocytes of the soles of wild type mice. The deletion of the Krt2 and Krt10 genes abolished these proteins and resulted in a massive increase in the amounts of K1 and K16. Immunofluorescence analysis confirmed that K1 and K16 accumulated in the suprabasal layers of the epidermis of K2/K10-deficient mice, and quantitative reverse transcription-PCR demonstrated that K1 and K16 were upregulated at the mRNA level. Electron microscopy showed that suprabasal keratinocytes of K2/K10 double knockout mice contained only short keratin bundles which were associated with desmosomes. In summary, this study suggests that the loss of the keratin pair K2/K10 is partly compensated by the upregulation of K1 and K16.

P051 (O01/03)

The psoriasis-associated IL-17A interferes with keratinocyte differentiation in 3D organotypic skin models

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Psoriasis is an immune-mediated inflammatory skin disorder, which affects up to 3% of the population worldwide. Recent findings suggest that the pro-inflammatory cytokine IL-17A plays a key role in the pathogenesis of psoriasis. Therefore we were interested to determine whether IL-17A affects the formation and the functionality of the skin barrier in psoriasis. To determine the effects of IL-17A, a cytokine expressed by Th17 cells, on the differentiation of human epidermal keratinocytes (NHEKs), we treated 3D skin equivalents developed with keratinocytes and dermal fibroblasts from psoriatic lesions of patients or healthy donors with or without IL-17A. This resulted in changes in skin morphology including parakeratosis and reduced epidermal thickness. Microarray and immunohistological studies of IL-17A treated control and psoriasis 3D models revealed down-regulation of genes and proteins important for epidermal differentiation and skin barrier formation, including filaggrin, involucrin, loricrin and repetin. In addition, we detected increased expression of a series of antimicrobial peptides (AMPs), including the human beta defensins (hBDs) hBD-2 and hBD-3 as well as members of the S100 calcium binding family such as S100A7, S100A7A and S100A12. Furthermore a significant up-regulation of different IL-36 cytokines could be detected in IL-17A treated 3D models. The expression of these genes was markedly increased in 3D models from lesional keratinocytes and fibroblasts compared to models containing cells from healthy donors. Thus it was tempting to speculate that IL-17A effects on skin cells were at least in part mediated by the induction of IL-36 cytokine expression in keratinocytes. Indeed the application of IL-36 cytokines was sufficient to induce the expression of genes encoding different AMPs, including S100A7A, S100A12 and hBD-2, in NHEKs. In addition, at least part of the IL-17A response was inhibited in NHEKs when IL-36alpha signaling was blocked by neutralizing antibodies. Signaling by IL-17A resulted in activation of several downstream effectors. Inhibiting the p38 MAPK was sufficient to interfere with the induction of the expression of IL-36alpha and AMPs in NHEKs. In conclusion we were able to establish 3D organotypic skin equivalents with keratinocytes and fibroblasts of psoriasis patients, which indicated an enhanced response to IL-17A in comparison to control models. Moreover IL-36 cytokines were identified as key downstream effectors of IL-17A signaling.

P052

LASP-1 in melanoma – no tumor marker but melanin regulator

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LIM and SH3 protein (LASP1) is a nucleo-cytoplasmic signaling protein and predominantly present at focal contacts. In various cancer entities, LASP1 expression increases with disease progression and the protein serves as a prognostic tumor marker.
Therefore, we investigated LASP1 expression in normal skin and melanoma. Surprisingly, immunohistochemistry data showed LASP1 expression in the basal membrane of normal skin and in melanocytic nevi while the protein level is significantly reduced in melanoma. Western blot experiments revealed the expression of LASP1 in HEMS (human epidermal melanocytes) as well as in various melanoma cell lines (e.g. MaMel2, M14, SKMel2).
Immunofluorescence staining in HEMS and MaMel2 cells demonstrated colocalization of LASP1 with the melanosome marker enzyme tyrosinase in dendrite tips and along dendrites. LASP1 knockdown experiments in MaMel2 cells resulted in a moderate but significant increase in melanin pigmentation without affecting enzymes involved in melanin synthesis like TRP1 and tyrosinase.
Pull-down experiments identified zyxin, LPP and dynamin as novel LASP1 binding partners in melanoma cell lines – with dynamin being a protein involved in vesicle trafficking and exocytosis.
In conclusion, we are proposing a model that LASP1 plays a role in melanosome vesicle budding by recruiting dynamin and other proteins at the dendrite tips in melanocytic cells.

Chemokines/Cytokines

P053 (O04/04)

Inflammatory caspases and IL-1 in pemphigus vulgaris

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Pemphigus vulgaris (PV) is an autoimmune bullous skin disease which is associated with IgG antibodies directed against desmogleins, cell surface proteins on epidermal keratinocytes. Binding of the specific antibodies to the desmogleins leads to loss of keratinocyte adhesion and suprabasal acantholysis of epidermal cells, resulting in the formation of skin blisters and erosions, both on the skin and mucosa.
Even though PV is considered to be a disease caused by the adaptive immune system via specific IgG antibodies and antigen-directed T cells, pro-inflammatory cytokines of the innate immune system such as IL-1α, IL-6 and TNF, cytokines involved in innate immune responses, have been detected in the blister fluids and sera of these patients. This regional activation of innate cytokines might explain the discontinuous disease manifestation as not the entire body is affected by blister formation. However, the precise mechanisms of acantholysis still remain cryptic. Putative modalities

also include inflammatory cell death mediated by innate cytokines, known as pyroptosis. Here, we aim to determine the pathogenetic role of the innate immune system and in particular of inflammasomes which lead to the activation of inflammatory caspases and consecutive activation of the Interleukin-1 pathway. Using a monoclonal antibody which is directed against human desmoglein 3 (AK23), we could increase IL-1 secretion in primary human keratinocytes. Moreover, in an established in-vitro acantholysis assay, epidermal monolayers cocultured with AK23 were subjected to shear stress and the time and extent until anti-desmoglein 3-induced disruption was measured. Surprisingly, pharmacological blocking of both inflammatory and apoptotic caspases leads to an accelerated acantholysis in the presence of AK23. Upon activation of the AIM2-inflammasome in primary keratinocytes via the transfection of dsDNA, caspase-1 is activated. In the acantholysis assay AIM2-inflammasome activation protects the keratinocytes from anti-desmoglein induced acantholysis, confiming the results of the caspaseinhibitors. In an attempt to explain the mechanisms of acantholysis, keratinocytes were subjected to osmotic stress, which leads to IL-1 secretion in a dose-dependent manner. Addition of anti-desmoglein antibody reduced the threshold of osmotic stress, implicating a putative role of osmotic stress and IL-1 signalling in desmoglein induced acantholysis.
Our results introduce inflammasomes and inflammatory caspases as important co-factors in skin blistering and osmotic stress as one putative IL-1-activating mechanism accelerating antibody-mediated acantholysis.

P054

IL-1 signalling is up-regulated in chronic venous leg ulcers and represents a therapeutic target that is counteracted by dermal ABCB5+ mesenchymal stem cells via secretion of interleukin-1 receptor antagonist

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Chronic wounds such as diabetic foot and venous leg ulcers are difficult to treat and impose a considerable burden on both the patient and national health care institutions. We and others have previously shown that chronic wounds are marked by a persistent and exacerbated inflammatory response, mediated by an M1 activated macrophage population and that impedes proper healing and tissue repair. In line with most chronic inflammatory disorders, the IL-1 pathway that has detrimental effects on tissue repair mechanisms plays a central role in the physiopathology of chronic wounds. Mesenchymal stem cells (MSCs) feature many characteristics, such as tissue regeneration capacity and immune modulation, beneficial for therapeutic applications in injury and trauma. Interestingly, MSCs are able to suppress inflammatory macrophage M1 activation and to promote prowound healing M2 type macrophages. Here we describe the isolation of an ATPbinding cassette sub-family B member 5 (ABCB5) positive plastic-adherent dermal cell subpopulation from the dermis and its characterization as bona-fide MSCs. We show that ABCB5+ dermal MSCs accelerated resolution of the inflammatory phase in the iron-overload mouse model for chronic venous ulcers and contributed to fullthickness excisional skin wound healing. Furthermore, we demonstrate that ABCB5+ dermal MSCs secreted interleukin-1 receptor antagonist (IL-1RA) in response to inflammatory stimulation, which dampened classical macrophage activation with TNF-α release *in vitro* and mediated a switch from an M1 towards M2 macrophage activation profile *in vivo* in the wound bed. The importance of the anti-inflammatory effects on wound macrophages by MSC-secreted IL-1RA for cutaneous chronic wound healing was substantiated by siRNA-mediated gene-silencing. In conclusion, human dermal ABCB5+ sorted MSCs represent an easy accessible source for cellbased therapy of chronic skin wounds that accelerates healing at least in part by the secretion of IL-1RA.

P055

Orai1 and STIM1 control IL17a-induced calcium signals in human primary keratinocytes

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Psoriasis is an inflammatory and hyperproliferative skin disease. The pathogenesis of psoriasis involves pro-inflammatory signals, such as IL-17a secretion by inflammatory cells, which promote epidermal hyperproliferation and the development of cutaneous lesions. This model is confirmed by the efficiency of anti-inflammatory therapies, such as anti-IL-17 antibodies, in the management of psoriasis.
We have shown recently that Orai1-dependant calcium signals control keratinocyte proliferation. Orai1 is a plasma membrane calcium channel which is activated by STIM1, a calcium sensor of the endoplasmic reticulum (ER), upon depletion of the calcium content of the ER.
Here we show that IL-17a triggers cytosolic calcium signals in primary human keratinocyte *in vitro*. IL-17a activates Orai1 and STIM1 as Orai1-CFP and STIM1- YFP show colocalizing clusters and increased FRET signals following stimulation of keratinocytes with IL-17a. We also show that Orai1 and STIM1 silencing with small interfering RNA prevents the calcium influxes that are elicited by IL-17a.
We conclude that IL17-a-induced calcium signals are controlled by Orai1 and STIM1 in primary human keratinocytes. Pathological calcium signals induced by proinflammatory cytokines such as IL-17a may thus play a key role in the pathogenesis of psoriasis.

P056

Innate immune sensing of Toll-like receptor ligands modulates CXCL16 expression on monocytes and dendritic cells in psoriasis

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Psoriasis is a chronic inflammatory skin disease characterized by a cutaneous infiltrate, containing activated dendritic cells and monocytes orchestrating the T cell response. We previously showed that the chemokine CXCL16, that can be expressed on and secreted by antigen presenting cells, mediated homing of CXCR6+ CD8+ T cells into human skin. However, the regulation of CXCL16 expression on antigen presenting cells in psoriatic patients was not investigated. It is known that activation of Toll-like receptors (TLRs) by bacterial or viral infections contributes to the initiation and maintenance of psoriasis. We therefore asked how such stimulation could influence the expression of CXCL16.
In blood and skin of psoriasis patients monocytes and dendritic cells expressed higher amounts of CXCL16 on their surface compared to healthy controls. Upon maturation, especially dendritic cells upregulated CXCL16. Likewise, stimulating monocytes from psoriasis patients with the synthetic TLR ligands Pam2 (TLR2/6 agonist), Pam3 (TLR2/1 agonist) and R837 (TLR7 agonist) significantly enhanced the expression level of CXCL16 on the monocyte subpopulations (CD14+CD16low and CD14lowCD16+). This TLR induced upregulation of CXCL16 was much more pronounced in monocytes from patients with psoriasis compared to healthy controls.
In contrast, stimulation by the TLR4 agonist lipopolysaccharide and TLR8/7 agonist R848 significantly inhibited the CXCL16 expression level by 60% on monocytes and dendritic cells from psoriatic patients.
As CXCL16 can also be induced by TNFalpha stimulation, we further analysed the influence of TNFalpha blockers on CXCL16 expression. Etanercept and adalimumab similarly ameliorated the TLR mediated induction of CXCL16 expression by 40% on dendritic cells and monocytes indicating that TNFalpha blockade can inhibit CXCL16 upregulation by infectious stimuli ligating TLR2/6, TLR2/1 or TLR7.
In conclusion, our data suggest that bacterial or viral infection triggering TLR2/6, TLR2/1 or TLR7 but not TLR4 or TLR8 can enhance CXCL16 expression on monocytes and thereby stimulate the inflammatory response in psoriasis. Our data further elucidated that potent anti-inflammatory TNFalpha blockers inhibit CXCL16 upregulation on proinflammatory dendritic cells and monocytes demonstrating an additional effector mechanism of TNFalpha inhibitors in psoriasis.

P057

PsoBiop – a clinical trial correlating the dermal signaling pattern with systemic metabolic and inflammatory parameters in psoriasis vulgaris

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There is growing evidence that psoriasis, a chronic inflammatory skin disease, extends beyond the skin and has a considerable systemic dimension. This might be explained by the fact, that psoriasis patients show a higher prevalence to exhibit conventional risk factors for cardiovascular diseases such as the metabolic syndrome, leading to an increased morbidity and mortality. Currently it is assumed that the systemic inflammation induces insulin resistance in metabolic and nonmetabolic tissues, which in turn can contribute to the development of diabetes and/or atherosclerosis resulting in the mentioned morbidity and mortality. In addition, there is growing evidence that the metabolic syndrome itself causes an inflammatory milieu, which induces different signaling cascades in the skin that contribute to the dermal changes in psoriasis. Specifically, we showed previously that the Akt/mTOR cascade is deregulated in psoriatic lesions and contributes to the psoriatic phenotype.
In order to better understand the interplay between the systemic and dermal dimension of the psoriatic inflammation, we aimed to investigate the cytokine and metabolic profile of psoriasis patients and to correlate the disease severity with the dermal signaling pattern.
27 patients with moderate to severe plaque-type psoriasis were enrolled in a prospective, observational cohort study before therapy initiation. Lesional and nonlesional biopsy samples of the participants were taken and stained for activation of the Akt/mTOR pathway. In addition, blood samples were collected and cytokine levels as well as diabetes and metabolic parameters were measured. Most patients (68%) presented with a BMI above 25 and a moderate disease (median PASI of 13.7, ranging from 4.0 to 23.7). We found that the disease severity correlated significantly with signs of metabolic imbalance as measured by increased levels of insulin and C-peptide. Interestingly we also found that activation of the mTOR pathway correlated with clinical disease assessment as well as levels of Th17 cytokines.
In summary these results not only support our previous findings on the activation of mTOR signaling by psoriatic cytokines, but also underline the close interrelation between the dermal and systemic dimension of psoriasis.

P058 (O03/06)

Silver-nanoparticles complexed with natural extract of cornus mass significantly inhibit inflammation *in vitro* and in human psoriasis plaques

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Pro-inflammatory molecules, especially TNFalpha and IL-12, are crucial mediators in the pathogenesis of human psoriasis and therefore first-line targets of effective therapeutic strategies. New biomaterials based on nanoparticles carrying polyphenols-rich natural extracts recently showed promising anti-inflammatory, anti-tumoral and anti-angiogenic activity. Herein we studied the effect of silvernanoparticles complexed with Cornus Mass (AgNPs-CM) extract on inflammatory macrophages *in vitro* and on human psoriatic plaques. AgNPs-CM incubated with bone-marrow-derived murine macrophages pro-inflammatory stimulated *in vitro* with LPS and IFNalpha significantly and dose-dependently decreased the release of the pro-inflammatory mediator nitric oxide as well as of the master pro-inflammatory cytokines IL-12 and TNFalpha as compared with non-treated macrophages.
Treatment of 8 patients with chronic stationary plaque-psoriasis (with comparable disease severity as assessed by PASI score) with AgNPs-CM based ointments once daily for 6 weeks resulted in subjective clinical improvement, reduced scaling, erythema and plaque thickness.
Immunfluorescence staining of cryosections from psoriatic plaques treated with AgNPs-CM revealed significantly decreased numbers of TNFalpha-positive, as well as of IL12-positive CD68-positive macrophages in the inflammatory infiltrate when compared with plaques treated with the null formulation.
Remarkably, high-frequency ultrasound assessment of the psoriatic plaques showed a statistically significant reduction of the plaque thickness (average of 50.78% less than control plaques treated with neutral formulations) as compared with hydrocortisone-treated control plaques of the same patient (42.22% % less than control plaques treated with neutral formulations). Furthermore, quantification of low echogenity pixels as a measure of inflammatory infiltrate density, showed that AgNPsCM primarily exerted their clinical effect by reducing the number of infiltrating inflammatory cells in the psoriatic plaques.
Ultrasound characteristics of psoriasis plaques treated with AgNPs-CM ointment confirmed the anti-inflammatory properties of the studied nanomaterials which were even superior to hydrocortisone standard control treatment.
Our *in vivo* and *in vitro* data demonstrates promising anti-inflammatory effect of this novel nanoparticle-based technology and offers a safe and efficient therapeutic tool in modern psoriasis therapy.

P059 (O03/02)

IL-29 induces unique chemokines and provokes T-cell infiltration into the skin

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IL-29 is a novel member of the IL-10 – interferon cytokine family that is produced by Th17-cells as well as dendritic cells and preferentially acts on epithelial cells. IL-29 plays a fundamental role in the elevated anti-viral defense in diseased skin of psoriasis patients. However, whether IL-29 contributes to further alterations of psoriatic lesions was unknown so far. Since acanthosis, parakeratosis, hyperkeratosis, and massive immune cell infiltration are key features of affected psoriatic skin, we systematically investigated in this study the IL-29 impact on keratinocyte proliferation, differentiation, and chemokine production. Regarding proliferation, rather minimal growth-inhibiting impact of IL-29 on keratinocytes was observed. However, it was overcome by influences of IL-17A and IL-22, cytokines

that had been associated with epidermal hyperproliferation and decreased differentiation in psoriasis, respectively. Next, IL-29 did not regulate the expression of molecules necessary for keratinocyte terminal differentiation, including K10 and CALML5. Accordantly, it did not induce thickening or psoriasis-like appearance of three-dimensional epidermis models. In contrast, analysis of chemokine production revealed that IL-29 induced chemokines that attract Th1-cells and IFN-γ/IL-17 double positive Th-cells: CXCL10 and CXCL11. No influence was found on the production of chemokines attracting neutrophils, eosinophils, monocytes, dendritic cells, or Th2 cells. Regarding other psoriasis-relevant cytokines, only IFN-γ shared IL-29's capacity to induce CXCL10 and CXCL11. Furthermore, IFN-γ, TNF-α, and IL-1β strengthened the IL-29-induced production of these chemokines. The IL-29-specific effect on the production of these CXCR3 ligands was also observed in epidermis models and explanted healthy skin. Furthermore, the murine IL-29 counterpart injected into mouse skin induced cutaneous expression of CXCL10 and CXCL11, provoked cutaneous T-cell infiltration, and, in consequence, skin swelling. Murine IL-29 and IFN-γ counterparts also showed clear synergistic effects. According to the elevated cutaneous expression of IL-29 in psoriatic lesions, we found strong upregulation of CXCL10 and CXCL11 in lesional skin compared to non-lesional skin of psoriasis patients. Importantly, neutralization of IL-29 reduced CXCL10 and CXCL11 levels in explant cultures of psoriatic lesions. Finally, elevated blood CXCL11 levels were found in psoriasis patients that might be useful for monitoring of IL-29 activity in psoriatic lesions. In summary, our study shows that IL-29 specifically induces unique chemokines and, in consequence, provokes infiltration of potentially pathogenic T-cells into the skin.

Clinical Research

P060

Raman spectroscopy as analytical tool for melanoma research

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Background: Raman spectroscopy is an optical non-invasive screening technology that generates individual fingerprints of living cells by reflecting their molecular constitution.
Aim and methods: Here, we applied this technology to discriminate melanoma cells from melanocytes, for the distinction of drug-induced melanoma cell death stages (apoptosis, necrosis, autophagy) and to depict the susceptibility of melanoma cells towards anticancer therapy.
Results: Using the multivariate principal component analysis of the Raman spectra, melanocytes were clearly distinguished from melanoma cells and wild type from mutated (BRAF, NRAS) melanoma cells were discriminated from each other. After using the apoptosis-inducer staurosporine, the necrosis-inducer 3-Bromopyruvate (3-BrPv) and the autophagy-inducer resveratrol to induce cell death in SKMEL28 melanoma cells, Raman spectroscopy clearly distinguished these three types of cell death, which was confirmed by immunoblotting. Finally, different melanoma cell lines could be discriminated according to their susceptibility towards high-dose ascorbate.
Conclusions: Raman spectroscopy is a powerful non-invasive tool for the distinction between melanocytes and melanoma cells. It can be used to analyze the specific type of cell death in melanoma cells, and it can predict the susceptibility of melanoma cells to anticancer drugs.

P061

Specific and sensitive detection of serum autoantibodies against novel recombinant forms of the BP180 ectodomain in patients with bullous pemphigoid and mucous membrane pemphigoid

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Bullous pemphigoid (BP) and mucous membrane pemphigoid (MMP) are the two most frequent autoimmune blistering diseases in Germany. In both diseases, BP180, also termed collagen type XVII, is the major autoantigen targeted in almost all patients. However, the immunodominant non-collagenous domain 16A (NC16A) of BP180 is recognized in only 90% of BP and about half of MMP patients leaving a relevant diagnostic gap. At present, non-NC16A anti-BP180 antibodies can be detected by immunoblotting with the soluble cell-derived ectodomain of BP180, LAD-1, extracted from conditioned supernatant of cultured keratinocytes, or with full-length BP180 in extracts of human epidermis or cultured keratinocytes. Latter methods are difficult to standardize, have relatively low specificities, and are only available in specialized laboratories. Previous attempts to apply the entire recombinant BP180 ectodomain were hampered by the low specificity that was hypothesized to be related to the large collagenous stretches within the protein. Here, we expressed two fusion peptides consisting of the first fifteen NC (NC1–15) and all NC domains (NC1–16A) of the BP180 ectodomain, respectively, in E. coli. Western blotting of a large number of BP sera (n = 50) as well as sera from healthy blood donors (n = 23) and patients with non-inflammatory dermatoses of >75 years of age (n = 30) with the two recombinant proteins revealed sensitivities and specificities of 84% and 96% (for NC1–15) and 98% and 94% (for NC1–16A) for IgG autoantibodies. The two proteins were then probed by immunoblotting with BP (n = 52) and MMP sera (n = 15) from patients with positive direct immunofluorescence (IF) microscopy and IgG antibodies against LAD-1 that showed no reactivity against BP180 NC16A and BP230 by ELISA. In 67% (NC1–15) and 58% (NC1–16A), respectively, of these BP and MMP sera, IgG reactivity was detected. Future studies will analyze IgA reactivity against NC1–15 and NC1–16A in sera from patients with MMP and linear IgA disease. Furthermore, the two proteins will be applied by ELISA and IF microscopy-based tests for their potential use in commercial assay systems.

P062

Low risk for digital ulcer development in SSc patients with increasing disease duration and lack of Scl-70 autoantibodies

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Background: Digital ulcers (DU) constitute an important component of disease burden in Systemic Scleroderma (SSc). The natural history, for the development of DU, including risk as well as protective factors, is only partially understood.
Objectives: To identify new clinical parameters as risk factors for the development of DU.
Methods: In a prospective study, patients with definite SSc were included in three groups: (i) group 1 for patients with active DU; (ii) group 2 for patients, who have no active DU at baseline, but had reliably documented SSc-related DU in the past; (iii) group 3 for patients, who neither have active DU at inclusion nor had DU in their past. Patients in group 3 were followed on months 6, 12, 24 for the development of DU.
Results: 89 patients were included in group 1, 146 patients in group 2 and 410 patients in group 3. Median disease duration at enrolment, as determined by the first non- Raynaud organ involvement, was 8.7 years in group 1, 2 and 6.3 in group 3. Patients in group 3 with no history of DU were significantly ($P < 0.001$) older (mean 59 vs 55 years in group 1, 2), had lower mRSS (median 7 vs 11), a lower frequency of lung fibrosis (48.0% vs 63.9%) and a lower frequency of Scl-70 autoantibody detection (23.9% vs 44.9%). In group 3 after a median follow up of 1.8 years, only 18/410 (4%) patients developed a DU, which was significantly associated with Scl-70 positivity. In general, the risk to develop DU was highest within 3 years after beginning of Raynaud phenomenon (RP). Risk for DU was reduced for patients within 3–6 years after beginning of RP [OR = 0.227 95%-CI (0.046; 1.132)] and >6 years [OR = 0.353, 95%-CI (0.126; 0.985)].
Conclusions: This prospective, longitudinal clinical study demonstrates, that independent of several risk factors (diffuse disease, high mRSS, Scl-70 antibody) a sizeable number of patients will not develop DU. Furthermore, the risk to develop DU for the first time was inversely correlated with disease duration and most pronounced in patients negative for Scl-70.

P063

Impact of a glycolic acid-containing pH4 water-in-oil emulsion on skin pH

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The acidity of the skin surface is crucial for physiological skin functions. A decline in stratum corneum (SC) acidity, as it is observed in aged, but also diseased skin, may negatively affect physiological skin functions, such as permeability barrier, integrity/ cohesion of the SC, and antimicrobial capacity. The aim of the different clinical studies was to investigate the overall impact of a glycolic acid-containing water-in-oil (W/O) emulsion adjusted to pH4 regarding its effect on skin pH.
First, the impact of the pH4 (W/O) emulsion was investigated on healthy subjects (29.9 $\pm$ 4.7 years, n = 6). Immediately after application for 10 min, a significant decline of skin surface pH (pH-SS) at test areal was observed. However, this effect diminished after 2 h. The pH of the SC (pH-SC) was also reduced after removal of the SC by tape stripping, which indicates an effect even on deeper layers of the epidermis.
Next, the efficacy of the pH4 (W/O) emulsion was assessed in diabetic patients (diabetics: 70.2 $\pm$ 2.6 years, n = 10; non-diabetics: 59.8 $\pm$ 3.5 years, n = 10) on the bottom of the foot, the dorsum of the foot and interdigitally. Application (twice daily for 14 days) led to a significant reduction of pH-SS in all test areas. Additionally, some patients showed a shift towards a rather physiological skin flora in the interdigital region as compared to baseline evaluation.
To evaluate long-term efficacy and safety the pH4 (W/O) emulsion, a 28 days halfside trial was performed (70.2 $\pm$ 5.2, n = 30). Compared to untreated test areas, the increased pH-SS decreased significantly by 0.38 after 2 weeks of application, and even by 0.52 after 4 weeks. Furthermore, mean corneometer readings showed a significant increase of skin hydration already after 2 weeks, further increasing after 4 weeks of application. No irritations or intolerance reactions were observed in any of the above studies.
In summary, application of a pH4 (W/O) emulsion reduced significantly the skin pH in healthy, elderly and diabetic subjects without irritation, very likely leading to improved functions of the epidermal barrier.

P064

The international cochrane skin group dermatology outcomes research initiative (ORI)

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The failure to rigorously translate research knowledge into clinical practice constitutes a major challenge for evidence-based healthcare [1]. The Cochrane Collaboration plays an essential role in building the bridge from knowledge generation to knowledge usage by systematically summarizing and updating relevant clinical trials and by making these summaries accessible at a global level. One of the four key goals of the Cochrane Strategy to 2020 is to inform health decision making and to become the leading advocate for evidence-informed health care [2]. The lack of appropriate outcome measures is an important barrier for reaching this goal. Specifically, dermatology trials frequently fail to assess the important outcome domains or use outcome measurement instruments with unclear validity. The lack of standardization of outcome measures across trials introduces bias in trials and makes evidence synthesis impossible. Furthermore, generalizability of trial evidence to routine care may be limited due to differences in (i) the setting / structure of care provision, (ii) differences in procedures of care provision, and (iii) differences in outcome assessment between clinical trials and routine care.
In response to these challenges, the Cochrane Skin Group has established an Outcomes Research Initiative (ORI) – a global network on outcomes research in dermatology and allergology. ORI is coordinated at the University of Dresden, Germany. The over-arching aim of the Cochrane Skin Group Outcomes Research Initiative may be summarized as measurement research to improve trials and to make trial information more useful. The ORI initiative has the main objective to develop and disseminate consensus-derived minimum sets of outcomes for major dermatological diseases to be assessed in a specific situation in clinical research or clinical care (known as 'core outcome sets'), and to standardize, validate and disseminate outcome measurement instruments in dermatology. In this context, the Harmonizing Outcome Measures for Eczema (HOME) roadmap [3] will be used as a methodological standard for developing core outcome sets in dermatology and allergology. In addition, ORI aims to strengthen, implement and evolve good practice in outcomes methodology in dermatology.
The Cochrane Skin Group Outcomes Research Initiative is intended to be a research group that is open for everyone with interest and enthusiasm to work on dermatological outcomes research and evidence-based dermatology. All members of the Cochrane Skin Group, all members of the HOME initiative [4–5], and other interested researchers, patients, and stakeholders are invited to get involved.
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P065

Treatment of therapy resistant alopecia areata with fumaric acid esters

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Alopecia areata (AA) is a T cell-mediated immune disease targeting hair follicles, clinically presenting with round or oval patches of hair loss. The most frequent treatments include topical or systemic steroids. Alternatively, immunosuppressive therapies with calcineurin inhibitors, methotrexate or topical diphencyprone may improve AA. Since the pathogenesis of AA is linked to an inflammatory immune response dominated by interferon γ-producing T cells, immune-modulating compounds inhibiting type I responses could also be helpful for patients suffering from AA. Interestingly, fumaric acid esters (FAE), which are well established in the therapy of psoriasis and inhibit IFN-γ* and interleukin 17* T cell responses, have been reported to improve AA in individual patients. Therefore, we conducted a monocenter trial investigating the efficacy and safety of FAE in patients with chronic moderate to severe scalp AA (≥20% involvement). We included 40 patients (34 female, 13 male; 18–65 years) in the study and treated them with a Fumaderm® dosing regimen adapted from the treatment in psoriasis. Systemic or topical treatment for AA had to be discontinued prior to study entry. The primary objective was the change in the severity of alopecia areata tool (SALT) score at week 24 compared to baseline. 22 patients (65%) completed the study, 4 patients had to discontinue FAE treatment due to abnormal laboratory parameters. In total, 411 adverse events (AE) were documented and one patient experienced a serious AE (not drug-related). The most common AE were lymphopenia (n = 84), elevated liver enzymes (n = 79), gastro-intestinal symptoms (n = 58) and flushing (n = 29). No relevant elevation of serum creatinine, GFR or proteinuria was observed during treatment. In patients that fully completed the study (n = 22) the average SALT score decreased from 60.4 at baseline to 41.5 at week 24 of Fumaderm® treatment and 68% of these patients showed at least 30% improvement (average SALT score 48.7 at baseline and 21.6 at week 24, $P < 0.05$). Patients self assessment of their individual hair status (score 1–7) significantly improved from 2.3 at baseline to 3.9 at week 24 and patients quality of life as determined by DLQI improved from 9.95 at baseline to 5.31 at week 24 (n = 22). In conclusion, FAE treatment is well tolerated in patients with AA and shows a similar safety profile as in psoriasis. A subgroup of patients showed significant hair growth beginning earliest at week 8 of treatment. Currently, we are analyzing immune and cellular parameters from blood and skin samples, which may help to better understand the molecular effects in responders. More extensive and placebo-controlled studies are needed to further assess the efficacy of FAE in immune-mediated hair loss.

P066

Extracorporal shock waves induce healing of chronic leg ulcers via activation of cell-cycle regulatory proteins and pro-inflammatory cytokines

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Chronic leg ulcer, a tissue disorder with high and increasing incidence, is mainly caused by chronic venous insufficiency. Its treatment is multidisciplinary and challenging, and over the years many conservative (e.g. wound dressings), interventional (e.g. vacuum therapy) or surgical therapeutic approaches (e.g. vein operations, skin transplantation) were proposed. Yet, until now, no clinically efficient therapy for chronic leg ulcers has been described. Extracorporeal shock wave therapy (ESWT) has recently attracted interest and publicity as treatment modality for several clinical indications.

Here, we report that patients suffering from chronic leg ulcer show accelerated wound healing after repetitive ESWT (CellSonic®); however, the underlying mechanisms of induced wound healing remain unclear. Therefore, we analyzed the molecular processes that induce and control wound repair after ESWT. The known key players for skin repair are human epidermal keratinocytes, fibroblasts and microvascular endothelial cells. We demonstrate that after ESWT *in vitro*, cellcycle regulatory proteins are activated in fibroblasts as well as pro-inflammatory cytokines secreted by keratinocytes with a known function in the control of wound repair. Additionally, we observed both a morphological re-organization and an increased migration of human fibroblasts and keratinocytes, and increased proangiogenic activity of microvascular endothelial cells after ESWT at the same treatment modalities as applied on ulcer patients.

Together, elucidating the impact of ESWT on chronic leg ulcers *in vivo* and the cellular players *in vitro* ('from bedside-to-bench') allows us to better understand, which mechanisms induce wound repair and thus restore tissue integrity.

P067

Ingenol mebutate for ano-genital warts

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Ingenol mebutate has been approved for treatment of actinic keratoses. Due to its mechanism of action leading to rapid cell necrosis and an inflammatory action, we tested whether the agent could be suited for treatment of ano-genital warts (concylomata acuminata). We set out to determine the effectiveness of ingenol mebutate gel for the topical treatment of genital warts.

20 patients were treated topically with ingenol mebutate gel (150 or 500 µg/g). Skin biopsies were performed to confirm clinical diagnosis. Clinical efficacy was also documented by photography. Moreover, a skin biopsy 18 h post treatment served to study the inflammatory reaction. The gel base served as a control

Clinical response rates showed rapid clearance of genital warts, in many cases after one treatment. Follow-up examination showed clearance persisted for 240 days. Side effects included local skin irritation. Skin pathohistology exhibited spongiosis, single cell necrosis and beginning confluence of destroyed tissue. None of the gentital warts treated with gel base only exhibited clinical improvement.

Conclusions: Following topical treatment, genital warts disappear after an inflammatory tissue reaction. Our results show for the first time that ingenol mebutate gel is highly active for the treatment of genital warts.

Dermato-Endocrinology

P068

Effects of isotretinoin on FoxO1 transcriptional regulation and molecular functions of SZ95 sebocytes

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Introduction and objectives: Isotretinoin (13-cis retinoic acid (13-cis RA)) is the most potent treatment against severe acne for more than 30 years. It has an ability to normalize all pathogenic factors of acne; however, its molecular mechanism of action has not been completely investigated. A recent hypothesis proposes that isotretinoin via retinoic acid receptor (RAR) up-regulates nuclear forkhead box- O1 (FoxO1) transcription factor and can suppress sebocytes proliferation, lipid production and inflammation. The aim of the present study was to investigate the effects of isotretinoin on nuclear FoxO1 expression and FoxO transcriptional activity as well as SZ95 sebocytes molecular functions. It has been proved that insulin and insulin-like growth factor 1 (IGF-1) down-regulate nuclear FoxO1 and thereby increase sebocytes lipogenesis and expression of toll-like receptor 2 (TLR2) and 4. Subsequently, we investigated the effects of isotretinoin on insulin and IGF-1-induced SZ95 sebocytes.

Materials and methods: SZ95 sebocytes were treated in the dark with 0.1 μM isotretinoin in the presence or absence of 1 and 0.1 μM IGF-1 and insulin for different time points and cytoplasmic expression levels of FoxO1 and p-FoxO1 were analysed by western blot. FoxO transcriptional activity was measured by dual luciferase assay and nuclear and cytoplasmic mobilisation of FoxO1 and p-FoxO1 were determined by immunofluorescence microscopy. Proliferation of sebocytes was measured by [3H] thymidine incorporation assay and differentiation by semiquantitative analysis of lipid droplet accumulation using Oil Red staining. Concerning TLR2/4 expression measurement, flow cytometry has been done.

Results: Western blot results showed that FoxO1 expression has not been significantly up-regulated in SZ95 sebocytes and IGF-1 and insulin-induced sebocytes after 0.1 μM isotretinoin treatment and nuclear content of FoxO1 has not been increased. FoxO transcriptional activity was not up-regulated as well. Isotretinoin supressed proliferation of SZ95 sebocytes and IGF-1 and insulinstimulated sebocytes time dependently. In addition, 0.1 μM isotretinoin normalized lipid production in IGF-1 and insulin-induced sebocytes. Our data determined that expression of pro-inflammatory TLR2/4 has not been decreased after isotretinoin incubation.

Conclusion: These data demonstrate that isotretinoin has no ability to increase nuclear content of FoxO1 in SZ95 sebocytes and therefore cannot up-regulate FoxO transcriptional activity. We further propose that isotretinoin restores acne pathological factors by receptor-independent manner in SZ95 sebocytes.

P069

Diabetes mellitus causes multiple dysfunctions in human keratinocytes

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Diabetes mellitus is the most common endocrine disorder and can affect the skin in multiple ways. Approximately 30% of patients with diabetes develop skin complications such as cutaneous infections, itching and impaired wound healing. Hyperglycemia-induced damage to nerves and blood vessels as well as chronic lowgrade inflammation are discussed as the cause, however, analyses of direct longterm influences of diabetes mellitus on skin homeostasis in humans are missing.

First insights into the influence of diabetes mellitus on skin cells were raised by stimulating cells with elevated glucose concentrations or using experimental diabetes models. In this study, we sought to determine direct diabetes-induced alterations in human keratinocytes and the impact of these alterations on cutaneous innervation. Under normal culture conditions, human primary keratinocytes isolated from diabetic donors displayed a stable diabetic phenotype *in vitro*: mRNA expression of the insulin receptor and insulin receptor substrate 2 (IRS-2) was reduced and alterations were accompanied by a reduced insulin-induced glucose transport capacity indicating that diabetic keratinocytes are insulin resistant and maintain this status *in vitro*.

Inflammation and oxidative stress are known to induce insulin resistance in different cell types. An investigation of the inflammatory state and antioxidant defense system revealed that both were aberrant in the epidermis of diabetic skin models. Levels of IL1, IL6, and TNF were significantly increased and epidermal mRNA expression of nrf2, hmox1, nqo1 and ahr were reduced, pointing to a pro-inflammatory state and an impaired antioxidant defense system in diabetic skin models. Moreover, diabetic keratinocytes displayed reduced NGF levels and, in comparison to healthy control cells, these keratinocytes induced a reduction of nerve fiber density in innervated skin models. These data demonstrate that diabetes mellitus causes direct alterations in insulin signaling, inflammatory state and cytoprotective potential of epidermal keratinocytes as well as neuro-cutaneous crosstalk. We suggest that diabetesinduced alterations in keratinocyte biology might significantly contribute to diabetic small-fiber neuropathy.

P070

Effects of relaxin on human fibroblasts and its key role in menopause

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Menopause as well as exposure to UV irradiation affect the biological activity of human skin fibroblasts. Relaxin, a polypeptide hormone produced in the human female by the corpus luteum of pregnancy and the decidua, has been shown to promote corneal wound healing and to improve renal function and histology of the ageing kidney. Therefore, we investigated the role of human relaxin-2 (RLX-2) on sun-exposed and sun-protected skin fibroblasts obtained from elderly female donors with no endocrinological disorders. The cells were treated in a hormone mixture consisting of growth factors (e.g. GH, IGF-I) and sex steroids (e.g. DHEA, progesterone, 17β-estradiol) at levels corresponding to those circulating in young (20-y old) and postmenopausal (60-y old) women with/without relaxin-2 at several concentrations. Subsequently, fibroblast lipid synthesis, proliferation, apoptosis and cytotoxicity were documented using nile-red microassay, 4-methylumbelliferyl heptanoate assay, cell death detection kit and lactate dehydrogenase assay, respectively. The production of reactive oxygen species were measured by the 2',7'- dichlorodihydrofluorescein diacetate assay. RLX-2 exhibited diverse effects on sunprotected versus sun-exposed cells. In particular, cell proliferation of sun-protected cells under 60-y old hormone conditions was significantly induced by RLX-2 ($P < 0.01$), neutral lipid production was stimulated only in fibroblasts under hormone conditions immitating those of 20-y old women ($P < 0.001$) and RLX-2 showed no effect on cell apoptosis, ROS production and cytoxicity under all treatment conditions tested. On the other hand, RLX-2 significantly stimulated lipid production in 60-y old hormone treated sun-exposed fibroblasts ($P < 0.001$) and induced proliferation in 20-y old hormone treated sun-exposed cells ($P < 0.05$). ROS production and apoptosis were significantly inhibited by RLX-2 in cells under 20-y old hormone conditions ($P < 0.001$ and $P < 0.01$, respectively). Taken together, our results indicate that RLX-2 may exert a preventive, anti-oxidant function particularly on sun-exposed skin. Therefore, RLX-2 may be a candidate molecule to ameliorate altered functions of aged skin after menopause.

P071

Tropisetron – an emerging anti-inflammatory and antifibrotic agent

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Tropisetron is an approved antiemetic drug for patients undergoing chemotherapy. Recently we reported that tropisetron, originally characterized as a serotonin (5- HT) receptor modulator, suppressed transforming growth factor-beta1-mediated collagen synthesis in normal human dermal fibroblasts (HDFs) as well as in dermal fibroblasts from patients with systemic sclerosis (SSc). This effect of tropisetron was independent of the 5-HT3 and 5-HT4 receptor but mediated via the alpha7 nicotinic acetylcholine receptor (alpha7nAchR) in HDFs. Importantly, tropisetron had antifibrogenic and antifibrotic effects in experimentally induced skin fibrosis of mice. Since lung fibrosis is a

common complication in patients with SSc we tested whether tropisetron has antifibrotic effects in extracutaneous organs. In a mouse model of lung fibrosis experimentally induced by a single pharyngeal aspiration of bleomycin (BLM) tropisetron likewise significantly reduced collagen type I and III mRNA expression and protein amounts in the lungs compared with BLM-treated mice. To assess the relevance of these findings in the human system we performed an expression analysis of the putative tropisetron receptors in human lung fibroblasts. Neither 5-HT3 nor 5-HT4 receptors were detected while these cells expressed the previously identified off-target receptor of tropisetron, alpha7nAchR. Since the BLM mouse models of skin and lung fibrosis are inflammation-driven models we further investigated whether tropisetron can counteract inflammatory cell responses in nonfibroblast cutaneous cell types. Accordingly, we examined the impact of tropisetron on tumor necrosis factor (TNF)-α-mediated expression of interleukin (IL)-6 and 8 as well as on ultraviolet B (UVB)-induced cytokine expression in human epidermal keratinocytes (NHK). Tropisetron suppressed both TNF-α- and UVB-induced expression and secretion of these proinflammatory cytokines in these cells. This effect of tropisetron was independent of canonical p65/NF-B signaling. In analogy to human dermal and lung fibroblasts, neither 5-HT3R nor 5-HT4R was detectable in NHK. In contrast, α7nAchR were present and mediated the anti-inflammatory effect of tropisetron in these cells. The *in vivo* relevance of these *in vitro* findings was confirmed in the imiquimod mouse model of psoriasis in which injections of tropisetron resulted in a significant reduction of cutaneous inflammation. In summary, our data show that tropisetron has antifibrogenic potential not only in the skin but also in the lung and acts anti-inflammatory in other cutaneous cell type beyond fibroblasts. Further studies on the α7nAchRs in inflammatory and fibrotic skin diseases will clarify the therapeutic utility of drugs specifically targeting these receptors.

P072

Pparα deficiency leads to an exaggerated inflammatory response after acute barrier disruption

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Pparα is a nuclear hormone receptor that exerts various functions in skin homeostasis. Ligand activation of Pparα promotes epidermal differentiation, induces epidermal lipid synthesis, enhances cutaneous barrier recovery and ameliorates skin inflammation. Barrier perturbation induces DNA, protein lipid synthesis leading to barrier recovery. This study aims to elucidate the role of Pparα in models of acute barrier disruption.

Pparα mRNA levels are decreased 6 h post tape stripping (TS) in mouse skin. An increase of Il1β and Tnfα precedes the decrease of Pparα levels. Il1β mRNA levels are upregulated after TS in Pparα−/− and littermate control mice. Yet, the fold increase in Il1β expression is significantly higher in Pparα−/− mice. Similarly, Tslp and Tnfα mRNA levels are enhanced in skin of Pparα−/− mice after TS as compared to littermate controls. Lipid analyses show a decrease in 5-Lox and 8-Lox pathwayderived metabolites in the steady state in Pparα−/− mice when compared to control mice. Further analyses of the cutaneous eicosanoid composition of Pparα−/− mice and littermate control mice after TS are underway.

In human skin organotypic cultures, acute barrier perturbation leads to reduced PPARα mRNA levels and to an increase in IL1β and TNFα mRNA levels. Treatment of human keratinocytes with IL1β results in a decrease of PPARα expression and an increase of TNFα and TSLP expression

These data demonstrate that in acute barrier perturbation, IL1β potentially modulates Pparα expression. Furthermore, they show that Pparα deficiency leads to an exaggerated inflammatory response after acute cutaneous barrier disruption and results in alterations in eicosanoid and leukotriene composition in the steady state.

P073

The antimicrobial peptide koebnerisin interferes with UVB-triggered IL-1 beta activation in epidermal keratinocytes

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The skin is the first line of defence 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 dependent inflammasomes that are required for IL-1 beta secretion. Here, we showed that UVB increased IL-1 beta activation is amplified by inflammasome-inducing IFN gamma in human keratinocytes. Furthermore, we observed a co-regulation of certain S100 antimicrobial peptides, which control the UVB-mediated IL-1 beta secretion. Here, Koebnerisin (S100A15) was able to interfere with pro-IL-1 beta and inflammasome regulation in keratinocytes and suppressed the UVB and IFN gamma-mediated IL-1 beta activation by these cells. Data suggest the UVB-mediated IL-1 beta production is controlled by antimicrobial peptides, which are co-regulated and dampen the UVB induced skin inflammation.

P074

Analogues of thyroid hormones (KB2115 and 3,3′,5-triiodothyroacetic acid) promote human hair growth

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Unwanted hair growth and loss affect quality of life of millions world-wide; therefore better understanding of the (patho)physiology of human hair follicles (HFs), and thereby identification of novel therapeutic approaches, are important fields of experimental dermatology. Human hair follicles (HFs) express thyroid hormone receptors (TRs) and respond to their stimulation, both *in vivo* and *in vitro*. We have previously shown that thyroid hormones (THs) are potent regulators of human HFs biology. For example, thyroxine prolongs the growth phase of the hair cycle (anagen), and stimulates HF pigmentation and mitochondrial energy metabolism. Therefore, TH analogues may be promising candidates for treating hair loss disorders based on premature HF regression (catagen entry), leading to telogen effluvium. In the current study we therefore investigated the effects of two TR-modulators, KB2115 and 3,3′,5-triiodothyroacetic acid (TRIAC) in organ-cultured human scalp HFs.

In striking contrast to THs, neither KB2115 nor TRIAC significantly affected HF immunoreactivity for mitochondrially encoded cytochrome C oxidase I, suggesting that these agents do not greatly affect HF energy metabolism. Interestingly, both KB2115 and TRIAC altered HF tyrosinase activity *in situ* in a highly donor-dependent manner, yet exerted only negligible effects on actual HF melanogenesis (Masson- Fontana). Although both substances tended to non-significantly inhibit hair shaft production *in vitro*, importantly, KB2115 and TRIAC significantly prolonged anagen (hair cycle histomorphometry). This corresponds to a slight increase in the number of proliferating cells in the hair matrix (Ki67/TUNEL), and was associated with the downregulation of transforming growth factor-β2, a key catagen-promoter.

Taken together, these findings strongly argue for that KB2115, TRIAC and probably other TR-modulators could be exploited, next in appropriate clinical trials, as promising, novel tools in the treatment of diseases characterized by premature catagen entry leading to telogen effluvium.

P075

Effects of extracellular calcium and 1,25 dihydroxyvitamin D3 on seborrhea and acne

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Calcium and 1,25-dihydroxyvitamin D3 are well-known promoters of epithelial cell functions; however their effects on the sebaceous gland and its diseases are not clearly elucidated. The *in vitro* part of our study was performed in order to evaluate extracellular calcium and 1,25-dihydroxyvitamin D3 effects on human sebocytes. In addition, a clinical study was conducted in order to evaluate Ca^{2+} and 1,25(OH)2D3 levels in acne patients and to elucidate the clinical relevance of the *in vitro* results. Morphology, ultrastructure, proliferation, lipid synthesis and apoptosis of SZ95 sebocytes were assessed *in vitro* under different concentrations of extracellular calcium (0.05–1.4 mM) with or without 1,25-dihydroxyvitamin D3 (10^{-9} and 10^{-7} M) at 24 and 72 h in culture. Serum Ca^{2+} and 1,25(OH)2D3 levels were assessed in 104 patients with acne (47 female and 57 male; 53% under the age of 25 years) by commercial assays. SZ95 sebocytes maintained at low extracellular calcium (0.05 mM) exhibited a rounded cell morphology, formed few loose colonies and tended to detach from culture plates. Numerous mitochondria, highly developed Golgi complex and several small to large lipid droplets consistent with active cell metabolism and lipogenesis were observed. In contrast, SZ95 sebocytes maintained at high extracellular calcium (1.4 mM) were polygonal, readily expanded and formed large compact colonies firmly adherent to culture plates, whereas lipid droplets were barely detected. Increasing extracellular calcium levels significantly enhanced SZ95 sebocyte numbers and reduced lipogenesis. Reducing extracellular calcium enhanced SZ95 sebocyte caspase 3/7 activity (apoptosis) and calcium chelation by EGTA resulted in enhanced lipogenesis. 1,25-dihydroxyvitamin D3 decreased sebaceous lipogenesis as shown by functional and ultrastructure studies. The latter also detected signs of autophagy in 1,25-dihydroxyvitamin D3-treated sebocytes. On the other hand, all patients tested exhibited serum Ca^{2+} levels inside the normal limits. In contrast, 81% of the acne patients presented at least 1,25-dihydroxyvitamin D3 insufficiency, whereas 47% of the patients were even deficient. More young acne patients presented 1,25-dihydroxyvitamin D3 deficiency (60%) in comparison to older ones (33%, Yates $P = 0.01$). In conclusion, extracellular calcium and 1,25-dihydroxyvitamin D3 regulate sebocyte morphology and increase cell growth but decrease sebaceous lipogenesis and induce cell autophagy *in vitro*. Interestingly, the majority of acne patients presented 1,25-dihydroxyvitamin D3 insufficiency and high rates of deficiency, especially the younger ones.

P076 (O01/06)

Vasoactive intestinal peptide (VIP) is a novel, complex neuroendocrine regulator of human HF melanocyte biology *in situ*

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Although vasoactive intestinal peptide (VIP) is one of the key neuropeptides released by perifollicular sensory nerve fibers, its role in human hair follicle (HF) biology is poorly understood. Here, we asked whether human scalp HFs express VIP receptors and whether VIP acts on human HF pigmentation *in situ*.

Indeed, VIP receptors are strongly expressed in the HF bulb but only slightly in the HF bulge, where VIP receptor 2 revealed the most prominent IR compared to VIP receptor 1.

When microdissected human HFs in serum-free organ culture were treated for 6 (mRNA analysis) or 72 h (protein analysis) with vehicle (supplemented Williams E medium) or VIP 10^{-7} M, quantitative (immuno-)histomorphometry and qRT-PCR analyses showed that VIP significantly decreases the melanin production in anagen HFs. However, intriguingly VIP up-regulates the total number of premelanosome protein (PMEL/gp100)+ HF melanocytes in the proximal human hair bulb but does not affect the number of MITF+ HF melanocytes, MITF transcription or tyrosinase activity *in situ* in the HF hair matrix. Interestingly, VIP significantly increases the number of intraepithelial c-Kit+ cells, including that of c-Kit+ HF melanocytes. Instead, VIP downregulates SCF protein expression *in situ* in the HF hair matrix. qRT-PCR analyses confirm these c-Kit and SCF expression responses to VIP on the transcriptional level.

Taken together, these results show that VIP operates as a novel, surprisingly complex neuroendocrine regulator of human HF pigmentation *in situ*, and likely impacts primarily on HF melanocyte survival via differentially regulating a key melanocyte growth factor (SCF) and its receptor (c-Kit) as well as PMEL. Therefore, VIP deserves to be fully explored as a novel HF melanocyte survival factor.

P077

Comparing patient-reported need for psychooncologic support and doctors' perspective: do they relate to disease severity in melanoma patients?

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An association between cancer outcomes and psychosocial strain may exist. However, directionality of this interaction and objective means to determine the possible association between psychosocial strain and disease onset, severity or progression have yet to be established. Here, we compare three patient-reported questionnaires with a doctor-reported questionnaire of psychooncologic strain, frequently used in routine cancer care. From October 2011 to December 2013 we enrolled 361 melanoma patients successively seen in outpatient care units at the universities of Giessen and Marburg, Germany. The naturalistic study included $n = 222$ recently diagnosed patients seen for the first time (Group I) and $n = 139$ patients diagnosed at least 6 months before enrolment (Group II). In our total melanoma cohort, hardly any association was seen between disease severity and self-reported need for psychooncologic support. Only a moderate association was seen between patient- and doctor-reported measures. Stratified analyses showed a small association between patient-report instruments and disease parameters commonly linked to more rapid cancer progression (tumour stage, positive lymph nodes in Group I; ulceration in Group II). In contrast, doctors reported a somewhat higher need for support for higher tumour stages in Group II. Our results suggest that physicians should discuss diagnostic elements linked to likely progression with their patients with great care. Further, patient self-report should be preferred over doctorreport to initiate psychooncologic interventions. However,

more precise instruments to measure psychosocial strain – preferably combined with biomarkers of stress and tumour immune surveillance – are required to determine a potential interaction between psychosocial strain and outcomes in melanoma patients.

Dermatopathology

P078

Stem cell factors and epigenetic modifications in cutaneous malignant melanoma: EZH2, SOX2, Oct4, H3K4me2 and H3K27me3 immunohistochemical expression patterns of inner tumor mass and tumor invasion front

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Accumulating evidence strongly suggests the presence and involvement of cancer cells with stem cell-like properties (CSCs) in the initiation, progression and metastasis of malignant melanoma. SOX2 and Oct4 represent crucial components of the reciprocal regulatory circuit that controls stemness. The misregulation of epigenetic modifications, such as H3K4me2 and H3K27me3 which constitute stem cell-like 'bivalent' domains, have been also identified to possess a crucial role in carcinogenesis. Polycomb-group proteins proteins and mainly EZH2 are responsible for maintaining the balance of the bivalent chromatin domain through the methylation of H3K27. The discovery that the epithelial to mesenchymal transition (EMT) generates cells with stem cell-like properties and a more invasive and metastatic phenotype, brings a connection between metastasis and the stem-cell state. According to this model, cells with stem cell properties are located predominantly at the tumor invasion front (IF) and can derive through the acquisition of transient EMT phenotype. Taking these data into account, we performed this study to evaluate the immunohistochemical expression of the stemness markers SOX2 and Oct4 and of the histone methyltransferase EZH2, as well as to examine the cellular levels of the posttranslational modifications H3K4me2 and H3K27me3 in cutaneous malignant melanoma, investigating besides the potential identification of cancer cells with stem cells properties at the tumor IF.

Immunostaining was performed on sections of 89 melanomas derived from 79 patients. In cases where staining was heterogeneous, as at the tumor IF, immunostaining was evaluated both for the inner tumor mass and the IF. EZH2, H3K4me2 and H3K27me3 were identified in the cell nuclei of melanoma cells, nevus cells, and normal keratinocytes, while the stemness markers SOX2 é Oct4 showed, in contrast to previous studies, nuclear and cytoplasmic expression. In general, a specific distribution pattern of H3K4me2 and H3K27me3 was found, as stronger levels were localized at the tumor IF compared to the inner tumor mass. A similar trend was also observed for EZH2, without achieving however statistical significance, and similarly for SOX2 in a few sporadic cases. H3K4me2, H3K27me3, nuclear SOX2 and cytoplasmic Oct4 levels presented significantly higher values in primary with respect to metastatic melanoma lesions. Advanced melanoma demonstrated significantly lower H3K4 immunohistochemical expression than did cases of lowest Clark level (I) or low Breslow depth. Significantly increased EZH2 expression was observed in melanoma compared to nevus cells, while nuclear Oct4 expression was found increased in nevus compared to melanoma cells. Remarkably occasionally increased Oct4 expression in endothelial cells of the tumor microvasculature in melanoma tissues was observed. Furthermore, statistical analysis revealed a positive correlation in melanoma cells between expression levels of EZH2 and H3K27me3, H3K4me2 and H3K27me3, as well as of the stemness markers Oct4 and SOX2.

Our results suggest the possibility that combined immunohistochemical expression of EZH2, SOX2, Oct4, H3K4me2 and H3K27me3 might identify cancer cells with potential stem cell properties, particularly at the invasion front of this malignancy. This hypothesis should be substantiated, as many of the epigenetic changes are druggable and new CSC-directed therapies, may hold promise for the treatment of malignant melanomas.

P079

Over-representation of IL-17E producing keratinocytes and IL-17E positive macrophages in the lesional skin of psoriatic patients

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Background: Psoriasis is a relatively common chronic immune-mediated disease associated with a severe decrease in health-related quality of life. Targeting interleukin (IL)-17A or IL-17RA, the common receptor subunit shared by several IL-17 family members (i.e. IL-17A, IL-17C, IL-17E, IL-17F), has proven to be highly efficacious to treat moderate-to-severe psoriasis in phase II and III clinical studies. While the role of IL-17A as key factor in psoriasis pathogenies is being unraveled, little is known about the roles exerted by other isoforms of the IL-17 family. In the present study, we aimed at assessing the contribution of IL-17 isoforms signaling via the IL-17RA subunit to psoriasis pathogenesis.

Methods: Formalin-fixed paraffin-embedded biopsies were prepared from 10 psoriasis lesional, 6 psoriasis non-lesional, and 8 normal skins. The cells positive for IL-17A, IL-17C, IL-17E and IL-17F, and the type of cells expressing the different IL-17 isoforms were assessed by immunohistochemistry (IHC) and multicolor immunofluorescence (IF) analysis, respectively. IHC and IF images were quantified using MetaMorph® and Definiens Tissue Studio Imaging softwares. *In situ* hybridization was used to determine the *in vivo* transcription of IL-17A and IL-17E genes. Levels of IL-17E mRNA and protein in skin biopsies were measured by RTPCR and western blot, respectively. Primary keratinocyte cultures were obtained from healthy individuals undergoing plastic surgery and their ability to produce IL-17E assessed by ELISA.

Results: IL-17A expressing cells were more numerous in the dermis of psoriatic lesions when compared to the non lesional ($P = 0.0415$) and healthy skin ($P = 0.0044$). IL-17E positive cells were increased in the dermis of both psoriatic lesions ($P = 0.0002$) and non-lesional skin ($P = 0.0047$) when compared to control skin, with the non-lesional skin having intermediate levels. IL-17E but not IL-17A positive cells in lesional skin positively correlated with the PASI score. No differences in the number of IL-17F and IL-17C expressing cells were observed among the three study groups. Determination of IL-17E levels in total protein extracts from skin biopsies were higher in lesional psoriasis compared to control skins. In lesional skin, IL-17A and IL-17E expressing cells accumulated within 50 μm downward the epidermis. In the dermis, IL-17A was mainly expressed by tryptase positive mast cells while IL-17E was mainly found in CD68 positive macrophages. CD3 positive T cells and myeloperoxidase positive neutrophils constituted only a minority of IL-17A and IL-17E expressing cells. An altered distribution and sustained overexpression of IL-17E was observed in the epidermal layer in lesional skin when compared to both non lesional ($P = 0.05$) and normal skin ($P = 0.0479$). Keratinocytes represented the major source of IL-17E in lesional skin, as revealed by their high expression of IL-17E mRNA *in vivo*. Of interest, an enhanced production of IL-17E was observed when keratinocytes from healthy individuals were cultured in presence of sera from psoriatic but not normal controls.

Conclusions: Psoriatic lesions contain an increased numbers of IL-17A positive mast cells and IL-17E positive macrophages which accumulate in the papillary dermis. In psoriatic epidermis, keratinocytes actively synthetize and over-express IL-17E. The increased expression of IL-17E, in addition to IL-17A, may contribute to psoriasis pathogenesis.

P080

Immunhistological differentiation of cutaneous acute graft-versus-host disease versus other severe cutaneous reactions

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Graft-versus-host disease (GvHD) represents one of the major complications after allogeneic hematopoietic stem cell transplantation. In addition to liver, the skin and the mucous membranes are its predilection sites. Diagnosis of cutaneous GvHD is complex because it is hard to discriminate from drug reactions and viral infections that often evoke similar clinical and histological symptoms. Specific immunohistochemical markers for cutaneous GvHD are still lacking. GvHD occurs when the recipient's tissue is damaged and host antigen presenting cells (APCs) are activated by inflammatory cytokines. Donor T cells are activated and then attack host cells like professional APCs and non-hematopoietic APCs. We have examined skin biopsies from 54 patients with acute GvHD (aGvHD, grade 1–3 Lerner classification) after stem cell and organ transplantation immunohistochemically. For comparison, we examined 27 biopsies from patients with Stevens-Johnson syndrome (SJS) or toxic epidermal necrolysis (TEN), 26 biopsies from patients with maculopapular druginduced exanthema (MDIE) and 10 healthy controls. The aim of this study was to identify an immunohistochemical marker-panel, which can be used for diagnostic purposes in cutaneous aGvHD. Prompt diagnosis of aGvHD is pivotal as it requires specific treatment different from those for the mentioned differential diagnoses. The number of CD1a+ dendritic cells (DCs) in the epidermis (Langerhans cells) and dermis was lower in aGvHD, SJS/TEN and MDIE in comparison to healthy skin; there was no obvious difference between the diseases. CD11c+ dermal DCs were only decreased in MDIE. CD20+ B cells were hardly present in all skin biopsies. No alterations were found in the number of CD68+ macrophages compared to healthy skin and among the differential diagnoses. Interestingly, CD56+ NK cells were increased in aGvHD and hardly found in healthy controls or samples from the other diseases investigated. We detected higher numbers of CD3+, CD4+ and CD8+ lymphocytes in the epidermis and dermis of biopsies from patients with aGvHD, whereas in healthy controls, SJS/TEN and MDIE these were low. In aGvHD, the CD4+ lymphocytes stained positive for FoxP3 indicating differentiation into effector/ regulatory T cells. A staining for cytokines such as IL-5, TGFbeta and IFNgamma revealed only increased levels of TGFbeta in all diseases compared to healthy controls. In summary, these results illustrate that a specific immunohistochemical marker panel to identify and differentiate aGvHD from its differential diagnoses can be established; however, an even more clear cut differentiation of T cells in aGvHD compared to SJS/TEN or MDIE needs to be performed.

P081

IL-1-related cytokines as potential biomarkers in autoinflammatory skin diseases

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Background: Urticarial rash is a hallmark symptom of autoinflammatory skin diseases (AISDs) such as Cryopyrin-associated periodic syndrome (CAPS) and Schnitzler's syndrome (SchS). Clinically, the urticarial rash in AISD patients is very similar to that in chronic urticaria patients. Interleukin-1β (IL-1β) has been shown to play a pivotal role in the pathogenesis of CAPS and SchS. As of yet, IL-1ß and related cytokines have not been investigated for their potential as diagnostic biomarkers in patients with AISDs.

Materials and methods: Immunohistochemical stainings (IL-1ß, IL-6, IL-18) of lesional skin of patients with CAPS ($n = 3$), SchS ($n = 13$) and chronic spontaneous urticaria (CSU) ($n = 11$) as well as healthy control skin samples ($n = 10$) were analyzed by semi-quantitative histomorphometry and compared with cutaneous protein concentrations assessed by ELISA.

Results: Semi-quantitative histomorphometry revealed neutrophil-dominated dermal cell infiltrates in skin samples of CAPS and SchS patients, but not CSU patients. Infiltrating inflammatory cells in the skin of CAPS and SchS patients showed strong cytoplasmatic immunoreactivity for IL-1ß, IL-6, and IL-18, which was much less pronounced in CSU and absent in healthy control samples. In addition, protein concentrations of all three cytokines were significantly higher in the skin of AISD patients as compared to healthy controls.

Conclusion: Our study demonstrates that IL-1-related cytokines are upregulated in the skin of urticarial autoinflammatory diseases and suggests to further explore these cytokines as diagnostic biomarkers in larger patient samples.

P082 (O03/04)

Role of the cholinergic system in cutaneous stress response

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Recent evidences indicate that cholinergic signaling in partnership with the immune system is important for a successful adaptation to physical and psychosocial stressors alike. Mast cells are prominent targets of stress-induced worsening in chronic inflammatory diseases such as atopic dermatitis. In this study, we analyzed the skin's cholinergic system in mice under inflammatory stress [experimental allergic dermatitis (AID)] and under psychosocial stress (24 h noise-stress). Analysis of mRNA expression by microarray showed a dysregulation of cytokine receptors and associated downstream signaling in stressed skin supportive of a pro-allergic inflammatory state. RTPCR results confirmed the dysregulation. Next we investigated the gene expression of key elements of the cholinergic system like the rate limiting enzyme for acetylcholine (ACh) synthesis – choline acetyltransferase (ChAT), vesicular ACh transporter (VAChT), nicotinic ACh receptor alpha 7 (Chrna7) and its endogenous ligand Secreted Ly-6/uPAR-related protein 1 (SLURP1). Compared to control skin, Chrna7 and SLURP1 were upregulated in noise-stressed mouse skin while in AID skin ChAT was downregulated. The combined exposure to noise-stress and AID increased VAChT and Chrna7 expression compared to AID. Treatment of noise-stressed AID mice with brain derived neurotrophic factor (BDNF)-neutralizing antibodies dramatically further upregulated VAChT, while administration of nerve growth factor (NGF)-neutralizing antibodies downregulated Chrna7 and upregulated SLURP1 expression. By immunohistomorphometry, expression of ChAT, VAChT, Chrna7 and SLURP1 was found in mast cells. Compared to control, Chrna7 + mast cell number was upregulated in stressed skin while ChAT and Chrna7 + mast cell number was attenuated in AID skin. The combination of AID and stress abolished the decrease of ChAT and Chrna7 + mast cells. Analysis of the expression of the pro-inflammatory cytokines tumor necrosis factor alpha (TNFa) and interleukin-1 beta (IL-1b) and the anti-inflammatory cytokines tumor growth factor beta (TGFb) and IL-10 in mast cells in stressed skin showed increased expression of IL-1b. In AID mouse back skin TNFa and IL-1b were upregulated while the anti-inflammatory cytokines were downregulated. Noise-stress exposure in combination with AID revised this expression pattern. Additional blocking of

neurotrophins decreased TNFa positive mast cells to control levels. Number of IL-10 positive mast cells was downregulated under anti-BDNF, whereas TGFb was downregulated under anti- NGF. Hence cholinergic signaling elements are low under conditions of AID that enhance pro-inflammatory cytokines and high in stress worsend AID. In the light of these results we conclude that cholinergic signaling is involved in cutaneous immune response to inflammatory as well as perceived stress. Mast cells emerge as a key player in AID-mediated dysregulation of the cholinergic system of the skin.

P083

Biased Th17-cell function in patients with cutaneous T-cell lymphomas: reasons and consequences

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Primary cutaneous T-cell lymphomas (CTCL) are neoplastic disorders of skin-homing T-cells. Affected skin areas of CTCL patients show morphological similarities with alterations in common T-cell-mediated dermatoses such as atopic dermatitis (AD) and psoriasis. Interestingly, like in AD but in contrast to psoriasis, CTCL patients are frequently afflicted by cutaneous bacterial infections. Those infections not only support survival and expansion of lymphoma cells but – in the context of a CTCLassociated impaired systemic T-cell repertoire – may also become life-threatening. Therefore, we aimed to investigate the mechanisms of elevated susceptibility to cutaneous infections in CTCL patients.

We demonstrate for the first time that CTCL lesions compared to psoriatic lesions show significantly lower levels of antibacterial proteins (ABPs). Importantly, the cutaneous ABP levels in affected skin of CTCL patients were even below those in AD. Analysis of cytokines able to regulate ABP production revealed a relative deficiency in ABP-inducing interleukin (IL)-17 and a strong presence of ABPdownregulating IL-13 in CTCL lesions. *In vitro* and *in vivo* search for further Th17- cell cytokines disclosed selective production of IL-26 by Th17-cells and, interestingly, the presence of this mediator in CTLC lesions. This indicated that IL-17 deficiency in CTCL lesions results from partial inhibition of Th17-cell function and not from absence of these cells. Accordingly, IL-17 but not IL-26 production was inhibited in Th17-cells by IL-4 receptor α ligand *in vitro*. In contrast to IL-4, IL-10, whose expression was also elevated in CTCL lesions, did not regulate IL-17A or IL-26 production in Th17-cells. Levels of other ABP-inducers such as TNF-α, IL-1β, IFN-γ and IL-22 were comparable between lesions of CTCL and psoriasis. The same was true regarding further IL-22/TNF-α targets, including the keratinocyte hyperregeneration marker K16 and the matrix-degrading enzyme MMP1. Interestingly, all T-cell cytokines present in CTCL lesions appeared to be produced by the skininfiltrating reactive T-cells rather than the tumor cells themselves.

In summary, our study suggests that the cutaneous bacterial infections in CTCL are caused by an impaired ABP production as consequence of a Th2-mediated biased Th17-function.

P084

High prevalence of axial spondyloarthropathy in patients with acne inversa

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Acne inversa (AI, also referred to as Hidradenitis suppurativa) is a chronic inflammatory disease of terminal hair follicles, which affects the intertrigenous skin of axillary, genitofemoral and perianal sites. It causes painful, fistulating sinuses with malodorous purulence and scars and is frequently accompanied by profound metabolic alterations. Since some chronic inflammatory diseases affecting epithelial tissues like psoriasis, Crohn's disease, and colitis ulcerosa are frequently associated with spondyloarthropathies (SpA) that strongly reduce the quality of life of respective patients, we asked for the association of AI and SpA.

When conducting a questionnaire survey among 100 AI patients, surprisingly about 70% of these patients stated suffering from back pain. A third of them even reported about very frequent or permanent back pain. The evaluation of anamnestic and clinical parameters revealed that there were no differences in age at onset of AI, disease duration, BMI, or disease severity between AI patients without back pain, patients with occasional pain, and patients with very frequent or permanent back pain. In order to identify the reason for the back pain of AI patients, we evaluated all magnetic resonance imaging (MRI) scans of the pelvis in 26 men and 20 women suffering from AI. Importantly, about 32% of these patients showed clear signs of chronic SpA in MRI and a further 24% showed active inflammatory signs for SpA. There were no significant differences between patients with and without SpA regarding the following parameters: age at time of MRI, age at onset of AI, disease duration, smoking habits, BMI. Furthermore, there was no correlation between these parameters and the degree of SpA.

In summary our study demonstrates that SpA is very common among AI patients and that neither history nor clinic parameters of AI provide any hints for the presence of SpA. Furthermore, it strongly suggests that AI patients should be evaluated for SpA presence and affected patients should be systemically treated (e.g. TNF-alpha blockers) in order to avoid chronic joint alterations.

P085

Stem cells and cancer stem cells of palms and soles

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Introduction: The epidermis is maintaned by stem cells that residue in the pilosebaceous unit as well as the interfollicular epidermis with lineage plasticity upon regeneration. However, the palms and soles lack the pilosbaceous unit. It has been supposed that sweat ducts contain multipotent progenitors that contribute to homeostasis and regeneration in mouse paws. The biology in human palms and soles are poorly understood including which cells give rise to epidermal skin tumours that appear at palms and soles.

Methods: We used BrdU pulse injections to detect label-retaining cells within the paws of C57Bl/6 mice. A group of 6 10-day-old mice were injected with 50 mg/kg bodyweight BrdU every 12 h for a total of four injections to label mitotic cells. Another group of 6 mice were not injected. 66 days later mice were collected and the group that did not receive BrdU pulse treatment at day 10 received BrdU 50 mg/kg bodyweight 1 h prior sacrifice. In another experiment we painted either TPA 6 nmol/l in Acetone or Acetone onto tail, paws and back skin of 2 × 6 two months old C57Bl/6 mice 3×/week for 3 weeks. Finally we stained treated mouse tail, back skin and paw skin as well as human Morbus Bowen, squamous cell carcinomas and HPV acanthamas and unaffected skin of palms and soles. We stained label retaining cells for CK14, CK15, Lrig1, a6- and b4-Integrin, b1-Integrin, Lgr6, CK7, EMA and CEA.

Results: We observed that mainly CK14 and CK15 which can be found in the interfollicular epidermis and in sweat gland ducts were highly upregulated in proliferating mouse paw skin and detected label retaining cells in mouse paws and proliferative skin induced by TPA. Typical markers for sweat glands such as EMA and CEA did not label LRC or was detected in human skin tumours.

Conclusion: Biology of skin cancer and skin homeostasis is different between skin of palms and soles from skin conatining the pilosebaceous unit.

Epidemiology

P086 (O05/06)

How to assess infant atopic dermatitis in a birth cohort study – experiences from the Ulm SPATZ Health Study

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Introduction: Previous systematic reviews on instruments assessing clinical signs and on definition of an incident case of atopic dermatitis (AD) in controlled trials have aimed at providing guidance on how to assess AD. Patient-assessed instruments for AD severity were found to have limited validity and the suggested questions to assess AD symptoms cover locations of the body which are not necessarily typical for the disease in the first year of life. Most observational studies in infancy will use these questions on symptoms and/or reported doctor's diagnosis to assess AD. We aimed to determine the agreement between parent- and physician-reported AD diagnosis in infancy in a population-based birth cohort study. A further objective was to investigate the potential of an additional clinical examination to reduce misclassification of reported diagnoses.

Methods: In the Ulm SPATZ Health Study, 1006 newborns and their 970 mothers (49% of eligible families) were recruited during their hospital stay after delivery in the University Medical Center Ulm, Southern Germany, between 04/2012 and 05/2013. Follow-ups at 6 months and 1 year were conducted. In the first year of life, AD symptoms were assessed by self-administered parental questionnaires during both follow-ups, a reported doctor's diagnosis only at 1 year. Children, for whom parents reported a constant or variable itchy rash over at least 4 weeks, generally dry skin, or flexural rash were invited for a dermatological physical examination by trained dermatologists. At age 1 year, in addition to the parents, the children's primary care physicians answered a separate self-administered questionnaire on diagnoses in the first year of life including AD.

Results: The agreement between parent- and physician-reported diagnosis was moderate with a kappa of 0.62. None of the assessed symptoms (itchy rash, flexural rash, or generally dry skin) had high sensitivity and specificity for either parent- or physician-reported diagnosis of atopic dermatitis. Fifty-five (46%) children attended the dermatological examination after invitation following the 6 month follow-up at a median age of 7.9 months. These children were seen prior to sending their 1 year questionnaire. In total, 38% of those examined were diagnosed with atopic dermatitis by the trained dermatologists. This diagnosis of atopic dermatitis was not reported by 40% of the parents and 40% of the physicians in the 1 year questionnaire.

Discussion: The moderate overlap between parent- and physician-reported diagnosis as well as the high proportion of parents who did not recall a diagnosis made about 4 months prior to answering the questionnaire suggests important potential for misclassification using either method to assess AD. Given the heterogeneity of symptoms of AD and location on the skin in the first year of life, screening for AD assessing self-reported symptoms also seems an incomplete option. Future followups in the Ulm SPATZ Health study using all modes of assessment may allow determination of specific and sensitive combinations of indicators for AD.

P087

Psoriasis and cardiometabolic risk: independent association but distinct genetic architectures

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Psoriasis has been linked to cardiometabolic traits, but results from epidemiological studies are inconsistent, and it has been suggested that the co-occurrence is due to shared genetic risk factors. Therefore we investigate associations and a potential genetic overlap of psoriasis and cardiometabolic outcomes.

The association between psoriasis and cardiovascular risk was analysed in the KORA population-based cross-sectional study (n = 4185) and in a prospective cohort of German National Health Insurance beneficiaries (n = 1 811 098). To study a potential genetic overlap genome-wide data from >22 000 coronary artery disease (CAD) cases and >4000 psoriasis cases were explored, and a dense genotyping study of cardiometabolic risk loci on 927 psoriasis cases and 3717 controls was performed.

Controlling for major traditional risk factors, in the cross-sectional analysis psoriasis was significantly associated with type 2 diabetes (T2D, OR = 2.36; 95%CI = 1.26–4.41) and myocardial infarction (MI, OR = 2.26, 95% CI = 1.03–4.96). Likewise, in the longitudinal analysis patients with psoriasis (n = 44 623) were at increased risk for T2D (RR = 1.11; 95%CI = 1.08–1.14) and MI (RR = 1.14; 95%CI = 1.06–1.22) during the 6 year observation period. Risk increments were significantly higher in psoriasis patients who received a systemic therapy. There were no significant associations of established psoriasis susceptibility loci with CAD nor vice versa, and the dense genotyping study did not indicate a role of cardiometabolic risk loci in psoriasis.

Psoriasis, in particular severe psoriasis, is a risk factor for T2D and MI, its genetic architecture is distinct from cardiometabolic traits.

P088 (O04/06)

Chronic itch in hemodialysis: new data from the German Epidemiological Hemodialysis Itch Study (GEHIS) study

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Chronic itch (CI) is a frequent and often underestimated symptom in hemodialysis (HD) patients but epidemiological data are still missing. As the number of HD patients will continuously increase due to the demographic situation and increased survival of patients with end-stage renal disease, CI in dialysis is a field of particular interest. GEHIS (German Epidemiological Hemodialysis Itch Study) was established in 2012 as a prospective observational prevalence study. This cross-sectional study investigated the prevalence of CI in HD patients and identified associated factors. 860 HD patients from a randomly selected cluster-sample were included. The primary outcome measures were current CI, CI occurring during the last 12 months and CI ever in life. Secondary outcome measures were characteristics of CI such as severity [measured by visual analogue scale (VAS)] and localization, generic healthrelated quality of life (HRQOL), itch-related quality of life (assessed by ItchyQol) as well as dialysis characteristics (dialysis efficiency and membranes, laboratory parameters). The mean age of HD patients was 67.2 years (standard deviation ± 13.5 years). The point prevalence of CI was 25.2% with a 95% confidence interval (95%-CI) of 22.4 to 28.1, the lifetime prevalence was 35.2% (95%-CI 31.9–38.3). A history of dry skin and eczema were significantly associated with CI. There was a significant association of the time since HD treatment started and the occurrence of CI. CI correlated significantly with two body locations (back and lower limb). Using the clinical classification for CI according to the international forum for the study of itch (IFSI), 18.6% (n = 33) of 177 patients showed specific dermatoses (IFSI I), 43.5% (n = 77) had a clinically normal appearing skin (IFSI II) and 37.9% (n = 67) showed secondary scratch lesions on the skin (IFSI III). There was no significant association between the duration of CI and the clinical status of the skin according to the IFSI classification. Interestingly, 22.6% of all affected patients had ever consulted a dermatologist, 80% of whom were classified according to IFSI III. Those patients had the highest VAS scores and the worst ItchyQoL scores. Patients dialyzed with polyarylethersulfone-membrane showed significantly more CI in all prevalence estimates, while those with polysulfone-membrane were significantly less affected by CI. Xerosis cutis was the most frequent dermatological finding (89.8%). We found evidence that the time since dialysis treatment started was associated with a higher prevalence of CI. GEHIS also showed HD patients suffering from CI to have a significantly reduced HRQOL and a significantly reduced general health status. More than half of the HD patients with CI reported difficulties with falling asleep more than once a week. Impaired quality of sleep was significantly associated with CI, but not with itch severity. This is the first representative cross-sectional study investigating precise prevalence estimates of CI and associated factors in HD patients. Also GEHIS is the first study ever that classified HD patients suffering from CI according to the IFSI classification. The study clearly demonstrates that CI is still a major burden in this patient population.

P089

Frequency of malignant tumors in the acute hepatic porphyrias

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The porphyrias are a group of predominantly inherited metabolic diseases resulting from an enzymatic deficiency of one of the eight enzymes along the heme biosynthetic pathway. They can be classified in acute and non-acute or in cutaneous and non-cutaneous types. Clinically, the acute hepatic porphyrias (AHPs) can manifest with life-threatening acute neurovisceral attacks and blistering photosensitivity on the sun-exposed areas of the body. An important complication of the AHPs is the development of hepatocellular carcinoma (HCC), the most common malignant primary liver tumor. However, systematic studies on the occurrence of other malignancies in patients with the AHPs have not been performed to date. Here, we studied the development of HCC and distinct malignant tumors in patients with the AHPs registered in a single European porphyria specialist center. A questionnaire was designed and sent to all individuals (n = 122) diagnosed between 1970 and 2012 of whom a valid address was available (n = 82), requesting information on their personal and family history of cancer. Statistical analysis was performed to calculate incidence, prevalence and relative risk of HCC. To calculate confidence intervals, a Poisson distribution was assumed. 49 patients (59.8%) returned a completed questionnaire. Overall, HCC was diagnosed in one female (2.1%) and the remaining patients reported on six distinct malignancies. We were able to confirm that HCC is an important complication in AHP. The patients in our cohort had an approximately 35-fold increased risk of developing HCC. In addition, we detected colon, breast, uterine and thyroid cancer as well as lymphoma and a liver metastasis in patients with AHP. Given the relatively small number of patients and tumors studied here, further studies of HCC and other cancers in AHP patients should be performed in a multicenter setting.

P090

Antihistamine use in patients with chronic hand eczema: an analysis based on data from the German carpe registry

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Antihistamines (AH) are often used to treat chronic skin diseases, especially when they are related to allergy and/or itch. There is no evidence base for the use of AH in patients with hand eczema (HE). Nevertheless, data from the carpe registry (German acronym: Chronisches Handekzem-Register zum Patienten-Langzeitmanagement) show that about one quarter of patients with chronic hand eczema (CHE) report having used AH. It is unclear which factors are related to the use of AH in these patients. Therefore, the aim of the analysis presented here was to identify factors associated with AH use in patients with CHE.

Relevant data of CHE patients were assessed by means of a patient and a physician questionnaire as well as a dermatological examination. AH use in the past 12 months was considered as outcome variable. Clinical, demographic and treatment-related variables were considered as predictive factors. Initially, univariate analyses were conducted. Variables that showed significant associations in univariate analysis were entered into a multivariable binary logistic regression model. Odds ratios (OR) with corresponding 95% confidence intervals (CIs) were computed. Analyses were done using SPSS.

A total of 1255 patients with CHE were eligible for this analysis (54.1% female; mean age: 47.1 (±13.6) years; mean disease duration: 6.3 (±8.0) years). Significant positive associations with AH use were identified for moderate (OR = 3.05, 95% CI 1.81–5.15) or severe (OR = 4.27, 95% CI 2.40–7.59) pruritus, a history of systemic treatment excluding AH within the last 12 months (e.g. corticosteroids, cyclosporine, alitretinoin) (OR = 2.85, 95% CI: 2.06–3.96), UV phototherapy (OR = 1.78, 95% CI 1.28–2.46), flexural eczema (OR = 1.89, 95% CI 1.32–2.71), allergic rhinitis/ conjunctivitis (OR = 2.41, 95% CI 1.71–3.39), and female gender (OR = 1.58, 95% CI 1.16–2.14) in multivariate analysis (N = 1184). Significant inverse associations were found for an eczema localization besides the hands (OR = 0.66, 95% CI 0.46–0.94) and for patients being recruited in hospital (versus dermatological practice; OR = 0.72, 95% CI 0.33–0.67).

This cross-sectional study suggests that AH use is frequent in patients with HE and mainly related to reported systemic and UV treatment, pruritus and co-morbid atopic disease. Further studies are required to define a subset of HE patients that receive AH for the treatment of HE (and not an atopic comorbidity) and to investigate the effectiveness of AH in this population.

P091

UV dependence – an artifact?

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Recently more and more studies have reported high prevalence rates for a 'UV dependence' among sunbed users. The authors of these studies base their argumentation on a modified (m) version of the CAGE (Cut-down, Annoyed, Guilty and Eye-opener) Criteria, initially used as screening instrument for alcohol addiction. By means of cognitive interviews and a large population survey, we tested the validity of the mCAGE Criteria and the abovementioned prevalence that was deduced on the basis of rather small collectives.

Firstly, it seems that the mCAGE Criteria wording used so far is inconsistent, misleading and intrinsically invalid. Secondly, our population-based data show a much lower percentage (15%) of current sunbed users with potential dependence symptoms than the abovementioned previously published studies. Thirdly, the usage parameters for most of the supposed 'addicts' do not indicate a substance addition. 38% of the users with positive scores reported not having visited a tanning studio at all in the previous month, 39% did not use sunbeds regularly and 89% did not show objective signs of tolerance to UV radiation. The mCAGE Criteria do not seem suitable for assessing tanning dependence.

P092

Comparison of different methods for the assessment of skin aging parameters: exploratory study in 83 female patients

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Background: Assessment of skin aging is mostly done by clinical inspection which is poorly reproducible. The goal of our study was to establish a simple, but, highly reproducible method to determine the condition of the skin based on objective biometric data.

Methods: We evaluated different methods for measuring skin aging parameters: elastometry, corneometry, sebumetry (Cutometer MPA580, Courage + Khazaka), colorimetry (CR 300 Chromameter), and sonography (Esaote MyLab2, Esaote) in a group of female test persons. Inclusion criteria were: skin type I–IV, age 20–60 years, BMI <28, no cosmetic procedures in the past. Smokers or former smokers were excluded. We defined 16 subgroups, one for each decade and skin type. The total of all test persons was distributed evenly among these subgroups. Parameters were assessed in well-defined areas: temple (T), cheek (C), volar site of the upper arm (A), and buttock (B). Before measurement the test persons cleaned the skin with Octenisept (Schülke & Mayr) and ran through an acclimatization period of 30 min (T = 24.8°C, relative humidity = 39.6%). Elastometry was measured, both, as elastic deformation after 1suction step and as stiffness by a repetitive course of 10 suction steps. Skin surface moisture and sebum is given as technical unit calibrated for cutometer MPA580. By using a 20 MHz ultrasound transducer we measured overall thickness of the cutis (mm) and sub-epidermal low echogenic band (SLEB in mm). The colour of the skin in the test areas was measured in three different positions and is given as individual typology angle (ITA).

As a second part of the study we invited 8 women (4 experts, 4 lay) to form a jury. After 1 min inspection time, the jury had to estimate age, degree of aging and aging parameters had to be specified by using a 4 point aging scale. All areas except the face of the test person were covered.

Results: 83 subjects participated: 22 in subgroup 20–29 (mean 23.3) years, 20 each in the subgroups 30–39 (mean 34.6) and 40–49 (mean 42.6), and 21 in subgroups 50–60 (mean 54.4) years of age. The respective numbers for the sub-groups with skin type I, II, III, and IV were: 20, 25, 22, and 16 test persons. Clinical examination showed that coarse wrinkles, fine wrinkles and lentigines increased significantly with age, especially in T and C and decreased significantly in subjects with darker skin types (skin type III, IV). Elasticity decreased with increasing age in all measured areas and for all skin photo types, significantly in area C and A. However, we found no correlation between the number of coarse/fine wrinkles and elasticity. Surprisingly, corneometry showed higher skin moisture values with increasing age, statistically significant in B. The SLEB could only be detected in the UV-exposed areas (T, C) most significantly in patients with high cumulative UV exposure. The skin colour decreased significantly with age in C. 17 of the 83 subjects participated in the jury review. The number of participants was well-balanced with respect to age and skin type. Although experts listed more skin aging parameters, especially coarse/fine wrinkles and lentigines, in term of the overall assessment we found no significant difference between experts and laymen. Both, experts and laymen overestimated the age of test persons with sun-damaged skin.

Conclusion: Elastometry and sonography are excellent skin aging parameters. Loss of elasticity describes overall skin aging whereas SLEB is very specific for actinic skin damage. However, to get a universally universally applicable numeric equivalent for skin aging it appears necessary to combine different parameters.

Genetics

P093

Analysis of binding characteristics of RNA trans-splicing molecules using a fluorescence-based screening system

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Epidermolysis bullosa (EB) is a monogenetic mechano-bullous skin disease which occurs due to mutations in genes expressed in the basement membrane zone of the skin, including COL7A1, COL17A1, PLEC, K5 or K14. These alterations on genomic level lead to absence or misexpression of the respective protein with subsequent blister formation of the skin and mucous membranes. To correct a wide range of disease-associated mutations, we use a pre-mRNA-based repair strategy, called RNA trans-splicing, with the aim to replace 5′, 3′ or internal pre-mRNA regions of selected EB-associated genes. For this, we developed a fluorescence-based screening method which allows us to test the efficiency of randomly designed RNA trans-splicing molecules (RTMs) out of a large pool of variants. Co-transfections of gene-specific target molecules and individual RTMs into HEK293 cells lead to the recombination of both molecules by RNA trans-splicing manifested in the expression of full-length GFP which was provided in parts by both screening molecules. In our system, a high GFP expression due to accurate trans-splicing correlates with a high efficiency of the RTM, which can be measured by flow cytometric analysis. In order to improve our RTM screening system we have generated cell lines stably expressing a target minigene containing the RTM binding regions of interest. The stable expression of target minigenes of the EB-associated genes PLEC, COL17A1, COL7A1 and K5 significantly facilitates the selection and design of highly efficient RTMs prior to endogenous experiments. After RTM library introduction into the target cell lines we compared and analysed cells

expressing GFP in low and high levels. RTM binding domains, which are predominantly expressed in GFP-high positive cell populations, were sequenced and analysed revealing a tendency of preferred target regions for RTM binding. We assume that these regions represent the most potent binding sequences for efficient trans-splicing. Our screening system thus accelerates the construction of highly functional RTMs for endogenous RNA repair approaches.

P094

Functional characterization of the multifunctional XPG protein during nucleotide-excision-repair

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Xeroderma pigmentosum (XP), a cancer model disease, is the perfect proof for the existing model of carcinogenesis activated by mutations. All patients share a defect in Nucleotide excision repair (NER). The gene, which is disease-causing for XP complementation group G (XPG) patients, encodes for the multifunctional endonuclease XPG. This enzyme has many binding partners like TFIIH, RPA and PCNA, and acts at a crucial step at the very end of NER. Several functional domains of XPG were mutated to investigate the behavior of the respective mutants during NER intermediates of dual incision, using DNA repair synthesis (UDS) and Host cell reactivation (HCR) assays. Furthermore, a new XPG patient with implications for the functional XPG-TFIIH interaction has been studied. By genotype-phenotype correlation of a XPG patient (XP172MA), this study greatly suggests to narrow down the functionally important XPG interaction domain between TFIIH and XPG to the XPG amino-acids 30–85. This study demonstrates that the functional PCNA-XPG interaction is more important for NER than the endonuclease function of XPG. The C-terminally located PIP-box of XPG is required for immediate UV response but not for the functionality of XPG during NER in transiently transfected primary fibroblasts. The N-terminal PIP-UBM ubiquitin binding domain is more important for integrity of NER than the C-terminal PIP-box. I raise the model of an NER intermediate state that involves obligatory ubiquitination during NER and the blocking of error-prone translesion polymerases by XPG. This study excludes XPG as the responsible factor for PCNA recruitment and designates XPG as the factor as restrictive element for UV-damage dependent activation of translesion polymerases to S-phase. The results obtained with the endonuclease defective E791A XPG mutant confirm the actual 'cut-patch-cat-patch' model of dual incision during NER. Moreover, this study clearly demonstrates the ability of endonuclease defective XPG to perform accurate NER in living cells. This accounts for the existence of a cellular backup mechanism for the XPG endonuclease function.

The proposal for a nuclear backup mechanism is supported by the investigation of a physiologically relevant (evolutionary developed) XPG splicevariant with NER activity (IsoVI). The severely truncated XPG isoform is able to structurally complement a XPG defect. This complementation is dependent on the endonuclease function of Fen1. This suggests the existence of an evolutionary developed backup mechanism for XPG during NER.

P095

Establishment of two mouse models for CEDNIK syndrome reveals the pivotal role of SNAP29 in epidermal differentiation

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Loss-of-function mutations in the SNAP29 gene cause CEDNIK (CErebral Dysgenesis, Neuropathy, Ichthyosis, Keratoderma) syndrome, a rare human genodermatosis, associated with a severe ichthyosis phenotype. In this study, we created two Snap29 deficient mouse models using the Cre-loxP system to investigate the role of SNAP29 in epidermal differentiation. Knock-out mice exhibited a congenital ichthyotic phenotype resulting in neonatal lethality. Histological examinations revealed para-hyperkeratosis and acanthosis as well as a reduced number of hair follicles. Using immunohistochemistry we found altered expression of keratinocyte differentiation markers, accelerated levels of proliferation, decreased amounts of lamellar granules, an altered level of a trans-Golgi network and lamellar body markers, and a disturbed epidermal barrier. Examination by electron microscopy showed a disturbed maturation and secretion of lamellar bodies. Our mouse models provide insights into the contribution of SNAP29 to the development of a functional epidermis.

P096

Identification of putative gene networks caused by variation of mitochondrial ATP8 gene in an inflammatory skin disease mouse model

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Recent studies have shown that not only mutations in protein-coding genes contribute to disease phenotypes. Therefore to better understand the molecular basis of disease phenotypes it is necessary to study the gene networks involving proteincoding genes and non-coding regions due to their intertwined relationship.

Mitochondria are cellular organelles that control many cellular pathways, including cellular proliferation, activation and apoptosis. Mitochondria carry their own genome (mitochondrial DNA; mtDNA) and variations in the mtDNA are known to cause mitochondrial dysfunction which results in disease phenotypes. The mitochondrial ATP8 gene, one of 13 protein-coding mitochondrial genes, has been reported to be associated with various autoimmune diseases. Polymorphisms in the ATP8 are associated with autoimmune blistering skin diseases and multiple sclerosis in humans. In addition experimental evidence demonstrated that mice carrying an ATP8 mutation showed altered disease susceptibility in rheumatoid arthritis and lupus nephritis models. However, the involved pathways and gene regulation networks are yet to be elucidated.

Therefore, in this work we aimed at identified putative pathways connected with variations in the mitochondrial ATP8 gene in a murine skin inflammation model using integrative genomics and genetics.

We obtained gene expression data for miRNA (265 miRNAs, 100 mice) and mRNA (1276 mRNAs, 190 mice) from 4-way advanced inter-cross mouse line immunized with type VII collagen. Additionally, the mice cohort was genotyped using an Illumina SNP Array carrying 1400 SNPs covering the full mouse genome. An eQTL study was performed in order to identify associations between genotype and expression data (miRNA and mRNAs) using the additive model implemented in R package 'happy'. Moreover, using the same gene expression data we generated clusters of genes by co-expression analysis (R package 'WGCNA'). Each gene cluster was correlated with the ATP8 genotype. We found two clusters of genes that were significantly correlated with the wild type allele of the ATP8 gene (one positively and one negatively correlated). The group of genes that had higher gene expression in ATP8 wild types was significantly associated with immune cell-signaling pathways such as EIF2 signaling, CD28 signaling in T-helper cells and fMLP signaling in neutrophils. Next, we integrated our eQTL data with an externally curated gene interaction database (Ingenuity Pathway Analysis, IPA) to identify a putative network related to the gene cluster in the co-expression analysis and found that the EED gene, TFAM gene, Akt gene and the PPARA gene play a central role to connect the ATP8 gene with the identified pathways of the network.

In summary, for the first time, we present a putative gene network integrating the mitochondrial ATP8 gene in the signaling pathways of an inflammatory skin disease in mice.

P097 (O01/05)

Meta-analysis of genome-wide association studies in alopecia areata resolves HLA associations and reveals two new susceptibility loci

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Alopecia areata (AA) is one of the most prevalent autoimmune diseases, with ten known susceptibility loci so far. Here, we performed a first meta-analysis in AA by combining data from two GWAS, and replication with supplemented ImmunoChip data for a total of 3253 cases and 7543 controls. The strongest region of association was the MHC, where we fine-mapped 4 independent effects, all implicating HLA-DR as a key etiologic driver. Outside the MHC, we identified two novel loci that exceed statistical significance, containing ACOXL/BCL2L11(BIM) (2q13); LRRC32 (GARP) (11q13.5), as well as a third region that achieved nominal significance SH2B3(LNK)/ATXN2 (12q24.12). Expression analysis of candidate susceptibility genes in these three regions demonstrates expression in relevant immune cells, as well as in the hair follicle. Finally, we performed a cross phenotype meta-analysis integrating our meta-analysis with data from seven other autoimmune diseases, providing insight into the molecular taxonomy of autoimmune diseases and the alignment of AA within this class of disorders. Taken together, our findings have uncovered novel functional pathways that are disrupted in AA, including autophagy/apoptosis, TGFß/Tregs and JAK kinase signaling, lending further support the causal role of aberrant immune processes in AA.

P098

Whole-exome sequencing identifies NIPAL4, SPINK5 and FLG genetic variants associated with ichthyosis and episodes of dermatitis

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The barrier function of the skin is mainly dependent on terminally differentiated keratinocytes, which are dynamically and regularly desquamated. This complex process is regulated by numerous molecular players, and mutations in the corresponding genes result in ichthyoses or atopic dermatitis. Using whole-exome sequencing, we performed a comprehensive genetic analysis in a young male displaying ichthyosis with allergic sensitizations, high IgE levels and episodes of dermatitis. Histopathologic and ultrastructural findings of the patient's skin were not specific. We showed that besides the NIPAL4 mutation c.527C>A, p.A176D which caused the ichthyosis, additional disease modifying variants in the genes encoding LEKTI and filaggrin contribute to the allergic manifestations in this case. Importantly, although the identified variants, p.E420K and p.S368N in LEKTI, and p.P478S in filaggrin, have been associated with atopic dermatitis in large-scale studies, they did not cause any cutaneous disease in the parents or siblings of our patient, but became relevant modifiers only in the context of the major keratinization defect present in the patient. To functionally characterize this mutation constellation, we performed in situ and *in vitro* studies. We observed an irregular distribution of LEKTI and filaggrin within the granular and upper spinous layers of the patient's skin, contrasting with the well-demarcated localization in the control skin. *In situ* zymographies demonstrated a moderate increase in protease activity in the patient's epidermis as compared to the healthy control, but lower than in LEKTI-negative skin of a patient with Netherton syndrome. In line with these results, immunoblot and quantitative real time PCR demonstrated strong reduction of LEKTI protein and mRNA, and FLG mRNA in the patient's keratinocytes compared to control cells. The decrease of the LEKTI expression combined with the heterozygous variant p.E420K results in strong reduction of the LEKTI proteolytic fragment D6D9 which was associated with susceptibility to atopic dermatitis. Our findings support the idea that a complex interplay exists between mutations and functional variants in genes for proteins involved in epidermal differentiation, resulting in a spectrum of barrier function defects and allergic manifestations.

P099

Targeted resequencing and finemapping identifies low-frequency missense variants in LRRC32 as risk factors for atopic dermatitis

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Genome-wide association studies (GWAS) on atopic dermatitis (AD) and related traits consistently identified a common intergenic susceptibility variant on chromosome 11q13.5. In order to refine the association signal we performed targeted resequencing of the 11q13.5 region (chr11:75 800 000–

76 070 000) in 31 earlyonset and severe AD cases enriched for low frequency risk haplotypes. 2 missense single nucleotide variants (SNVs) within LRRC32 without annotation in dbSNP and/ or a MAF<0.001 in 1000G were identified. Extended mutational screening of the coding regions of LRRC32 in 100 AD cases identified an additional 4 rare missense SNVs. Association analysis in 1000 AD cases vs. 1000 controls revealed a significant excess of low-frequency LRRC32 variants in individuals with AD ($P = 0.0007$; OR 1.98). Structural protein modelling of GARP, the protein encoded by LRRC32, predicted effects of these SNVs on protein stability and/or conformational changes. Functional overexpression assays of wildtype and mutated GARP in different cell lines are currently being performed. Our data link LRRC32 as causative gene to the etiopathology of AD, and indicate that low-frequency variants in LRRC32 compromise the function of GARP.

P100

Comparative genomics identifies filaggrin-deficient species of mammals

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Filaggrin is a critical protein of the mammalian skin barrier. Deleterious mutations of the filaggrin (FLG) gene are associated with ichthyosis vulgaris and atopic dermatitis. To get insights into the biological roles of filaggrin, we determined the conservation or loss of FLG during the evolutionary adaptation of animals to distinct environments and lifestyles. By comparative genomics, we show that FLG is absent from non-mammalian species and present in phylogenetically diverse mammals, indicating an evolutionary origin of filaggrin in primitive mammals after their evolutionary divergence from the lineage leading to modern reptiles. Among mammals, filaggrin was conserved in all species with sequenced genomes except for whales. Interestingly, other fully aquatic species such as dolphins and the orca have retained an apparently intact FLG gene. Both whales and dolphins have lost caspase-14, a protease implicated in the processing of filaggrin to components of the so-called natural moisturizing factor of the stratum corneum in terrestrial mammals. Our study suggests that comparative genomics can complement gene knockout studies in mice to correlate gene deficiencies and skin phenotypes.

Health Services Research

P101

Patient preferences for treatment of psoriasis with biologicals: a discrete choice experiment

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Background: Treatment dissatisfaction and non-adherence are common among patients with psoriasis, partly due to discordance between individual preferences and recommended treatments. However, patients are more satisfied with biologicals than with other treatments.

Objective: Our aim was to assess preferences of patients with moderate-to-severe psoriasis for treatment of psoriasis with biologicals by using conjoint analysis, a method closely reflecting clinical decision-making.

Methods: All biologicals currently approved for treatment of psoriasis in Germany were decomposed into outcome (probability of PASI 50 and PASI 90 response, time until response, sustainability of success, probability of mild and severe adverse events (AE), probability of ACR20 response) and process attributes (treatment location, frequency, duration and delivery method) and attribute levels. Theoretical treatment options based on random combinations of the identified attributes and levels were created using commercially available software (www.sawtoothsoftware.com). Participants ($n = 200$) were repetitively asked to choose their preferred option among pairwise presented scenarios. Relative Importance Scores (RIS) of each attribute were calculated individually for each participant and averaged across the sample. Associations of sociodemographic and socioeconomic characteristics, disease severity and comorbidities with RIS were assessed by analysis of variance, *post hoc* tests, and multivariate regression.

Results: Probability of severe AE was regarded as most important (RIS = 17.3), followed by probability of PASI 90 response (RIS = 14.0) and probability of mild AE (RIS = 10.5). Process attributes reached intermediate RIS (8.2–8.8). Women attached less importance to PASI 50 response (RIS = 6.9 vs. 9.5, $P = 0.008$; $\beta = -0.191$, $P = 0.011$ in multivariate regression models) and PASI 90 response (RIS = 12.1 vs. 15.4, $P = 0.002$; $\beta = -0.197$, $P = 0.009$) than men. Older participants judged the probability of PASI 50 and 90 response less relevant than younger ones (PASI 50: Pearson's Correlation (PC) = −0.161, $P = 0.022$; $\beta = -0.219$, $P = 0.017$; PASI 90: PC = −0.155, $P = 0.028$; $\beta = -0.264$, $P = 0.004$) but worried more about severe AE (PC = 0.175, $P = 0.013$; $\beta = 0.166$, $P = 0.082$). 22.5% of the participants had psoriatic arthritis, 15% cardiovascular disease, 14.5% diabetes and 12.5% depression. Participants with psoriatic arthritis were particularly interested in probability of ACR 20 response (RIS = 10.3 vs. 5.0, $P < 0.001$; $\beta = 0.278$, $P < 0.001$) and sustainability (RIS = 5.8 vs. 5.0, $P = 0.032$). Those with cardiovascular disease worried more about mild AE (RIS = 12.8 vs. 10.0, $P = 0.027$; $\beta = 0.170$, $P = 0.027$) and severe AE (RIS = 23.2 vs. 16.2, $P = 0.001$; $\beta = 0.203$, $P = 0.007$) but cared less about most process attributes.

Conclusions: Our results underscore the importance of discussing safety and efficiency with candidates for biologicals while considering that preferences vary dependent on age, gender and comorbidities.

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Immunology

P102 (O02/06)

Mechanistic analysis of CD3-driven T cell functions reveals a CD32a polymorphism associated with melanoma emergence

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Interaction of the T cell receptor (TCR) with antigen presenting cells causes T cell activation solely in the presence of additional co-stimuli like CD28-ligands. Since CD3 receptors are required for TCR signaling, immobilized anti-CD3 in combination with anti-CD28 monoclonal antibodies (mAb) are commonly used to mimic T cell stimulation *in vitro*. In the present study, the mechanism of T cell activation by anti- CD3 mAb was investigated comprehensively using two different anti-CD3 mAbs, CD3/OKT3 (mouse IgG2) and CD3/UCHT-1 (mouse IgG1). Both antibody clones were found to trigger T cell proliferation even in the absence of co-stimuli and when applied solubly. Incubation of PBMC (peripheral blood mononuclear cells) with CD3/ UCHT-1 or CD3/OKT3 leads to T cell stimulation dependent on the presence of monocytes. Both mAbs can induce T cell proliferation and the secretion of various cytokines such as IFNγ. Remarkably, in contrast to CD3/OKT3 treatment there was no detectable IL-2 release from T cells treated with CD3/UCHT-1. Also, we gained evidence that CD3 mAbs do not signal via the long established TCR signaling cascade including proteins like ZAP-70. During functional analyses, we recognized that T cells within PBMC from all healthy donors ($n = 91$) respond with proliferation to CD3/OKT3 treatment. However, T cell proliferation was only detectable in 59% (54/91) of PBMC from healthy donors treated with CD3/UCHT-1. Analyses of demographic data showed that 80% of male but only 39% of female donors were found to be CD3/ UCHT-1 Responder ($P < 0.01$). Surprisingly, the rate of CD3/UCHT-1 Responder was higher in melanoma patients than in healthy donors (74.5%, 76/102, $P = 0.031$). As opposed to healthy donors, no gender association of CD3/UCHT-1 responsiveness was found in melanoma patients. In a preliminary analyses of clinicopathological data of melanoma patients, we found that CD3/UCHT-1 Non- Responder were diagnosed with melanoma at a significantly younger age than CD3/ UCHT-1 Responder ($P = 0.04$) while the Breslow-index of primary tumors was similar in both groups ($P = 0.14$). Further studies showed that the state of responsiveness is genetically determined by a polymorphism in the Fc receptor CD32a. Whereas CD32a from CD3/UCHT-1 Non-Responder bear histidine residues (H/H) at amino acid position 166, Responder show expression of an arginine residue (R/R or R/H).

Our results illustrate that anti-CD3 mAbs might not mimic physiological TCR stimulation, suggesting that antibody isoforms for *in vitro* as well as *in vivo* applications need to be chosen carefully. Further, CD3/UCHT-1 responsiveness is genetically determined by a CD32a polymorphism and shows a gender-dependent distribution. Our preliminary results indicate that the CD32a genotype might by associated with an increased susceptibility to melanoma. The pathogenetic role of this polymorphism in melanoma development warrants further studies.

P103 (O03/01)

The Aryl hydrocarbon receptor is up-regulated in murine as well as human systemic lupus erythematosus and seems to be involved in disease progression

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The pathomechanisms underlying the development and progression of autoimmune diseases like systemic lupus erythematosus (SLE) are still elusive. However, it has been shown that SLE is caused by a combination of genetic and environmental factors. Signaling via members of the tumor necrosis factor (TNF) superfamily, such as CD40 and its ligand CD40L, has been proposed to be involved in SLE development. Accordingly, in a transgenic mouse model the epidermal overexpression of CD40L (K14-CD40L tg mice) resulted in the induction of a SLElike autoimmune disease including dermatitis, nephritis and proteinuria as well as the presence of autoantibodies in the serum. In K14-CD40L tg mice autoreactive CD8 + T cells are crucial for the onset of disease, since adoptive transfer of pathogenic CD8 + T cells from autoimmune prone K14-CD40L tg mice induced disease in wildtype (wt) recipients. Autoreactive CD8 + T cells from tg mice are characterized by an upregulated expression of IL-17. In CD4 + T cells the IL-17 expression can be controlled by the ligand-dependent transcription factor aryl hydrocarbon receptor (AhR). AhR is known to sense environmental stimuli, such as UV light or dioxin-like chemicals, both of which have been described as risk factors for SLE. Hence, we investigated whether AhR might be involved in the pathogenicity of IL-17 expressing CD8 + T cells and the progression of CD40L-induced SLE. Interestingly, we could show that AhR is up-regulated in lesional skin from tg mice compared to non-lesional skin of the same mice or wt controls. To assess the role of AhR for disease development *in vivo* K14-CD40L tg mice were crossed to AhR deficient animals. Surprisingly, the double mutants showed a delayed onset of disease and moreover a significantly reduced severity of autoimmune dermatitis compared to tg controls. Additionally, double mutants exhibited a rescued renal function as evidenced by the decreased protein excretion in the urine and the reduced immunoglobulin depositions at the kidney tissue. In contrast to tg controls, activated B cells and autoantibodies were not detectable in the serum of double mutants, suggesting an important role of the AhR during the pathogenesis of CD40L-induced systemic autoimmunity. To characterize the impact of AhR on the pathogenicity of autoreactive CD8 + T cells in more detail, the IL-17 and IL-22 expression as well as the expression of STAT-3 and ROR-γt, both transcription factors, which have been linked to Tc17 development, were analyzed in CD8 + T cells from tg mice and double mutants. Interestingly, we observed a significantly decreased expression of IL-17 and IL-22 as well as reduced expression of the transcription factors in CD8 + T cells from double mutants compared to tg animals. The down-regulated expression of proinflammatory cytokines and characteristic transcription factors in CD8 + T cells from double mutants finally resulted in a reduced migration of CD8 + T cells to lesional skin and the amelioration of dermatitis. To investigate whether AhR might be involved in the pathogenesis of LE in humans as well, we analyzed the AhR expression in the lesional skin of LE patients. In line with our mouse model, AhR was up-regulated in lesional skin from individuals with SLE as compared to healthy donors. Moreover, we observed increased numbers of AhR expressing dendritic cells and CD8 + T cells in isolated peripheral blood mononuclear cells from individuals with SLE versus healthy donors suggesting that AhR might be involved in the development and progression of LE in humans as well.

P104

Cutaneous RANK-RANKL signaling impairs anti-bacterial immunity but does not affect defense against parasites

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Infections are among the most common diseases of the skin and *Staphylococcus aureus* (*S. aureus*) is known to cause the majority of bacterial skin infections. Infections are controlled by the immune system and since RANK-RANKL signaling is critical for dendritic cell - T cell communication as well as for the regulation of immunity by inducing regulatory T cells (Treg) we investigated if it might also play a role in cutaneous anti-bacterial immunity. Therefore, transgenic mice over-expressing RANKL (CD254) in basal keratinocytes (K14-RANKL tg) and wildtype (wt) controls were intracutaneously infected with 2×10^7 CFU of the *S. aureus* strain SH1000. Whereas disease progression was similar in both groups, tg mice developed significantly larger skin lesions compared to wt mice suggesting reduced antibacterial immunity or impaired skin barrier function. However, immunofluorescence staining as well as quantitative realtime-PCR revealed a decreased bacterial load in lesional skin from tg compared to wt mice. To characterize the impact of the RANKL-mediated peripheral expansion of Treg during anti-bacterial immunity flow cytometry analysis was performed and numbers as well as function of Treg were assessed in infected mice. As compared to mock-infected mice Treg expanded in *S. aureus*-challenged mice and the proliferation of Treg was even more pronounced in tg versus wt mice pointing to an efficient inhibition of anti-bacterial immunity in tg mice. Moreover numbers of macrophages in draining lymph nodes as well as lesional skin from tg mice were reduced, known to be key players in first line defense against bacterial infections. Since protective adaptive immunity to *S. aureus* in mice is mainly mediated by Th1 and Th17 cells, we further analyzed these cells in infected wt and tg mice. Notably, total numbers of activated CD4 + T cells were significantly decreased in tg compared to wt mice as quantified by the expression of CD44 and CD69. Moreover, the levels of T-bet and IFNg expressing Th1 as well as Ror-gt and IL17 expressing Th17 cells were reduced in tg compared to wt mice. To investigate the role of Treg *in vivo* we crossed K14-RANKL tg mice to DEREG mice expressing a diphtheria toxin (DT) receptor in the FoxP3 gene locus. Hence, injection of

DT resulted in the selective ablation of Treg in K14-RANKL tg × DEREG double mutants. Interestingly, Treg depletion reduced skin lesion size in tg mice to wt level and normalized the numbers of anti-bacterial effector cells like macrophages, neutrophils, Th1 or Th17 cells, indicating that the RANKL-mediated expansion of Treg might have indeed impaired anti-bacterial immunity in tg mice. We next investigated whether the RANKL-mediated inhibition of anti-microbial immunity is a general phenomenon and therefore, infected wt and K14-RANKL tg mice with the parasite *Leishmania major* by injecting $1 \times 10e^4$ (low dose) or $2 \times 10e^7$ (high dose) promastigotes into the hind foot pad. Whereas wt and tg mice on a C57BL/6 background did not develop a significant foot pad swelling Balb/c mice showed a marked swelling and initial ulcerations 10 (low dose) or 3 weeks (high dose) after infection. However, in both models we did not detect any differences concerning the course or severity of disease between wt and tg mice. Thus, our data indicate that RANK-RANKL signaling might play diverse roles in cutaneous anti-microbial immunity, whereas anti-bacterial immune responses are impaired by expanding immunosuppressive Treg this signaling pathway did not affect defense against parasites.

P105

PDE4 inhibition as potential treatment of epidermolysis bullosa acquisita

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Autoimmune bullous dermatoses (AIBD) such as epidermolysis bullosa acquisita (EBA) are notoriously difficult to treat. In all AIBD (muco)-cutaneous blistering is caused by autoantibodies directed against (hemi)-desmosomal antigens; in EBA the autoantibody response is directed against type VII collagen (COL7), the main constituent of the anchoring fibrils. Despite the growing insights into the pathogenesis of AIBD, use of corticosteroids is still the mainstay of treatment. In experimental models of EBA, neutrophil activation has been identified as a key event leading to blistering. In parallel, neutrophil activation has been documented to depend on phosphodiesterase (PDE) 4 activation. PDE4 is an enzyme regulating amount of cAMP, an important second messenger involved in regulating antiinflammatory and proinflammatory cytokine production. Thus, its PDE4 inhibition has anti-inflammatory effects. We here therefore evaluated the potential of PDE4 inhibition in experimental models of EBA. First, the PDE4 inhibitors rolipram (ROL) and roflumilast (ROF) were tested for their effects on immune-complex (IC)-induced reactive oxygen species (ROS) release from neutrophils. Both, ROL and ROF, dose-dependently impaired IC-induced ROS release from neutrophils. Furthermore, both compounds prevented dermal-epidermal separation of normal human skin incubated with antibodies to COL7 and neutrophils. The inhibitors also reduced shedding of CD62L and decreased CD11b expression on IC-stimulated neutrophils as determined by flow cytometry. To validate these *in vitro* findings, ROF was selected for further *in vivo* validation. For this, experimental EBA was induced in mice by transfer of anti-COL7 IgG into C57BL/6J mice. While injection of normal IgG did not lead to a blistering phenotype, mice injected with anti-COL7 IgG and solvent (4% methylhydroxylpropylcellulose with PEG400) developed severe subepidermal blistering. In contrast, mice treated with 5 mg/kg of ROF showed significantly reduced blistering compared to the vehicle treated group, while 1 mg/kg of ROF had no effect on the clinical disease manifestation. These results support the notion that PDE4 inhibition may be a drug candidate for treatment of EBA as well as other neutrophildriven AIBDs.

P106

Comparison of mouse models of chemically induced scleroderma: analysis of early inflammatory processes in skin fibrosis by multi-color flow cytometry

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Systemic sclerosis (Scl) is a chronic autoimmune disease which among other clinical symptoms manifests in severe fibrosis of the skin. While a multitude of data have been reported in terms of fibroblast activation and the late phase of fibrosis, the specific contribution of macrophages and DC in the early phase of fibrosis induction remains unaddressed. Scl can be induced in mice by intradermal application of the cytostatic drug bleomycin. In this model, the cellular infiltrate mimics the early histological features of human Scl, in which an important role of antigen-presenting cells (APC) in the development of fibrosis is suggested. As the bleomycin model does not match all clinical manifestations of the disease in humans, we decided to perform a comparative study with the hypochloric acid (HOCl)-induced Scl model, which is known to induce different systemic manifestations.

Bleomycin and HOCL were administered s.c. in the neck area every day and skin punches were analyzed for quantification of skin thickness, collagen deposition, myofibroblast activation (alpha-SMA), inflammatory infiltrate (H&E, flow cytometry) and for expression of inflammation and fibrosis related mediators (qRT-PCR).

At day 28 both models resulted in a significant increase in dermal thickness, total collagen levels (hydroxyproline) and a prominent appearance of collagen fibers (Goldner's trichrome staining). Mice treated with HOCl exhibited the most prominent skin thickness versus the bleomycin-treated animals which was accompanied by a thick layer of subdermal fat in the HOCl-group. Moreover, TGF-β1 levels in plasma of both treatment groups were elevated, with significantly higher levels in bleomycin-treated mice. Histological analysis revealed an increase in cellular infiltrates in both Scl models which were characterized by using 7-color flow cytometry for CD45, CD11b, CD11c, MHCII, Ly6C, Ly6G and F4/80. Cellular infiltrates peaked at day 7 in bleomycin-/ HOCl-treated skin, to decrease at day 14 and being absent at day 28 of continuous bleomycin or HOCl application. There was a significantly increase in the number and percentage of total CD11b+ cells in the skin after HOCl-/ bleomycin-treatment when compared to PBS-treated mice. A detailed analysis revealed higher numbers of CD11b+MHCII+ cells in the HOCl-model which points to an activated antigen presenting myeloid cell population. In addition, the percentage of CD11c + MHCII+ representing mostly DC, of Ly6C +MHCII+ and of F4/80+MHCII+ monocytes/ macrophages was significantly elevated in the skins of HOCl-injected animals. In both models, we found an upregulation of profibrotic parameters with a prominent induction of procollagen alpha1(I) in HOCl and preferentially for alpha-SMA in bleomycin treated mice.

Given the differences in the percentages of infiltrating APC populations and certain fibrosis parameters, we will continue to define dissect innate immune parameters relevant in the progression of Scl.

P107

The protective effect of skin microbiota on pathogen infections is dependent on the integrity of the epithelial barrier

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Human skin as the first barrier to the environment is constantly exposed to various potential pathogens, but at the same time allows commensal bacteria to colonize and form a tissue specific microbiota. This skin resident microbiota plays an important role in innate and adaptive immune responses against pathogen infections. Keratinocytes, as the most abundant cell type in the epidermis, actively participate in the innate immune response towards pathogens by cytokine production or expression of antimicrobial peptides or proteins (AMPs). Skin commensal bacteria are able to amplify this immune response of keratinocytes and create a protective environment by immune conditioning of the epithelial surfaces. We show that skin resident bacteria are not only able to induce defensive immune responses against pathogen infections in human keratinocytes but also have a protective effect on pathogen attachment and invasion *in vitro* and *in vivo*. Using an epicutaneous mouse skin infection model, we demonstrate that skin colonization by pathogens is associated with profound cutaneous inflammation and therefore promoted by epithelial barrier defects. Furthermore, we reveal an adverse impact of skin barrier defects on the protective effect of commensal bacteria against pathogen infections *in vivo*.

Current experiments address this interaction between commensal and pathogen bacteria and the skin immune system *in vitro* and *in vivo* to gain insight into this complex interplay.

P108

Neutrophil granulocytes from individual healthy donors demonstrate a distinct predisposition for extracellular trap formation: introduction and application of a refined method for extracellular trap quantification

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Neutrophil extracellular trap (NET) formation is a recently discovered mechanism of innate immune defense. It describes the process by which neutrophil granulocytes produce net-like structures of decondensed chromatin strands decorated with antimicrobial peptides to trap microorganisms and is referred to as 'NETosis' if this process leads to cell death. Alterations of this mechanism were reported not only to beneficially contribute to immune defense but also to be involved in the pathogenesis of autoimmune and autoinflammatory diseases including psoriasis and systemic lupus erythematodes. It would therefore be interesting to precisely quantify the predisposition of neutrophil granulocytes to produce NETs in different diseases or in different settings. The available methods to quantify differences in NET formation *ex vivo* and *in vitro* are, however, either prone to interference and do not reliably differentiate between viable and dead cells (i.e. fixing and processing of cells prior to staining, thereby inevitably disturbing the delicate morphology of individual NETs), do not differentiate between modes of cell death (i.e. quantification of the DNA content of a culture medium, thereby potentially including apoptotic/necrotic material), or require expensive automatized setups or software. We report a refined method for NET quantification that does not require extensive processing of cells or expensive dyes and can be easily performed in most laboratory settings. Briefly, neutrophil granulocytes were isolated and stimulated with PMA in a regular culture plate following a strict protocol. Subsequently, the cells were stained with a membrane permeable and impermeable DNA-dye and the culture plate was carefully transferred to an inverted microscope stage where images were acquired at preset positions. Finally, images from individual channels were segmented and the information from 3000 to 5000 individual cells per condition was automatically processed to give a ratio of alive cells, dead cells, and NETs. The results obtained from repeated measurements from the same individual were in good agreement ($n = 3$). Furthermore, the response of neutrophil granulocytes from different healthy individuals to PMA stimulation was remarkably heterogeneous and, interestingly, these interindividual differences were still present on retesting after a period of at least 4 weeks ($n = 5$). In conclusion, we report a refined method to quantify NET formation and, using this method, identify the predisposition of neutrophil granulocytes to undergo NETosis as an individual quality that remains constant over time. Our results and the related methods can help investigators to reliably quantify disease related differences in NET formation.

P109

Pollen and UV-B: a couple causing enhanced inflammasome activation in human primary keratinocytes

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Skin and mucosa are the first contact sites for environmental factors. Therefore their primary response, including release of cytokines, is crucial for setting the course for follow-up immune responses like allergic reactions. One possible source of inflammatory cytokines is the inflammasome complex with Interleukin (IL)-1 beta and Interleukin-18 as hallmark products. Pathways of inflammasomes are known to be triggered by several different environmental factors e.g. UV-B irradiation, but recent studies also pointed to an involvement in allergic reactions e.g. against house dust mite or contact allergens. Similar to this, plant pollen is an environmental factor directly impacting skin epithelia and by this a promising candidate for inflammasome activation in epithelial cells. To expand knowledge in this area we are showing in this study not only an influence of pollen derived mediators on the inflammasome system in human primary keratinocytes (KC), but also an additive effect to the effects oft UVB treatment.

KC were stimulated with aqueous pollen extracts (APE) of birch, ragweed and timothy grass in different concentrations and in combination with a second environmental factor: UV-B irradiation. Cell-free supernatants were analyzed for IL-18, IL-1 beta and IL-1 alpha release. Protein level of active Caspase-1 and IL-1 beta / IL-18 was determined by Western Blot. Influences on the mRNA level were tested in 2D culture as well as in a 3D skin model and analyzed by qPCR.

Results revealed that treatment of KC with pollen extract leads to elevated mRNA levels of IL-1 beta and IL-18 proform which is accompanied by increased protein levels of active Caspase 1 as well as a rapid release of mature IL-1 beta, IL-18 and IL-1 alpha. Furthermore we observed an additive effect of APE on UV-B induced inflammasome pathways with the tendency to be stronger in atopic individuals than in healthy controls.

In summary, our results support the hypothesis that pollen influence the immunological barrier of the skin by triggering the inflammasome of human keratinocytes *per se* and aggravating the effects of UV-B irradiation. Thus, pollen themselves can provide a danger signal alone but also excite additive effects which may be important for the initiation and persistence of inflammatory allergic skin reactions. The 'head and neck' type of atopic dermatitis might thus not only be due to contact to pollen but also the combination of sun and pollen exposure.

P110

Integrin αE(CD103) - a modulator of regulatory T cell function in allergic contact hypersensitivity

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Allergic contact dermatitis (ACD) is a T-cell mediated inflammatory type IV reaction and a major cause of occupational skin disease worldwide with significant impact on life quality of affected patients. Murine contact hypersensitivity (CHS) models in many aspects ACD with important players taking part such as CD8 + T cells being effectors and CD4 + cells being mainly regulators in those immune responses towards various haptens.
Integrin αE(CD103) is expressed among others on T cells and dendritic cells (DC). It takes over functions in localization of epithelial lymphocytes and retention of CD4 + regulatory T (Treg) cells into inflammatory lesions. In this work we describe how CD103 determines Treg cell function in CHS. During CHS, integrin αE(CD103)-deficient mice showed significantly increased ear swelling and leukocyte infiltration of hapten challenged skin compared to wild-type mice. In contrast, reactions during acute irritant inflammation showed no objective differences. Studying sensitization and elicitation phase during CHS revealed normal DC function, indicating that CD103 expression on DC is not required for optimal stimulation of antigen-reactive T cells in CHS. However, adoptive transfer experiments with sensitized lymph node cells clearly indicate that elevated CHS responses in CD103−/− mice are primarily, if not exclusively, transferred by primed T cells and are independent of resident cells in recipient mice. Albeit general T cell counts were elevated in challenged skin of αE(CD103)-deficient animals, FoxP3 was expressed at significantly lower levels in CD4 + CD25 + Treg cells pointing to a role of CD103 in Treg function within the skin. Furthermore CD103 on FoxP3 + Treg cells was involved in Treg localization to inflamed skin in bone-marrow chimeras.
Together, our results cleary demonstrate that αE(CD103) is important for sufficient regulation of allergic CHS. Here, CD103 on FoxP3 + Treg cells functions as a fine regulator of CHS responses by affecting both, Treg function and Treg retention. Unrevealing the mechanisms behind this connection between CD103 and Treg cells will further augment our understanding of cutaneous allergies and open new therapeutic options.

P111

Induction of regulatory T cells by the antimicrobial peptide human betadefensin 3

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Antimicrobial peptides (AMPs) are small molecules which are released by a variety of cells including keratinocytes. AMPs were initially described according to their antimicrobial activity. They are essential components of the innate immune response and responsible for antibacterial defense. AMPs exert additional activities beyond their antimicrobial capacities, e.g. influencing the adaptive immune system by modulating antigen presenting cells. We observed that the UV-inducible murine AMP beta-defensin 14 (mBD14) is able to induce regulatory T cells (Treg) by inducing the transcription factor Foxp3. To clarify whether this applies also for the human system, it was studied whether human beta-defensin 3 (hBD3), the human orthologue of mBD14, exerts similar features. Human peripheral blood mononuclear cells (PMBC) obtained from buffy coats of healthy donors were separated into CD4 + CD25 + (Treg) and CD4 + CD25− T cells by magnetobead separation. The nonregulatory CD4 + CD25− fraction was incubated with hBD3. FACS analysis 24 h later revealed significant upregulation of the Treg characteristic molecules Foxp3, neuropilin, CTLA-4 and GARP, a transmembrane protein which is present only on the surface of activated Treg. To address the functional relevance, freshly isolated CD4 + CD25− T cells were treated with hBD3 for 48 h and cocultured with CD4 + CD25- responder T cells in the presence of anti-Biotin MACSiBead particles that were pre-loaded with biotinylated anti-CD2−, anti-CD3− and anti-CD28− antibodies. After 4 days, cell proliferation was measured using the MTT assay. hBD3 treated CD4 + CD25− T cells significantly suppressed the antibody triggered proliferation of the responder cells, indicating induction of suppressive feature of T cells upon incubation with hBD3. These data provide evidence that hBD3 similar to its murine analogue may change phenotypic and functional properties of nonregulatory T cells towards suppressive Treg. Through this ability, human AMP may protect the host from microbial attacks on the one hand, but tame T-cell-driven reactions on the other hand, thereby enabling an antimicrobial defense without collateral damage by the adaptive immune system.

P112

GM-CSF regulates pro-inflammatory and immunomodulatory activities of mesenchymal stromal cells

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Cells of mesenchymal lineage are important regulators of tissue homeostasis and repair. Among these, multipotent mesenchymal stromal cells (MSC) have the ability to release mediators of inflammation upon damage and/or infection and at the same time secrete powerful immunoregulatory molecules necessary for control and resolution of inflammation such as Prostanglandin E2 (PGE2), Kynurenine and Tumor Necrosis Factor stimulated Gene Protein (TSG-6). This has raised the interest for the use of these cells for the therapy of a growing number of conditions including defects in skin wound healing. Despite intensive research, the signals and mechanisms responsible for this immunoregulatory 'activation' of MSC remain largely unknown and therefore attractive and potential therapeutic targets are undefined and call for investigation. Here we identify Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) as a potent autocrine signal for the activity of MSC. Recombinant GM-CSF activates the gene expression of COX-2 (responsible for the synthesis of PGE2), IDO-1 (necessary for Kynurenine) and TGS-6 in a dose and time dependent manner. Functionally, MSC pre-treatment with GM-CSF is able to improve the capacity of the cells to reduce inflammatory functions of activated macrophages as seen e.g. by the reduction of the release of IL-12p40 and TNF. Additionally, a macrophage alternative pro-repair phenotype characterised by release of IL-10 and upregulation of CD206 and CD163 expression is induced upon coculture of MSC with differentiating monocytes in the presence of GM-CSF. We propose a model where MSC are rapidly stimulated by damage signalling to secrete GM-CSF which is in turn necessary for monocyte chemoattraction and support of their survival and differentiation in the early context of wounding and infection, but also acts as an important autocrine activator of the immunomodulatory activity of MSC which is important in the later context of inflammatory resolution. Thus, this cytokine links the pro-inflammatory with the anti-inflammatory program in MSC - representing a crucial check point for the control of inflammation. The *in vivo* spatio-temporal expression and activity of GM-CSF, whether imbalances in GM-CSF signalling might have a role in defective wound healing and inflammatory diseases, and how and when modulation of GM-CSF signalling could be of therapeutic significance remain to be studied.

P113

The tripeptide KdPT and its chemically modified derivative WOL074-019 ameliorate ongoing inflammation in the skin and the gut

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Alpha-melanocyte-stimulating hormone (α-MSH) as well as KdPT, a tripeptide that is closely related to the C-terminal amino acids of α-MSH are known to exhibit anti-inflammatory and immunomodulatory effects, which are mainly mediated by a reduction of nuclear factor B (NF B) activation and translocation. Previously, we have shown that KdPT ameliorated ongoing imiquimod-induced psoriasis-like skin inflammation in mice by inducing tolerogenic dendritic cells and expanding regulatory T cells (Treg). However, due to its unfavourable PhycChem properties KdPT is not suitable for topical application. Hence, we chemically modified the tripeptide at the C- and N-terminal positions (WOL074-019, 19) to improve its PhysChem properties and thus, its ability to penetrate the skin barrier. To investigate the anti-inflammatory and immunomodulatory potential of 19 *in vivo* we induced a psoriasis-like skin inflammation in mice by topical application of imiquimod (IMQ) for 8 consecutive days. At day 4 and 6 after the start of treatment mice were intravenously injected with PBS, KdPT or 19. Interestingly similar to KdPT, 19 treatment ameliorated ongoing skin inflammation as shown by the reduced thickness of epidermal rete ridges and the decreased levels of pathogenic Th1 as well as Th17 cells in regional lymph nodes and lesional skin, which were quantified by flow cytometry, realtime-PCR and immunofluorescence staining. This effect was mediated by the reduction of pro-inflammatory cytokines like IL-1β, IL-6 or TNF-α and the expansion of immunosuppressive Treg in 19-treated mice versus controls. To assess whether the beneficial impact of 19 was limited to cells of the adaptive immune system we next induced a local Shwartzman reaction, an animal model that displays histopathological characteristics of vasculitis and is mediated by the infiltration of innate immune cells, like neutrophils, into the skin. Therefore, mice were subcutaneously injected with LPS and TNF-α at an interval of 24 h and locally treated with PBS, KdPT or 19. Notably, mice that received 19 showed a reduced ear thickness, decreased bleeding as well as significantly down-regulated neutrophil counts as compared to mice that were treated with PBS. This effect was paralleled by a reduced expression of TNF-α, myeloperoxidase or the damage-associated-molecular pattern (DAMP) molecules S100A8 and A9, thus indicating that the anti-inflammatory capacity of 19 was not restricted to adaptive immune cells but rather general. To investigate whether 19 was able to ameliorate inflammation in other epithelial tissues than the skin we induced colitis in mice by adding 2.5% dextrane sodium sulphate (DSS) to the drinking water resulting in severe weight loss and the induction of rectal bleeding in PBS-injected control animals within 7 days. Interestingly, mice that were intraperitoneally injected with 19 at day 4–7 were protected from weight loss and moreover, did not show any signs of diarrhoea or rectal bleeding. Additionally, quantitative real-time PCR as well as immunofluorescence staining of colonic tissue revealed decreased levels of IL-6, IL-1β, TNF-α and IFN-γ. Besides, the numbers of neutrophils and macrophages were significantly reduced in mesenteric lymph nodes and the colon from 19-treated mice versus PBS-injected controls pointing to a potent anti-inflammatory effect of 19 in the model of DSS-induced colitis. Together, these data indicate that 19, similar to the original tripeptide KdPT, is able to efficiently ameliorate ongoing inflammation in the skin and the gut. Our data might suggest 19 to be a potential therapeutical option for patients.

P114

Cutaneous innate immune sensing of TLR2/6 ligands suppresses T cell immunity by inducing myeloid-derived suppressor cells

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Skin is constantly exposed to bacteria and antigens, and cutaneous innate immune sensing orchestrates adaptive immune responses. Otherwise, skin pathogens can expand, entering deeper tissues leading to life-threatening infectious diseases. To characterize skin-driven immunity better, we applied living bacteria, defined lipopeptides and antigens cutaneously. Surprisingly, cutaneous infection with Grampositive *S. aureus* caused suppression of immune responses, which was based on bacterial lipopeptides. Interestingly, skin exposure to TLR2/6- but not TLR2/1-binding lipopeptides suppressed immune responses. Investigating underlying mechanism, we found Gr1 + CD11b+ myeloid-derived suppressor cells (MDSCs) to be responsible for the diminished immune response. Experiments with chimeric mice revealed that TLR2 on skin resident skin induced production of cutaneous IL-6 and this cytokine was responsible for induction and development of suppressive MDSCs. Moreover, in contrast to MDSC induction, we excluded a role for IL-6 in cutaneous MDSC recruitment and suppression of the effector immune responses and collected evidence that chemokines CCL22 and CCL28 are involved in MDSC migration to the skin.
Ex vivo isolated MDSCs were able to inhibit unpolarized as well as Th1, Th2- or Th17-polarized T cells and their suppressive activities were mediated by inducible NO synthase (iNOS) and NO production, as treatment of MDSC with iNOS inhibitor abrogated the suppressive activity.
To take our findings further, we next analyzed AD patients, in which cutaneous TLR2 is constantly activated by Staphylococci derived substances. We observed a significant increase of human MDSCs and their suppressive activity in the blood and skin in AD in comparison to healthy individuals.
These data demonstrate that cutaneous recognition of TLR2/6 ligands orchestrates a unique pathway of cutaneous immune modulation mediated by MDSCs, indicating a new and yet unknown level of immune counter-regulation.

P115

Molecular mechanisms and functional consequences of blood monocyte recruitment and macrophage activation in tissue repair and fibrosis

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Tissue repair is characterized by the permanent deposition of extracellular matrix, replacing progressively the transient granulation tissue and resulting in the formation of a scar. Pathological healing conditions, as associated with chronic venous diseases, diabetes mellitus or autoimmunity, often cause excessive accumulation of fibrous connective tissue leading to fibrosis and organ malfunction. Inflammation is considered a key factor driving the development of progressive fibrotic diseases. However, detailed understanding how elements of the inflammatory cascade might induce and sustain a fibrotic response is elusive. In this study we are aiming to identify signaling pathways that direct the recruitment and activation of blood monocytes into damaged tissue and that initiate tissue fibrosis.
We focused our studies on the chemokine receptor CCR2, which has been implicated in the recruitment of inflammatory monocytes in various inflammatory and fibrotic diseases. In fact, based on genetic screens in fibrotic conditions e.g. scleroderma, CCR2 is considered as highly attractive anti-fibrotic therapeutic target. However, preclinical *in vivo* studies that unambiguously demonstrate a direct functional relevance of CCR2 in fibrogenesis are scarce. To monitor recruitment of CCR2 + blood monocytes during fibrosis, skin of CCR2-eGFP reporter mice was injected daily with bleomycin and single-cell suspensions of the fibrotic lesions were generated after 2 and 4 weeks of bleomycin treatment. Analysis by flow cytometry revealed a robust accumulation of CCR2 eGFP+ monocytes/macrophages within the fibrotic lesion suggesting a functional role of CCR2-mediated recruitment of blood monocytes during fibrogenesis. Unexpectedly, macrophage numbers were not reduced in fibrotic lesions of CCR2−/− deficient mice when compared to controls. Moreover, CCR2 deficiency failed to protect mice from bleomycin-induced skin fibrosis. Thus, our findings provide evidence that expression of CCR2 on blood monocytes does not represent a prerequisite for the induction and progression of bleomycin-induced skin fibrosis in mice. Furthermore, in our study we are aiming at unraveling the functional impact of macrophage polarization during the development of skin fibrosis. For this purpose we investigated bleomycin-induced skin fibrosis in myeloid cell-restricted STAT3 deficient mice (STAT3 fl/flLysMCre). Interestingly, in mutant mice with STAT3-deficient myeloid cells the fibrotic response was significantly increased after 2 weeks of bleomycin challenge when compared to control mice. Accelerated development of skin fibrosis in STAT3 fl/flLysMCre mice did not show altered relative or absolute macrophage numbers within the fibrotic

lesion, but was characterized by a dysregulated macrophage activation phenotype. Analysis of the gene expression profile of F4/80 + CD11b+ FACS-sorted macrophages from fibrotic tissues revealed a robust upregulation of IL-10 in macrophages isolated from controls, whereas the expression in mutant animals was significantly reduced. Together, our findings identified STAT3 expression in myeloid cells as critical regulator of bleomycin-induced skin fibrosis. Of note, this process appears to be independent of CCR2-mediated recruitment of blood monocytes into the damaged tissue. Currently, we further investigate the origin and the phenotype of anti-fibrotic STAT3 expressing macrophages. Our findings provide new mechanistic insights into macrophage-mediated skin fibrosis which might be relevant for the development of novel anti-inflammatory therapies to prevent tissue fibrosis and scarring.

P116

9-cis-retinoic acid modulates dendritic cell differentiation to generate a Treg inducing phenotype

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9-cis-retinoic acid (9cisRA, Alitretinoin) is a high-affinity pan-agonist for the retinoic acid receptors (RAR) and retinoid X receptors (RXR). 9cisRA is highly effective for treating chronic hand eczema, which is often associated with delayed type allergy. There is limited data how 9cisRA exerts anti-inflammatory functions in the skin immune system. We here investigated the effects of 9cisRA on dendritic cell (DC) – T cell interactions in the context of allergic contact hypersensitivity.

Murine bone marrow derived DC were cultured by standard protocol in the presence of different concentrations of 9cisRA. We found that in comparison to untreated DC the highly CD11c expressing DC that are differentiated from murine bone marrow in the presence of 9cisRA (9cisDC) express less MHC-II, CD44 and CD86. Further, 9cisDC had an altered pattern of cytokine and chemokine expression, secreting less IL-1β, IL-12p70, CXCL9, CXCL10 and CCL-1. To investigate the functional characteristics of 9cisDC we performed allogeneic mixed lymphocyte reactions. 9cisDC were less potent in stimulating T cell proliferation, however, they were highly potent in converting naïve T cells into CD4 + /Foxp3 + /CD25 + Treg cells. This was mediated through 9cisDC, as 9cisRA treatment of CD3/CD28 activated T cells in the absence of 9cisDC did not induce Tregs. Finally, *in vivo*, we tested whether 9cisDC were able to inhibit established antigen specific contact hypersensitivity (CHS). When TNCB sensitized mice were treated with 9cisDC loaded with TNBS 6 days after sensitization they significantly inhibited CHS response in comparison to mice injected with untreated TNBS loaded DC. Further, 9cisDC treated TNCB sensitized mice showed elevated numbers of Tregs in skin draining lymph nodes 48 h after antigen challenge.

In conclusion our findings propose that 9cisRA modulates DC toward a phenotype that is able to suppress established contact hypersensitivity through the induction of Tregs.

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Wound healing defect of CD18−/− mice due to impaired beta2 integrindependent activation of the NADPH oxidase in macrophages

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Reactive oxygen species (ROS) released by the phagocyte nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX2) are key players in infection and inflammation. We here provide first evidence for the critical role of β2-integrinsmediated activation of NOX2 in macrophages for transforming growth factor-β1 (TGF-β1)-induced granulation tissue formation and proper healing of skin fullthickness excisional wounds. Targeting the NOX2 activator rotenone to wound macrophages fully restored reduced ROS levels, impaired TGF-β1 activation, granulation tissue formation and wound healing of β2 integrin-deficient CD18−/− mice; this effect was completely reversed by the NOX2 inhibitor ebselen. NADPH based fluorescence lifetime imaging (FLIM) revealed that NOX2 failed to assemble for its activation at the CD18−/− macrophages membrane upon adhesion to apoptotic neutrophils. Similar to CD18−/− mice, also mice lacking the p40phox domain of NADPH (p40phox−/−) with impaired oxidase function presented significantly reduced ROS levels and defective wound healing. Impaired TGF-β1 activation by p40phox−/− wound macrophages was causal for reduced angiogenesis, myofibroblasts differentiation and wound contraction of p40phox−/− mice. Injection of wildtype, but neither p40phox−/− nor CD18−/− macrophages, into wound margins restored the impaired wound healing of CD18−/− mice, supporting a central role of the β2 integrin-NADPH oxidase pathway in macrophages for tissue repair and inflammation.

P118

Mastcells are heterogeneous

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Mature human skin mast cells (HSMCs) are thought to be a homogenous population, based on the expression of typical activating receptors such as c-kit and FcRI and the mast cell proteases tryptase and chymase. To test for the presence of distinct subpopulations, we performed single cell protein profiling by RT PCR and subsequent immunohistochemistry analyses of adult HSMCs in paraffin samples and cytospins. We identified 5 proteins that are highly expressed (HBXIP, TXNIP, S100A11, WASF, CAPZA) by HSMCs. By immunohistochemistry, 20–50% of HSMCs expressed TXNIP, S100A11 or WASF, i.e. 50–80% of the HSMCs were negative for the proteins. These results point to distinct subpopulations of mast cells present in healthy human skin, but the role and relevance is still unclear. The three identified proteins have previously been described to be associated with faster tumor proliferation, higher rates of metastasis and greater rates of relapse. We are currently investigating the presence and function of TXNIP +, S100A11 + and WASF + HSMCs in skin tumors.

P119

Human monocyte-derived dendritic cells stimulated with specific nondigestible oligosaccharides mimicking the functionality of breast milk oligosaccharides induce CD4 + Foxp3high T cells

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Non-digestible oligosaccharides (NDO) alone or in combination with lactic acid bacteria (LAB) have been shown to harbor preventive effects towards immuneregulatory disorders like allergies. Therefore it is current practice to apply LAB and NDO derived from bovine milk (galacto-oligosaccharides) and plants (fructooligosaccharides) mimicking the functionality and molecular size of human milk oligosaccharides as a supplement in infant formulas. In previous studies we revealed direct effects of these NDO mixtures alone or in combination with certain LAB on human monocyte-derived dendritic cells (MoDC), showing an enhancing effect of NDO and LAB on IL-10 release by MoDC. The aim of this study was to further investigate and confirm a possible induction of foxp3high regulatory T cells by NDO and/or LAB- stimulated MoDC. Immature human MoDC prepared from peripheral blood of healthy non-atopic volunteers were screened *in vitro* after stimulation with specific NDO mixtures in the presence or absence of different LAB strains. Cytokine release by MoDC was analyzed after 24 h in cell-free supernatants by luminex-based assay and ELISA. To investigate the resulting T cell response, stimulated MoDC were further co-incubated with naïve T cells in allogenic stimulation assays and intracellular Foxp3 expression was assessed. NDO and LAB exert a significant enhancing effect on anti-inflammatory IL-10 secretion by MoDC, while no ability to increase pro-inflammatory IL-12p70 production was observed. T cells induced by these MoDC show a regulatory T cell phenotype characterized by Foxp3 expression. These results indicate immune-regulatory properties of the investigated NDO in the presence or absence of LAB *in vitro*. The tested combinations might represent a useful dietary supplement for the maintenance of intestinal hemostasis via the induction of regulatory T cells and therefore could be considered as allergy preventing ingredients in food.

P120

Myeloid cell-restricted insulin/IGF-1 signaling controls UV light-induced skin inflammation

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The function of Insulin/Insulin like growth factor-1 (IGF-1) signaling in myeloid cells in skin physiology or pathology is little investigated so far. Here we examined the role of cell autonomous Insulin/IGF-1 signaling in myeloid cells in skin inflammation and homeostasis by generating mice that lack both the Insulin and the IGF-1 Receptor in myeloid cells (IR/IGF-1RMKO). IR/IGF1RMKO mice developed normally without any obvious skin phenotype. The healing response of full thickness excision skin wounds revealed a temporary reduction in granulation tissue formation in knockout mice when compared to controls, however was otherwise similar in mutants and controls. In contrast, in a model of chronic dermatitis (induced by topical application of sodium dodecyl sulphate (SDS) IR/IGF1RMKO mice were protected from skin inflammation, whereas control mice developed a severe skin inflammatory response. Interestingly, whereas lesional dermis in control mice was dominated by a pro-inflammatory macrophage phenotype, macrophages in mutant mice rather showed the expression of immunosuppressive mediators. Furthermore, to investigate the role of myeloid cell-restricted IR/IGF1R expression in the UVlight induced inflammatory skin response we exposed IR/IGF1RMKO mice to a single application of UV-B light (600 mJ/cm^2). The inflammatory cell infiltrate was analyzed 7 days after irradiation. Consistent with our findings in the SDS model, myeloid cell-restricted deletion of IR/IGF1R rescued mice from UV-induced dermal influx of inflammatory cells and epidermal hyperproliferation. Analysis of the recruited macrophages showed a highly pro-inflammatory (M1) phenotype in control mice upon the UV response, whereas macrophages in mutants were characterized by attenuated expression of pro-inflammatory mediators and upregulated expression of IL-10. Gene expression analysis of macrophages stimulated *in vitro* with Insulin/ IGF-1 corroborated our *in vivo* findings, indicating a critical pro-inflammatory action of Insulin/IGF-1 in dermal infiltrating myeloid cells. Myeloid cell-restricted IR/IGF-1R signaling thus appears dispensable in acute inflammatory processes such as excisional wound repair, whereas it is critical to sustain prolonged inflammation induced by toxic triggers or UV irradiation. In conclusion, we provide evidence for a novel IR/IGF-1R-dependent pathway in myeloid cells that plays a critical role in skin inflammatory responses, and may add to the understanding of the molecular basis of inflammatory skin diseases.

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Analysis of the antigen-presenting cell compartment in pemphigus vulgaris

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In the potentially lethal autoimmune skin blistering disease pemphigus vulgaris (PV) autoantibodies (autoAb) predominantly directed against the desmosomal cadherin desmoglein-3 (Dsg3) cause loss of epidermal keratinocyte adhesion. It is still unknown which role cells of the innate immune system, especially of the antigen-presenting cell compartment play in the initiation and maintenance of this autoAb-driven disease. The goal of this study was to phenotypically and functionally characterize dendritic cells (DC) and monocytes in PV patients (n = 34). Patients suffering from myasthenia gravis (MG), another autoAb-mediated organ-specific autoimmune disorder, were used as controls (n = 31). Quantitative and qualitative alterations within the DC and monocyte populations of PV and MG patients were investigated using flow cytometry analysis of peripheral blood and compared with the results in healthy controls (n = 32). Our results reveal neither increased DC subpopulations such as myeloid DC (CD14−CD11c++), plasmacytoid DC (CD11c−CD303 +) and slan (6-sulfo LacNAc) DC (CD14−CD16 + HLA-DR+ and CD14−CD16 + CD86 +) nor elevated numbers of inflammatory CD14 + CD16 + monocytes in PV patients. In addition, no phenotypic signs of increased immunogenicity of antigenpresenting cells as shown by a higher expression of the maturation markers HLADR and CD86 could be found in PV patients by flow cytometry. Functional properties of monocytes such as the antigen uptake capacity of Dsg3 protein were tested by flow cytometry-based analysis of internalized fluorescently-labeled recombinant Dsg3 protein. After stimulation of DC with bacterial lipopolysaccharides (LPS) the proinflammatory cytokines IL-6 and tumor necrosis factor-alpha (TNF-α) were detected by intracellular cytokine staining. In ongoing experiments, levels of IL-6 and TNF-α are being detected in the serum of PV patients and the control cohorts. The results of the antigen-presenting cell compartment are correlated with CD4 + T helper cell profiles as well as with anti-Dsg3 IgG titers of PV patients. Therefore, peripheral blood mononuclear cells (PBMC) from PV patients and the control cohorts were stimulated with phorbol myristate acetate (PMA) and ionomycin and intracellular synthesis of the Th1, Th2, Th17 and regulatory T cell related cytokines IFN-γ, IL-4, IL-17 and IL-10, respectively, was detected by flow cytometry. Preliminary data show significantly increased populations of IL17- and IL-10-producing T helper cells in PV patients compared to MG patients and healthy controls. In summary, we did not detect significant differences in the antigen-presenting cell compartment in peripheral blood of PV patients compared with MG patients and healthy individuals, respectively. However, in PV we detected significantly higher numbers of IL17- and IL-10-producing T helper cells in PBMC upon mitogenic stimulation; the relevance of this finding is being investigated in more details in ongoing experiments. The comprehensive analysis of experimental, serological and clinical parameters will contribute to a better understanding of the role of DC and monocytes in the pathogenesis of PV.

P122

B-cell independent functions of T cells during immune-complex induced neutrophil-dependent inflammation

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Tissue injury during autoimmune diseases depends on binding of autoantibodies to the effector organs and a subsequent cellular response. During epidermolysis bullosa acquisita, a prototypic organ-specific autoimmune blistering disease, autoantibodies to type VII collagen, a major component of the hemidesmosomal adhesion complex of the dermal-epidermal junction are generated. Binding of antibodies leads to generation of a pro-inflammatory milieu in the skin and subsequent neutrophil extravasation and activation. Reactive oxygen species and proteolytic enzymes released from neutrophils then lead to subepidermal blister formation. By using an autoantibody-transfer model for EBA here we first provide evidence for further contribution of T cells during neutrophil-dependent tissue injury in a B cellindependent manner: T cell-deficient nude mice are almost completely protected from neutrophil-dependent tissue injury. Differences in the activity and number of neutrophils in nude mice could be clearly excluded. Reconstitution of nude mice with T cells from wild type mice regained the inflammatory phenotype, underscoring the importance of T cells for the modulation of neutrophil-dependent injury. Although the reconstituted T cells in nude mice induced production of antibodies, a B celldependent effect of T cells during neutrophil-dependent tissue injury could be excluded by using B and T cell deficient SCID.beige mice for experimental EBA. Again, SCID.beige mice are protected from experimental EBA and reconstitution with T cells from wild type or DO11.10 mice that predominantly possess a specificity for ovalbumin and lack a polyclonal T cell repertoire, regained clinical symptoms without any B cell involvement. In order to specify the responsible T cell-subclass involved in neutrophil-dependent tissue injury, we depleted different T cell-subsets in wild type mice and additionally used knockout mice in the autoantibody-transfer model for EBA. Here, we identified NKT and $\gamma\delta$T cells as the responsible subsets for susceptibility during neutrophil-dependent tissue injury during EBA.

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Lifespan and immune senescence in UCP2-deficient mice

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Uncoupling proteins (UCPs) are mitochondrial transporter proteins which dissipate the proton gradient on heat generation instead of ATP production. Therefore, activation of UCPs suppresses the oxidative phosphorylation, followed by reduction of reactive oxygen species (ROS) production. There are 4 homologues of UCPs (UCP1, 2, 3, and 4) that show a tissue-specific pattern of expression. Of those, UCP2 is highly expressed in immune cells, brain, intestine and lung. Our group and others have previously reported that ROS levels in immune cells (e.g. CD4 + T cells) obtained from mice lacking the UCP2 gene were significantly elevated compared with those of wild type controls. In addition, a number of studies suggest that the UCP2 gene is associated with chronic inflammation and autoimmune diseases, such as systemic lupus erythematosus, rheumatoid arthritis and multiple sclerosis. Since in patients, these diseases usually are adult-onset, we hypothesized that the alteration of immune phenotypes in aging, i.e. immunosenescence, is partially controlled by the UCP2 gene. So far, immune phenotyping of UCP2-deficient (UCP2−/−) mice has been limited to young animals, and the role of UCP2 in senescence of immune cells needs further investigation.

Therefore, in this study, we investigated the effect of UCP2 gene deficiency on lifespan and immune aging phenotypes in mice in a large colony of UCP2−/− mice and C57BL/6J (B6) wild type controls (n = approximately 60/sex/strain). For the evaluation of general aging phenotypes, the body weight was measured at different time points. In addition, 5–6 mice/sex/strain were randomly selected and evaluated for immune subpopulation profiling in peripheral blood at the age of 3, 6, 12, 18 and 24 months. Sixteen immune cell subpopulations, e.g. CD4 + T cells, B cells, and memory T cells, were evaluated by flow cytometry. At the date of the abstract submission, UCP2−/− mice showed a significantly shorter lifespan compared with B6 wild type controls ($P < 0.0001$, both sexes, respectively, Log-rank (Mantel-Cox) Test). The body weight of UCP2−/− mice was significantly less at all 5-time points in comparison to B6 mice ($P < 0.0001$, t-test). The composition of immune cells in mice of 3–12 months of age was similar in both strains. However, at 18 months, UCP2−/− mice demonstrated significantly higher levels of monocytes and NKT cells as compared with B6 mice ($P < 0.05$ and $P < 0.01$, respectively, t-test), while numbers of effector memory CD4 + T cells ($P < 0.01$), central memory CD4 + T cells ($P < 0.01$), naive CD4 + T cells ($P < 0.001$), central memory CD8 + T cells ($P < 0.001$) and naive CD8 + T cells ($P < 0.01$) were all significantly less when compared to controls (t-test).

Our finding suggests that the UCP2 gene has a strong impact on immune cell senescence at later stages of life and such skewed immune phenotypes might initiate the aging process, resulting in shorter lifespan and the onset of age-related diseases.

P124 (O03/05)

Characterization of circulating CD4 + CXCR5 + follicular helper T cells in pemphigus vulgaris

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Pemphigus vulgaris (PV) is an organ-specific autoimmune disease characterized by blister formation at the suprabasilar layer of skin and mucous membranes. IgG autoantibodies (autoAb) against the desmosomal cadherin desmoglein 1 (Dsg1) and Dsg3 play a crucial pathogenic role in inducing blister formation. Several studies have focused on the pathogenic effects of autoAb in PV, but the immune mechanisms leading to the development of autoreactive B cells in PV still need to be clarified. In this context the potential role of follicular helper T (Tfh) cells acting as important costimulators during B cell maturation in germinal centers is not yet understood in the autoimmune pathogenesis of PV. Our study aims at (i) identifying circulating Tfh cells in peripheral blood of PV patients and (ii) at characterizing the role of Tfh cells in the secretion of Dsg3-reactive autoAb by auoreactive B cells. Using fluorescently-labelled (Alexa 647) recombinant Dsg3 protein, we established a flow cytometry-based assay for the detection of Dsg3-specific B cells in peripheral blood of PV patients. The staining conditions were optimized using mouse B cell hybridomas specific for human Dsg3 or an irrelevant antigen, resulting in a high specificity and sensitivity for the detection of Dsg3-reactive B cells. Our preliminary results suggest higher numbers of Dsg3-specific CD19 + CD27 + memory B cells in peripheral blood mononuclear cells (PBMC) in PV patients (n = 20) compared with healthy controls (n = 3). The frequencies of Dsg3-reactive CD19 + CD27 + memory B cells could be further increased after *in vitro* stimulation with IL-2 and the Tolllike receptor 7 agonist R848 as analysed by flow cytometry and B cell ELISpot, respectively. Tfh cells (defined as CD4 + CXCR5 + T cells) were analysed in PV (n = 10) and in patients with myasthenia gravis (MG) (n = 13), another antibody-mediated autoimmune disease. So far, our results point towards an increased number (+40%) of circulating Tfh cells in PV and MG patients, compared with healthy controls (n = 3). Furthermore, a subset of PV and MG patients show higher expression of Tfhassociated molecules, such as inducible T cell costimulator (ICOS) and programmed cell death 1 (PD-1), suggesting a potential role of Tfh cells in the development of autoreactive B cell clones. Ongoing experiments address the functional relevance of circulating CD4 + CXCR5 + Tfh cells in PV by T-B cell assays in which mitogenand Dsg3-stimulated Tfh cells, respectively, are cocultured with CD19 + B cells and the secretion of Dsg3-specific autoAb is going to be quantified by ELISpot and ELISA. Finally, the frequency of circulating Tfh cells is going to be correlated with various disease parameters, such as disease activity of the PV patients and autoAb titers. The more defined understanding of the role and function of Tfh cells in PV will broaden our understanding of the autoimmune pathogenesis in this model disease and might pave the road for innovative targeted therapies in the future.

P125 (O04/03)

Immune complexes recruit proinflammatory human slanDCs *in vitro* and *in vivo*

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Immune complexes (IC) have strong pro-inflammatory function in autoimmune diseases such as in lupus erythematosus (LE). Cells equipped with a unique capacity to bind IC via the two IC receptors CD16 and CD32 are 6-sulfo LacNAc dendritic cells (slanDCs). slanDCs circulate in blood at high numbers, have an outstanding capacity to produce IL-12, IL-23, TNF-α and IL-1β, and can be found as inflammatory dermal DC in psoriasis and LE.

In this study we provide strong evidence for the molecular and functional specialization of slanDCs as proinflammatory cells in LE-nephritis and cutaneous vasculitis. Histological studies revealed a strong accumulation of slanDCs in lesions with dense IC deposition, e.g. in the glomerulus from LE-nephritis patients and in the intra-/perivascular areas from cutaneous vasculitis patients. There was a high correlation between numbers of glomerular slanDCs and histopathologic activity index of patients with lupus nephritis [International Society of Nephrology/ Renal Pathology Society (ISN/RPS) classes II and III] which corroborate the proinflammatory function of slanDCs. Given the results of the histologic studies and the high IC binding capacity of slanDCs we studied whether slanDCs in blood can be directly captured by immobilized IC *in vitro* and *in vivo*.

For *in vitro* studies, we applied a perfusion assay-based approach coupled with time-lapse video microscopy and measured the arrest functions of purified slanDCs on immobilized IC. The flow conditions were adjusted to provide physiologically relevant surface shear stress of human venous capillaries. Under these conditions we observed a pronounced recruitment of FcγRIII (CD16) slanDCs. Furthermore, when monolayers of dermal microvascular endothelial cells were preincubated with an endothelial cell-specific antibody, slanDCs adhered to IC under physiological flow conditions in a CD16-dependent manner.

For our translational *in vivo* approach, immunodeficient Nonobese diabetic (NOD)- SCID interleukin-2 gamma chain receptor (NSG) mice were intravenously injected with preformed IC and subsequently with fluorescent labeled slanDCs. Using Fc receptors blocking monoclonal antibodies, we show that glomerular deposition of IC mediates recruitment of human slanDCs in a CD16-dependent manner.

Collectively, our findings demonstrate the IC capacity to recruit circulating slanDCs *in vitro* and *in vivo*. Modulation of IC-mediated slanDCs recruitment may offer therapeutic benefits in patients with IC-mediated inflammatory and/or autoimmune diseases.

P126

Kinome profiling: a closer look on human regulatory T cells

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Regulatory T cells (Treg) are essential for T cell homeostasis and maintenance of peripheral tolerance. They prevent activation of auto-aggressive T cells in the context of autoimmune diseases and allergy and, on the other hand are part of the tolerance-inducing tumor microenvironment. Foxp3, CTLA-4 or GARP are Tregassociated molecules, known to play a central role in differentiation and function of Treg. However, several studies suggest the involvement of additional regulatory elements such as kinases which seem to play an important role in Treg fine tuning. Nevertheless, our knowledge regarding the complex intracellular signaling pathways controlling phenotype and function of Treg is still limited and based on single kinase activity research so far. To obtain a more comprehensive knowledge into the pathways of Treg function we performed kinome profiling of human Treg at different activation stages compared to T effector cells (Teff). Extensive bioinformatical analyses indicated important quantitative but also qualitative differences in both populations. Resting and activated Treg showed an altered pattern of CD28− dependent components and of kinases involved in cell cycle progression such as CDK2/Aurora kinase B and cytoskeletal reorganization such as PAK2, also described as a positive regulator of T cell activation that interferes with NFAT expression and IL-2 production. Additionally, significant up-regulation of kinases in activated Treg but not in Teff such as TGF-β-R, EGFR, Akt1 or CK2 demonstrate that a specific molecular activation pattern defines the activation state of human Treg. To validate the data of the kinome profiling we performed investigations on mRNA and protein level. Taken together, kinome profiling that reveals insight into the functional properties and pathways of human Treg opens the possibility to identify new molecular targets for the development of effective immunotherapies against unwanted T cell responses in allergy, autoimmunity and cancer.

P127

Neutrophil extracellular trap-derived cathelicidin antimicrobial peptide: contribution to macrophage host defense

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Neutrophil extracellular traps (NETs), which are casted by dying neutrophils, are large web-like structures composed of chromatin and proteins of both nuclear and granular origin. NETs trap and immobilize bacteria and a characteristic feature of NETs is the expression of cathelicidin, as well as other antimicrobial peptides. Nevertheless, whether NETs kill entrapped pathogens remains highly controversial. Here, we hypothesize that NETs and NET-derived cathelicidin:DNA complexes contribute to the cooperative antimicrobial responses by human neutrophils and macrophages against intracellular pathogens. Primary human M-CSF differentiated macrophages were incubated with whole NETs, isolated from human neutrophils, and with *in vitro*-generated cathelicidin:DNA complexes. Analyses by immunofluorescence showed that human macrophages internalized cathelicidin as part of whole NETs and cathelicidin:DNA complexes. Internalized cathelicidin colocalized with the lysosomal marker lysotracker, indicating that the internalized cathelicidin reaches lysosomal compartments. Studies to determine whether cathelicidin as part of whole NETs and cathelicidin:DNA complexes kills intracellular pathogens after uptake by macrophages are ongoing.

P128 (O04/05)

The role of the mitochondrial genome in autoimmune blistering skin diseases

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Mitochondria play a central role in many cellular processes and mitochondrial dysfunction contributes to autoimmunity. Mitochondria carry their own genome (mitochondrial DNA; mtDNA). Mutations in the mtDNA are known to cause functional alterations in mitochondria, causing various pathologies, including inflammatory diseases, metabolic disorders as well as autoimmune conditions.

Our group has recently generated conplastic mouse strains carrying defined mutations in the mtDNA, e.g. a mutation in the ATP8 gene, which encodes a subunit of the ATP synthase (complex V). In previous mouse studies, a mutation in the ATP8 gene was found to be associated with autoimmune conditions such as autoimmune diabetes, lupus nephritis and autoimmune pancreatitis. We hypothesized that the mutation in the ATP8 gene impacts on epidermolysis bullosa acquisita (EBA), an autoimmune blistering skin disease and a prototype of autoantibody-mediated autoimmune diseases. EBA is characterised by the presence of autoantibodies targeted against type VII collagen, a skin structural protein. To evaluate the impact of the mutation in the ATP8 gene, we induced experimental EBA in mice carrying the ATP8 mutation (B6-mtFVB) and the wild type controls (C57BL/6J) by pathogenic antibody transfer. We observed a marked decrease in clinical score in B6-mtFVB compared to control mice ($P < 0.05$). In functional studies, the mitochondrial levels of reactive oxygen species (ROS) in stimulated immune cells were significantly reduced in the ATP8 mutant strain compared with the wild type ($P < 0.05$). The ATP/ADP ratio in isolated mitochondria and the oxygen consumption rate in lymphocytes were significantly reduced in the ATP8 mutant strain ($P < 0.05$) compared with the wild type. Measurements of the respiratory chain enzyme activities in isolated mitochondria showed an increased activity of complex V, which contains the ATP8 subunit, in B6-mtFVB mice compared to wild type mice. Furthermore, the activities in other OXPHOS complexes (I, III and IV) were also found to be altered.
In addition, we are currently investigating associations between polymorphisms in the mitochondrial ATP8 gene and AIBD patients, e.g. bullous pemphigoid.
In summary, we defined the effect of an mtDNA mutation on mitochondrial functions and subsequent effects on the immune cells leading to alterations of the disease severity in an AIBD model.

P129

Hydroxyethyl starch nanocapsules as a potent drug-delivery system for targeted cancer immunotherapy

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Cell-type specific targeting by use of nanoparticles is a promising approach for induction of efficient anti-tumor responses in cancer immunotherapy. However, nanoparticle-based carriers often exhibit a high non-specific uptake by antigen presenting cells (APC) like dendritic cells or macrophages and can alter the function of immune cells, thereby counteracting a cell-specific nanoparticle-based drug delivery in selected immune cells. In the present study, we demonstrate the generation of hydroxyethyl starch (HES) nanocapsules (NC) using selective interfacial cross metathesis by an inverse miniemulsion process, in which the cross metathesis took place between acrylated HES and an unsaturated phosphoester. The non-functionalized NC revealed a size of around 190 nm in diameter with a low size-distribution, measured by transmission electron microscopy and dynamic light scattering. They were fluorescent labelled by encapsulation of the dye Cy5. Additional, kinetic studies excluded the release of the incorporated dye, demonstrating the stability of the HES NC over time.
In order to test the non-specific uptake and effect of the HES NC on immune cells, in a first approach we used human immature (iDC) and mature dendritic cells (mDC) which exert a pronounced capacity for antigen uptake as the most potent APC of the immune system. For this purpose, we generated monocyte-derived DC from buffy coats and subsequently performed flow cytometry analysis. Intriguingly, these experiments demonstrated a very low non-specific internalization (<5%) in immature and mature DCs, independent of NC concentrations and time points. In addition, HES NC did not alter the phenotype and viability of iDCs and mDCs, excluding an immune stimulatory or toxic effect of the NC. Moreover, we incubated isolated peripheral blood mononuclear cells (PBMCs) with HES NC to analyze interaction with a variety of other immune cell populations. Here as well we did not find a functionally relevant uptake of HES NC in T cells (CD3 +), B cells (CD19 +) or phagocytes (CD16 + , HLA-DR+), respectively.
As a positive control and to exclude technical reasons for the low internalization of HES NC, we used the immortal cancer cell line HeLa because of its known highly enhanced non-specific phagocytosis. These experiments revealed a time- and dosedependent uptake of the non-functionalized HES NC in HeLa cells (up to 60%).
In summary, we generated non-functionalized HES NCs that display a functionally irrelevant uptake in various immune cell populations and are therefore a potent tool for functionalization to generate a cell-specific drug-delivery system for immune cells in cancer immunotherapy.

P130

T lymphocytes from patients with lichen planus (LP) recognize the NH2- and COOH-termini of bullous pemphigoid antigen 180 (BP180) ectodomain

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LP is a chronic inflammatory autoimmune disease of the skin with a prevalence of 1%–4% in the general population. Clinically, LP poses a major challenge as it affects not only skin, but also mucosal surfaces, scalp and nails and is frequently hard to treat. Typically, the inflammatory skin infiltrate shows an accumulation of lymphocytes at the dermoepidermal junction and destruction of basal epidermal keratinocytes. T lymphocytes isolated from LP skin lesions are predominantly CD8 + and cytotoxic against human keratinocytes. Oral LP shows clinical similarity with pemphigus vulgaris (PV) while cutaneous LP with secondary dense bullae resembles bullous pemphigoid (BP). In the present study, we examined the specificity of peripheral T lymphocytes from 12 LP patients regarding recognition of the autoantigens of PV, desmoglein (Dsg) 1 and 3 and BP, BP180, utilizing ELISPOT assays. *Ex vivo* stimulated T cells were monitored for the release of interferon γ (IFNγ), granzyme B, interleukin-5 (IL-5) and interleukin 17 (IL-17). Peripheral T cells from the LP patients showed a pronounced IFN-γ dominated cytokine profile upon co-culture with BP180 ($n = 6$) which was associated by granzyme B secretion ($n = 5$). Of note, markedly lower numbers of IL-5-secreting T cells ($n = 5$) and IL-17-releasing T cells ($n = 4$) were also detected upon *ex vivo* stimulation with BP180. In contrast to the preferential T and B cell recognition of the BP180-NC16a domain in BP, T cells from the LP patients recognized the NH2- ($n = 6$) and COOH-termini of the BP180 ectodomain ($n = 6$) to a similar extent. In summary, the findings strongly suggest that LP is associated with an IFN-γ-dominated, cytotoxic T cell response against BP180, the major autoantigen of BP. These findings identify a well-known autoantigen of the skin as a potentially relevant target antigen of LP.

P131

Regulation of GM-CSF production by human T helper cells

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GM-CSF is a hematopoietic growth factor with pleiotropic functions. Previous studies on experimental autoimmune encephalomyelitis (EAE) have demonstrated an essential pathogenic role for T cell derived GM-CSF in autoimmunity. However, the role of GM-CSF in human inflammatory diseases has not been established yet nor has its regulation in human T helper cell subsets been addressed. In this study we report the existence of GM-CSF producing human T helper cells *in vivo* that lack co-expression of other lineage defining cytokines such as IFN-γ, IL-4 and IL-17 and their respective transcription factors T-bet, GATA-3 and ROR-γt. Although this suggests the existence of a novel T helper cell subset we could demonstrate that the classical Th1, Th2 and Th17 cell subsets could acquire GM-CSF production abilities. Therefore GM-CSF production was not restricted to a separate T cell subset but also a universal feature of other polarized human T helper cell subsets. To address its role in autoimmune pathogenesis, we isolated T helper cells from psoriasis plaques and healthy skin and compared their cytokine profiles. T cells from diseased skin had higher expression of IL-17 and IL-22, two cytokines reported in the pathogenesis of psoriasis, but lower levels of GM-CSF than T cells in healthy skin.
Our results suggest that GM-CSF cannot be considered a major driver of autoimmune tissue inflammation in humans in contrast to mice due to its expression by all T helper subsets and its reduced production in inflammatory skin tissue. The physiological role of memory T cell derived GM-CSF remains to be identified.

P132

Identification of heterogeneous human Treg cell subsets with implications in the pathogenesis of Acne inversa

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Immune responses are tailored to protect against particular types of pathogen encounters. A successful immune defense strategy however also requires intricate negative regulation to restrict inflammation induced host damage. FOXP3 + regulatory T (Treg) cells are a broadly acting and potent anti-inflammatory population of CD4 + T cells essential for maintaining immune homeostasis and preventing autoimmune inflammation. Although Treg cells are generally considered to be a separate lineage of CD4 + T cells, recent murine studies have indicated that they use different transcriptional programs to regulate Th1, Th2, or Th17 responses, and that these are associated with the expression or activation of specific T helper cell-associated transcription factors. This implicates phenotypic and functional heterogeneity within the Treg compartment. We therefore set out to analyze if Treg cells in humans also display functional specialization. We could demonstrate the existence of distinct human Treg subsets with different migration capacities that correlated with different types of immune functions such as cytokine production. These Treg subsets matched their Th1, Th2 and Th17 effector cell counterparts (Tcon), but retained their suppressive function. Treg as compared to Tcon cells showed a homing bias towards the peripheral body surfaces such as the skin where they are expected keep microbiota induced immune responses in check. We could also demonstrate that in inflammatory diseases the relative composition of subsets within the Treg compartment is altered. In Acne inversa, skin homing Tregs are reduced whereas IL-17 producing Treg cells are increased.
Thus, we could demonstrate a so far unrecognized functional heterogeneity.

P133

RNase 7 promotes the uptake of bacterial and self-DNA and production of IFN-α by human plasmacytoid dendritic cells

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Keratinocytes are an important source for antimicrobial peptides which represent a chemical defence system against bacteria, fungi and viruses.
RNase 7 is one of the major antimicrobial peptides (AMPs) secreted by keratinocytes. It is constitutively expressed in the epidermis of healthy human skin and upregulated in chronic inflammatory skin diseases such as atopic dermatitis and psoriasis. Recently, immunregulatory functions have been published for several AMPs produced by keratinocytes. To date no immunregulatory function has been described for RNase7.
In the current study we demonstrate that RNase 7 enables rapid sensing of bacterial and self-DNA by human plasmacytoid dendritic cells (pDCs) which leads to strong IFN-α production. The induction of IFN-α production was mediated by activation of TLR9 and was sufficient to induce the upregulation of antiviral proteins as well as to inhibit herpes simplex virus type 1 (HSV-1) infection of human keratinocytes. Furthermore, experiments with a ribonuclease-inactive recombinant RNase 7 mutant show that RNase 7 ribonuclease activity is dispensable for the induction of IFN-α production.
Plasmacytoid dendritic cells (pDCs) are a dendritic cell population highly specialized for sensing viral and microbial DNA through endosomal Toll-like receptors to produce type 1 interferons and have been described to accumulate in the skin of patients with inflammatory skin diseases such as psoriasis.Our data indicate that RNase 7 has immunmodulatory functions and supports the efficient recognition of microbial infection by human pDCs.

P134 (O03/03)

Enhanced MHC class I molecule expression on Merkel cell carcinoma by HDAC inhibitors

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Introduction: Merkel cell carcinoma (MCC) is strongly associated with the Merkel cell polyomavirus (MCPyV) in most patients. Despite the expression of MCPyV derived viral antigens, advanced MCC escapes immune responses. Cytotoxic T cells only recognize processed viral antigens in the context of MHC class I molecules. Indeed, loss of MHC class I expression is a common immune escape mechanism of transformed and/or virus infected cells.
Methods: In situ expression of MHC class I and MCPyV large T antigen was evaluated in 57 paraffin embedded MCC tumors of 41 patients by immunohistochemistry. MCPyV DNA was detected by PCR. MHC class I expression of six MCPyV+ MCC cell lines was determined by immunoblot and flow cytometry, either without or after treatment with epigenetic modifiers (vorinostat, mithramycin A). Chromatin immunoprecipitation (ChIP) assay was performed to determine histone acetylation at the MHC class I promoter region. A flow cytometry based cytotoxicity assay, with lymphokine activated killer (LAK) cells was performed with or without an MHC class I blocking antibody. Finally, the effect of epigenetic modifiers on MHC class I expression was tested *in vivo* in a preclinical xenotransplantation model.
Results: MHC class I molecules were not or only weakly expressed in 75% of MCC tumors and their expression was significantly lower in MCPyV+ compared to MCPyV- MCC tumors ($P = 0.003$). Similarly, only two out of six MCPyV+ MCC cell lines expressed high levels of MHC class I molecules. Treatment of MCC cell lines with the HDAC inhibitor vorinostat in combination with mithramycin A increased histone acetylation at the MHC class I promoter region, resulting in a significantly increased MHC class I expression ($P < 0.05$). Vorinostat and mithramycin A displayed synergistic effects. Notably, increased MHC class I expression allowed an increased LAK-cell mediated lysis of MCC cells. This effect was fully blocked with a MHC class I blocking antibody. Treatment of mice bearing xenotransplanted MCC tumors with the combination of vorinostat and mithrmaycin A induced an increased MHC class I expression *in vivo*.
Conclusion: MHC class I down-regulation is a common immune escape mechanism in MCC. MCPyV+ tumors express particularly low levels of MHC class I molecules to prevent viral antigen presentation. Here, we report an efficient way to increase MHC class I surface expression on MCPyV+ MCC cells *in vitro* and *in vivo* by combinatory treatment with two FDA approved epigenetic modifiers, i.e. vorinostat and mithramycin A. Increasing MHC class I expression and thus viral antigen

presentation appears as an attractive strategy to boost immune therapeutic approaches for MCCs, such as checkpoint blocking antibodies

P135

Targeting tumor-associated M2-macrophages using nanoparticles to improve cancer immunotherapy

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Tumor-associated, M2-polarized macrophages form a significant part of the inhibitory tumor microenvironment and were shown to promote tumor growth by suppressing immunocompetent cells as well as inducing neoangiogenesis. They are increasingly becoming principal targets of novel therapeutic approaches such as nanoparticles that can be functionalized for therapeutic applications in tumor medicine.

In the present study we compared distinct features of human differentially polarized inhibitory M2 macrophages and immunostimulatory M1 macrophages and their targeting by nanoparticles. We used dextran particles that are biodegradable, show a high loading capacity, provide the possibility of a targeted release of their cargo and thus seem to be ideal candidates for immunotherapeutic intervention. The particles used in our study were all between 100 and 150 nm in diameter. In *in vitro* experiments we could show that incubation with nanoparticles is non-toxic for monocytes and macrophages. As they are cells of the myeloid lineage that function as natural phagocytes, NP uptake was over 90% in both populations. Additionally, we did not found any influence of non-charged particles on the characteristic phenotyp of CD206highCD80low M2 and the CD80highCD206low M1 macrophages respectively. In order to test the functional influence of nanoparticles on human macrophages, we cocultured nanoparticle-loaded macrophages with allogeneic CD4 + T cells and analyzed their T cell stimulatory capacity. As expected M1 macrophages induced a stronger T cell proliferation compared to M2 macrophages. Nevertheless, incubation with nanoparticles slightly upregulated the T cell stimulatory capacity of both macrophage populations.

The results obtained show that the nanoparticular approach itself is a feasible method to target human macrophages in order to reprogram inhibitory M2 into immunostimulatory M1 macrophages. The presented *in vitro* system allows the validation of different nanoparticular formulations before particles will be validated *in vivo* using murine models of cancer metastasis to lungs and liver.

To conclude, the development of engineered nanocapsules as drug delivery systems offers enormous potential for breaking immune tolerance to tumors that is mediated by (innate) stromal suppressor cells. Especially the targeting of molecules such as siRNA via biodegradable nanoparticles is an attractive emerging option for the treatment of (metastatic) cancers.

P136 (O02/04)

Normal steady-state, but increased cutaneous immune responses in barrierdisrupted filaggrin/hornerin (FLG/HRNR)-deficient mice

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An intact skin barrier is a prerequisite to maintain homeostasis in healthy skin. Several barrier gene mutations lead to a diminished skin barrier function and therefore predispose to develop skin diseases. Among them is filaggrin (FLG) which was identified as the most important risk factor for skin barrier disruption leading to atopic dermatitis and eczema-related asthma. Another genetic risk locus, namely hornerin (HRNR), has been implicated in atopic eczema in a FLG-independent manner. HRNR is very similar to FLG with respect to gene and protein structure, its close localization within the epidermal differentiation complex, and its expression pattern and localization in the epidermis.

In our mouse model with combined FLG/HRNR-deficiency we can assess the role of these two proteins in skin barrier formation and cutaneous inflammation in barrierdisrupted skin as seen in patients with atopic predisposition.

Constitutive knockouts for FLG and HRNR were performed in BL/6 embryonic stem cells with targeting vectors carrying a loxP-flanked neomycin/puromycin resistance cassette. Further breeding of chimeras with Cre-deleters led to the completely excision of the genetic loci encoding for both genes including the intergenic region. Homozygous FLG/HRNR-deficient mice have a normal litter size and neonates appeared normal at birth, but phenotypic aberrations became apparent at day 4. Mice show an overall flaky appearance with dry skin and peeling all over the body, strictures around limbs and the tail, and partial abduction of the paws. Starting at day 10, the phenotype improves until at day 21 mice appear quite normal except for the smaller ears and auto-amputated tails. With age mice do not show an overt skin phenotype with regard to spontaneous atopy or eczema under specific pathogen-free conditions.

Histologically, FLG/HRNR-deficient mice display a markedly reduced granular layer compared to epidermis of wild type controls. The basal and spinous layers appear normal and no further alterations of classical differentiation markers could be detected neither at the level of transcription nor at protein level. The baseline integrity of the skin barrier function was tested for outside-in as well as inside-out barrier, but no abnormalities were found in FLG/HRNR-deficient mice. Further, the recovery of skin barrier functions after barrier-disruption by tape stripping is normal in these mice.

The immune status in FLG/HRNR-deficient mice in steady state is normal as determined by screening for cellular composition in skin and skin-draining lymph nodes as well as basic immunoglobulin levels in blood. However, inflammatory conditions e.g. during allergic and irritant contact dermatitis induced elevated cutaneous immune reactions indicating for a barrier-deficiency allowing enhanced allergen-penetration, sensitization and finally higher inflammation.

Together, we assessed the consequences of barrier disruption by the two epidermal barrier proteins, filaggrin and hornerin, on subsequent skin inflammation in a mouse model of FLG/HRNR-deficiency. Unrevealing the pathway by which this deficiency affects cutaneous immune reaction will be the next step in understanding the importance of impaired barrier in the development of skin diseases and further opens new strategies for therapeutic interventions.

P137

The retinoid-related orphan receptor alpha is essential for the end-stage effector phase of experimental epidermolysis bullosa acquisita

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Genetic studies have added to the understanding of complex diseases. Here, we used a combined genetic approach for risk-loci identification in a prototypic, organspecific, autoimmune disease, namely experimental epidermolysis bullosa acquisita (EBA), in which autoantibodies to type VII collagen (COL7) and neutrophil activation cause mucocutaneous blisters. Anti-COL7 IgG induced moderate blistering in most inbred mouse strains, while some showed severe disease or were completely protected. Using publicly available genotyping data, we identified haplotype blocks that control blistering and confirmed two haplotype blocks in outbred mice. To identify the blistering-associated genes, haplotype blocks encoding genes that are differentially expressed in EBA-affected skin were considered. This procedure identified nine genes, including retinoid-related orphan receptor alpha (RORα), known to be involved in neurological development and function. After anti-COL7 IgG injection, RORα+/− mice showed reduced blistering, and homozygous mice were completely resistant to EBA induction. Furthermore, pharmacological RORα inhibition dose-dependently blocked reactive oxygen species (ROS) release from activated neutrophils but did not affect migration or phagocytosis. Thus, forward genomics combined with multiple validation steps identifies RORα to be essential to drive inflammation in experimental EBA.

P138

Radiosensitive cells determine clinical disease manifestations in the effector phase of epidermolysis bullosa acquisita

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Animal models enhanced our understanding of the pathogenesis of autoimmune diseases. Inbred mouse strains, however, show a high variability in disease manifestation. Identifying the factors that influence this disease variability could provide unrecognized insights into pathogenesis. We established an antibodytransfer model of epidermolysis bullosa acquisita (EBA), an autoimmune disease characterized by (muco)-cutaneous blistering caused by anti-type VII collagen (COL7) autoantibodies. Blistering after anti-COL7 IgG transfer showed clear variability among inbred mouse strains. The transfer of anti-COL7 IgG into irradiated, EBA-resistant MRL/MpJ mice rescued by transplantation with bone marrow from EBA susceptible B6.AK-H2k induced blistering. To the contrary, irradiated EBAsusceptible B6.AK-H2k mice that were rescued using MRL/MpJ bone marrow were devoid of blistering. Immune complex-activation of neutrophils from C57Bl/6J or MRL/MpJ mice showed an impaired ROS release from the latter, whereas no differences were observed after PMA activation. This finding was comparable relative to the major differences in mRNA expression. Collectively, we demonstrate that radiosensitive cells determine the varying clinical disease manifestations in the endstage effector phase of EBA.

P139

Prevalence and functional characteristics of pemphigus and pemphigoid autoantibodies in the general population

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Mucocutaneous blistering is the clinical characteristic of autoimmune skin blistering dermatoses (AIBD). In AIBD, blistering is caused by autoantibodies directed against structural components of the skin. Hence, the detection of specific autoantibodies has become a hallmark for AIBD diagnosis. Studies on prevalence of AIBD autoantibodies in healthy individuals are contradictory. To clarify this, samples from 7063 normal blood donors were tested for presence of anti-BP180-NC16A, anti- BP230 and anti-Dsg1/3 IgG by indirect immunofluorescence (IF) using a biochip. Cumulative prevalence of these autoantibodies was 0.9% (CI: 0.7–1.1%), with anti-BP180-NC16A IgG being the most prevalent. Validation of IF findings using commercially available ELISA kits confirmed presence of autoantibodies in 7/15 (anti-Dsg1), 6/7 (anti-Dsg3), 35/37 (anti-BP180-NC16A) and 2/3 (anti-BP230) cases. Moreover, in 16 samples, anti-BP180-NC16A autoantibody concentrations exceeded the diagnostic cut-off. Interestingly, these anti-BP180-NC16A autoantibodies from healthy individuals formed immune complexes with the recombinant antigen and dose-dependently activated neutrophils. Fine-epitope mapping within the NC16A domain, however, showed a different binding pattern of anti-BP180-NC16A autoantibodies from healthy individuals compared to bullous pemphigoid patients, while IgG subclasses were identical. Collectively, we report a low prevalence of AIBD autoantibodies in healthy individuals. Furthermore, functional analysis shows differences between autoantibodies from healthy donors and AIBD patients.

P140 (O06/04)

Curcumin protects from inflammatory autoimmune disease by suppressing IL-12/IL-23p40

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Curcumin (diferuloylmethane) is a naturally occurring yellow pigment isolated from the rhizomes of the plant Curcuma Longa. It has been reported that curcumin may possess anti-inflammatory activities and therefore is traditionally used in inflammatory disorders in some regions of Asia. However, the underlying mechanisms that could explain its beneficial activity during inflammation are not fully understood. In this study we analyzed whether curcumin is able to modify immune responses *in vitro* and *in vivo*. First, we investigated the potential anti-inflammatory activities of curcumin on mouse dendritic cells (DC) *in vitro*. Gene expression of curcumin treated DC or control DC activated through TLR4 was analyzed by PCR-arrays. Curcumin treated DC showed an upregulation of genes of the TNF superfamily, certain caspases and NFκB inhibitory genes. Interestingly, curcumin impaired the production of the cytokines interleukin (IL-)12 and IL-23 by TLR4-activated DC. To further investigate the effects of curcumin on immune responses *in vitro* we co-cultured isolated CD4 + T cells from mice immunized with a myelin peptide in CFA together with fresh APC and PLP peptide in the presence or absence of curcumin. After re-stimulation, curcumin treated cultures showed an inhibition of the Th1 and Th17 cytokines IFN-γ and IL-17, while the expression of the Th2 cytokines IL-4 and IL-10 increased. To assess the *in vivo* effects of curcumin, mice were immunized with myelin peptide in CFA, treated with curcumin or vehicle and followed for the development of autoimmune encephalomyelitis (EAE). Control mice developed severe symptoms of EAE, while mice treated with curcumin remained healthy or developed only mild disease. The protection from EAE by curcumin treatment was associated with a suppression of IL-12/IL-23p40 and subsequent Th1 and Th17 responses *in vivo*. Moreover, curcumin treatment induced IL-4 and IL-10 expression in CD4 + T cells after active immunization. Taken together curcumin treatment seems to be a promising approach for improving IL-12/IL-23-dominated autoimmune diseases by using a natural extract.

P141

Does human skin might contain macrophage progenitors?

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From the point of view of their origin, in mice, macrophages (MΦs) consist of two different classes: tissue-resident (trM) and infiltrating MΦs. The latter ones derive from monocytes, whereas the trM originate from the yolk sac. In non-inflammatory environment the monocytes do not show any contribution to the macrophage population and the 'physiological' macrophage number in the peripheral tissues of mice is thought to be maintained by self-renewing trM. However, although selfrenewing trMΦ have been reported in murine skin, it is unknown whether such cells exist in human skin. Therefore, we aimed to search for evidence that trMΦ precurors may also exist in human skin using microdissected and organ-cultured full-thickness human skin, in which local inflammation was induced by the pro-inflammatory neuropeptide, substance P (SP). In the absence of perfused vasculature or bone marrow, SP treatment significantly increased the number of MHC class II+ cells (quantitative immunohistomorphometry). The likelihood that the majority of these of these are likely to represent trMΦs was supported by the finding that the number of CD68 + MΦs was also increased in the test group. However, this does not appear to result from cell proliferation, as no CD68 + /Ki-67 + were visible in the dermis in either test or control skin. These pilot data suggest that trMP progenitor cells are present in human skin, and that their differentiation can be triggered by pro-inflammatory stimuli such as SP. Additional immunomorphometric analyses are underway to further explore this hypothesis (e.g. using phospho histone 3/CD68double-staining…). We are also currently analyzing the phenotype of this newly generated macrophage population. Preliminary data shows an increase in the number of CD206 + cells in SP treated compared to untreated samples, indicating a generation of new anti-inflammatory (M2) MΦ population. This investigation will contribute to the understanding of the modulation of macrophage precursors and their progeny in human skin, with potential relevance for inflammatory dermatoses characterized by a persistence of altered, pro-inflammatory macrophages, such as in atopic dermatitis and helminth infections.

P142

Blocking the activating Fc gamma RIV enhances neutrophil extravasation into the skin in autoantibody-induced tissue injury

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Autoimmune diseases have become a major medical burden. Many autoimmune diseases are characterized by the presence of autoantibodies, and in some, e.g. autoimmune bullous dermatoses (AIBDs), autoantibodies directly cause clinical disease manifestation. Recently, we established imaging techniques to visualize interactions of autoantibodies, target tissue and effector cells (neutrophils) *in vivo*. This led to the identification of checkpoints for autoantibody-induced tissue injury exemplified in an animal model of epidermolysis bullosa acquisita (EBA), an AIBD caused by autoantibodies to type VII collagen (COL7): (i) Immediate autoantibody binding to the target tissue, influenced by mechanical trigger factors, (ii) rapid neutrophil recruitment into the vicinity of autoantibody deposits and (iii) delayed and short-term neutrophil localization to these deposits and subsequent autoantibodyinduced tissue damage. Following up these findings, we here aimed to unravel the molecular control of these checkpoints. As the activating Fc gamma RIV (FcgRIV) is crucial for blistering in EBA, we evaluated the impact of FcgRIV inhibition on neutrophil recruitment to the immune complexes bound to the dermal-epidermal junction (DEJ). Therefore, fluorescently labeled, fully pathogenic, anti-COL7 IgGs were injected into LysM-EGFP mice (EGFP transgenic mice under the lysozyme M promotor) in the presence of either function blocking FcgRIV antibody (clone 9E9) or corresponding isotype control antibody. Using two-photon microscopy, we studied autoantibody binding and neutrophil extravasation into the dermis and their recruitment to the DEJ *in vivo* following a defined time course (1, 3 and 8 days after the initial anti-COL7 IgG injection for the induction of EBA). Surprisingly, we observed a rapid extravasation of LysM-EGFP+ cells into the skin which was significantly higher in mice treated with the FcgRIV-blocking antibody compared to the isotype control-treated mice at all of the three time points. In contrast, localization of LysMEGFP+ cells along the DEJ was, with the exception of day 8, significantly reduced in anti-FcgRIV antibody treated mice. These findings are in sharp contrast to the previously reported role of activating FcgRs, which were shown to promote leukocyte extravasation, as well as our own findings reporting a complete protection from skin blistering in experimental EBA after blockade of the FcgRIV. We hypothesize that following the failure of being 'appropriately' activated, i.e. at the site of immune complex deposition, uncontrolled neutrophil activation occurs, which leads to the perpetuation of further neutrophil recruitment into the skin. These unexpected findings underscore the importance of advanced *in vivo* imaging techniques to fully understand the complexity of antibody-mediated neutrophil-dependent tissue injury.

P143 (O06/06)

Contribution of IgE auto-antibodies to the pathogenesis of bullous pemphigoid

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Bullous pemphigoid (BP) is an auto-immune disease typically associated with old age. It is characterized by bullae at the dermal-epidermal junction (DEJ) that are thought to be induced by the binding of auto-antibodies. These antibodies can recruit inflammatory cells through complement activation, culminating in the proteolytic destruction of cell adhesion structures. While IgG has been the class consistently associated with the disease, more recent studies point to a potential involvement of IgE. Consistent with previous literature, we have detected IgE in perilesional skin of 22 out of 33 (67%) BP patients. This IgE was not found at the DEJ, but instead on the surface of mast cells, dendritic cells and eosinophils, most likely bound as an immune complex. We have evidence that the high-affinity receptor for IgE is the primary molecule involved in this interaction and that eosinophils are expressing FcεRI in BP patients. Furthermore, using whole skin lysates for immunoblotting, we have demonstrated peripheral BP IgE reactivity against antigens with approximately 60, 120, 180 and 230 kD. These likely represent intra- and extra-cellular domains of BP180 and the full-length BP180 and BP230 proteins, respectively. Given that the clinical picture of BP consists of erythema and bullae, appearing alone or concomitantly, an association between self-reactive IgE and urticarial-like lesions is therefore plausible and suggests an alternative pathway of disease pathogenesis. Uncovering the dominant epitopes for both IgG and IgE in different presentations of the disease could further clarify this question and additionally argue for the development of new IgE-based therapeutic approaches.

P144

Prevalence of laminin 332-specific autoantibodies, detected by a novel enzyme-linked immunosorbent assay, in patients with mucous membrane pemphigoid

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Mucous membrane pemphigoid (MMP) is an autoimmune blistering disease characterized by autoantibodies directed against anchoring proteins at the basement membrane zone. The disease affects both the skin and mucosal surfaces and associates with significant morbidity. The several diagnostic tools commonly used are not specific enough in differentiating MMP to other autoimmune subepidermal blistering diseases. The aim of the present study was to develop an accurate immunoassay for assessing the presence of autoantibodies against laminin 332 in a large cohort of patients with mucous membrane pemphigoid. Enzyme-linked immunosorbent assay (ELISA), using commercially available laminin 332, was performed in 200 patients with mucous membrane pemphigoid and 70 controls. The results were validated by immunoblot using extracellular matrix extract from HaCaT cells. The developed assay could represent a useful tool for the diagnosis of patients with mucous membrane pemphigoid.

P145

RAGE signaling drives the initiation and maintenance of the psoriatic inflammation

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Psoriasis is a common complex genetic disease of the skin characterized by epidermal hyperproliferation and chronic inflammation. The cause of psoriasis is unknown, and the relative contribution of keratinocytes and immune cells to disease initiation and maintenance remains 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. Our study aims at elucidating the role of RAGE and its ligands S100A8/A9, S100B, and HMGB1 on myeloid cells for cutaneous inflammatory responses regarding quality and quantity of the immune response using mouse models and material from psoriatic patients. Here, we show that RAGE ligands are highly upregulated in biopsies of human psoriatic lesions and in serum specimens of affected patients. Moreover, RAGE is found to be expressed in psoriatic lesions by keratinocytes, endothelial cells and dermal inflammatory cells, e.g. CD11c-positive dendritic cells (DC), suggesting an epidermal-dermal cross-talk of RAGE signaling involving DC. RAGE-deficient mice were resistant to development of a psoriasiform inflammation upon topical imiquimod treatment. This effect was rescued by injections of recombinant IL-23 suggesting IL-23 as putative target of RAGE signaling. In summary, our data point towards a central role of RAGE signaling in the initiation and maintenance of psoriatic plaques, therefore representing potential therapeutic target in psoriasis.

P146

IgE reactivity against the NC16A domain of BP180 in bullous pemphigoid: influence of total IgE serum levels

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IgE autoantibodies against the non-collagenous domain of BP180 (NC16A) have previously been detected in the sera from patients with bullous pemphigoid (BP) and were suggested to play a pathogenic role in two mouse models of the disease. Recent reports about the successful treatment of BP with the anti-IgE antibody omalizumab have further fuelled the detection of IgE autoantibodies in this disease. However, the extent to which BP patients develop IgE autoantibodies against BP180 NC16A varied in different reports between 22% and 77%. For further clarification we here developed an ELISA for the detection of serum IgE antibodies against BP180 NC16A based on the recombinant NC16A tetramer as previously established for IgG reactivity (Euroimmun, Lübeck). Serial dilutions of serum (pure, 1:10, 1:100) in sample buffer and secondary antibody (pure, 1:10 and 1:50) were employed. Analysis of consecutive BP patients ($n = 65$) as well as sera from age-matched patients with chronic non-inflammatory dermatoses ($n = 49$) resulted in a sensitivity of 78.5% with a specificity set to 98%. Since elevated total IgE levels have been reported in BP patients we assessed our BP cohort and found elevated total IgE levels in 58.5% patients in contrast to only 16.3% in the age-matched controls. As second control group we therefore included patients with inflammatory skin diseases with various total IgE serum levels ($n = 32$, IgE >500 IE/ml; $n = 16$, IgE 100–500 IE/ml; $n = 18$, IgE <100 IE/ml). Based on this control group, the sensitivity of IgE reactivity in our BP cohort was 41.5% with a specificity of 98%. When both control groups were combined to match total IgE levels with those of BP patients the sensitivity was 49.2%. This study found IgE anti-BP180 NC16A antibodies in about half of BP patients and highlights the importance of appropriate control groups for the detection of IgE anti-BP180 reactivity.

P147

Interaction of corynebacteria with skin-derived antimicrobial peptides

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Corynebacteria belong to the commensal skin microbiota and are often isolated from normal healthy skin. Since they are able to cause opportunistic infections their growth on the skin surface has to be tightly controlled and restricted. Antimicrobial peptides (AMP) are important effector molecules controlling the growth of microorganisms on the skin surface due to their potent antimicrobial activity. Since nothing is known about the interaction of AMP and corynebacteria we initiated a study to investigate the susceptibility of corynebacteria towards AMP and to analyze AMP expression in keratinocytes infected with corynebacteria.

Using an antimicrobial microdilution assay we found that the skin-derived AMP RNase 7, human beta-defensin (hBD)-2 and -3 exhibited potent antimicrobial activity in concentrations <1 μM against Corynebacterium amycolatum. In contrast, the AMP psoriasin (S100A7) required comparatively higher concentrations of 5–10 μM to restrict the growth of *Corynebacterium amycolatum*. *Corynebacterium xerosis* was also effectively killed by AMP indicating that AMP are in general active against corynebacteria. These findings were paralleled by a potent activity of stratum corneum against corynebacteria. Activity of stratum corneum against *Corynebacterium amycolatum* decreased by the use of a neutralizing antibody against RNase 7 indicating that RNase 7 contributes to the killing activity of stratum corneum against corynebacteria. Treatment of primary keratinocytes with living corynebacteria

induced the expression of RNase 7 as well as hBD-2 and-3. The use of a specific antibody directed against the epidermal growth factor receptor (EGFR) revealed that the activation of the EGFR is involved in the induction of RNase 7 and hBD-3 by *Corynebacterium amycolatum*.
In summary, our data indicate that keratinocytes are able to recognize corynebacteria leading to the induction of several AMP such as hBD-2 and -3 as well as RNase 7. The potent activity of these AMP against corynebacteria suggests that AMP contribute to control the growth of corynebacteria on the skin surface.

P148 (O04/02)

A new mucosal vaccine targets a distinct dendritic cell subset to convert a tolerogenic into a protective immune response against *Chlamydia trachomatis*

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Vaccines that are administered via non-mucosal routes are often poorly protective against mucosal pathogens, presumably because such vaccines do not generate memory cells that migrate to mucosal surfaces. Although mucosa-tropic memory cells are inducible by mucosal immunization, few mucosal vaccines are currently in clinical use because live vaccine vectors pose safety risks and killed pathogens or molecular antigens (Ags) are weak immunogens when applied to intact mucosa. This poor immunogenicity can potentially be overcome by adjuvants; however, most conventional mucosal adjuvants possess unfavorable safety profiles and the immune mechanisms of protection against many mucosal infections are not well understood. One case in point is *Chlamydia trachomatis* (Ct), a sexually transmitted intracellular pathogen that can cause mucosal infections resulting in female infertility and ectopic pregnancies.
In mice, genital Ct infection induces protective immunity that is thought to depend on interferon-γ (IFN-γ) producing CD4 T cells. By contrast, we observed that mucosal exposure to UV-inactivated Ct (UV-Ct) generates tolerogenic Ct-specific regulatory T cells, resulting in exacerbated bacterial burden upon subsequent Ct infection. Here, we show that mucosal immunization with UV-Ct complexed with charge-switching synthetic adjuvant particles (cSAP) did not exert the tolerogenic effect of UV-Ct alone but elicited long-lived protection against genital Ct infection. This differential effect of UV-Ct-cSAP versus UV-Ct was because the former was preferentially presented by immunogenic CD11b+CD103− dendritic cells (DCs), while the latter was primarily acquired by tolerogenic CD11b−CD103 + DCs. Notably, genital protection was achieved after either intrauterine (i.u.) or intranasal (i.n.), but not subcutaneous (s.c.) immunization with UV-Ct-cSAP. Only mucosal vaccination, like mucosal infection with live Ct, induced an early wave of Ct-specific CD4 memory cells that established longterm residence in the genital mucosa. Antibody inhibition experiments and studies in parabiotic mice showed that in the absence of early mucosal seeding by tissueresident memory cells, mice were poorly protected against Ct, even when circulating memory cells were abundant.
Thus, using a novel platform for mucosal immunization, we demonstrate that protection against Ct depends on the synergistic action of two discrete memory T cell subsets with distinct differentiation kinetics and migratory properties.

P149 (O02/03)

BAY61-3606, a novel spleen tyrosine kinase inhibitor, attenuates organspecific, autoantibody-induced tissue injury

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Autoimmune bullous dermatoses (AIBD) are chronic inflammatory, organ-specific diseases, characterized by mucocutaneous blistering and autoantibodies against desmosomal or hemidesmosomal antigens. AIBD have become a major medical burden with high morbidity and mortality. Spleen-tyrosine-kinase (Syk) is a crucial intracellular player in the modulation of the immune system. It acts downstream of Fc receptors and modulates several cellular subtypes. The role of Syk in the inflammatory disease manifestation is rather ambiguous and the role of Syk is yet to be evaluated in AIBD. Here, we describe the impact of BAY61-3606, a selective inhibitor of Syk, evaluated in experimental models of epidermolysis bullosa acquisita (EBA), an AIBD caused by autoantibodies to type VII collagen (COL7). *In vitro*, incubating freshly isolated neutrophils on immune complexes containing antibodies to COL7 together with BAY 61-3606, inhibited immune complex (IC)-induced reactive oxygen species (ROS) release in a dose-dependent manner. Additionally, BAY61-3606 treatment also inhibited IC-induced neutrophil activation by altering expression of carcinoembryonic antigen-related cell adhesion molecule 8 (CEACAM8 or CD 66b) and L- selectin (CD 62L). Inhibition of Syk did not show any changes on PMN cell viability, as measured by annexin-V and propidium iodide (PI) staining. In another experimental model of EBA, inhibition of Syk impaired dermal-epidermal separation induced by the incubation of human skin sections with anti-COL7 IgG and neutrophils *ex vivo*. Finally, *in vivo*, EBA can be induced in mice by repetitive injections of anti-COL7 IgG. Prophylactic treatment with BAY61-3606, however, impaired induction of skin blistering in a dose-dependent manner. To evaluate a cell lineage-specific impact of Syk inhibition during the effector phase of EBA, we used the cre-lox system to conditionally knock-out Syk. Mice that did not express Syk on myeloid cells [SYK (fl/fl) LysM-Cre (+)], in contrast to littermate controls, were completely protected from the induction of skin blisters. Interestingly, however, mice lacking Syk in lymphoid cells [(SYK (fl/fl) CD2-Cre (+)] did not show any protection from disease induction. This study demonstrates a key contribution of Syk to the effector phase of autoantibody-induced tissue injury in a prototypic, organ-specific autoimmune disease. Our results also identify Syk as a potential novel therapeutic target for EBA and related AIBD.

P150 (O05/02)

Deregulated production of reactive oxygen species by an age-dependently increased T cell subset leads to autoreactivity and immunosenescence

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A functional deficiency of the immune response is the result of an aging immune system termed immunosenescence. Since both the host defence and regulatory mechanisms decline with age, this leads to a higher prevalence of infection, tumor and autoimmune disease in the elderly.
We have previously characterized distinct age-dependent T cell subpopulations showing an increase in dysfunctional autoreactive T cells in peripheral lymphoid organs from young and adult to old mice (3–6, 9–12 and 18–24 months-old C57BL/6, $n > 4$). The majority of the CD4 + T cells showed an age-dependent increase in a CD27−/low CD28−/low CD5low subset indicating homeostatic expansion, which typically occurs due to a reduced thymic T cell output with age. The CD27−/low CD28−/low CD5low cell subset was also increased in CD8 + T cells and CD4− CD8− double negative (DN) T cells in an age-dependent fashion, suggesting these cells had an age-related increase in reactivity. Importantly these latter cells are characterized by an autoreactive TCR profile supporting autoimmunity while impairing regulation while age progresses. Recent reports indicate that imbalanced levels of reactive oxygen species (ROS) contribute critically to driving chronic inflammation and immunosenescence. Therefore, we used high-throughput eightchannel fluorescence FACS to further analyze the age-dependent CD27−/low CD28−/ low CD5low T cells regarding their ability of ROS production.
As a result CD27−/low CD28−/low CD3low CD5low T cells showed a reduced O_2^- radical and H_2O_2 production compared to phenotypically normal naïve and antigenexperienced control T cells. Such decreased levels of ROS result in reduced rather than oxidized cell surface proteins thereby increasing the activity of this autoreactive T cell subset. In comparison, phenotypically normal naive and antigen-experienced T cells showed a higher H_2O_2 production, turning down their activatability. The agedependently reduced H_2O_2 production of CD27−/low CD28−/low CD3low CD5low T cells was accompanied by increased intracellular O_2^- radical concentrations after treatment with the mitochondrial electron chain complex inhibitor rotenone *ex vivo*. This finding was even more dramatic in T cells from CD18 deficient mice, implying a role of CD18 deficiency in driving immunosenescence not only phenotypically but also on a molecular level. Our data reveal a substantial increase of pathogenic T cell subsets in old mice leading to an increased immune dysfunction. The agedependently increased O_2^- radical production *in vitro* was paralleled by an increase in lipopolysaccharid inducible peroxynitrite formation *in vivo*. This effect was ameliorated by CD18 deficiency, indicating a role of CD18 in immune suppression. Using immature dendritic cells (DC) and T cells of CD18-deficient and wildtype mice in crisscross stimulation/suppression assays, our data showed an impairment in ageand CD18-dependent suppression of prolifertation, which was antigen-specific.
Altogether, this indicates an age-dependent loss of DC-mediated immune regulation leading to an increase in autoreactive T cells driving immunosenescence. Our data may contribute to clarify important aspects of an age-associated dysfunction of T cells in an aging immune system responsible for infection, tumor and autoimmune disease in the elderly.

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Interleukin-10 receptor-1 expression in monocyte-derived antigen-presenting cell populations: dendritic cells partially escape from IL-10 inhibitory mechanisms

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Interleukin (IL)-10 is an immune mediator, of which strong anti-inflammatory and immunosuppressive effects were demonstrated *in vitro* and in mice models. On other hand, strongly elevated levels of IL-10 present in lesions of some chronic inflammatory diseases like atopic dermatitis are incapable to stop the inflammatory process. IL-10 mediates its effects via a transmembrane receptor complex consisting of two different chains, IL-10R1 and IL-10R2. While IL-10R2 is ubiquitously expressed and does not bind IL-10 primarily, the expression of IL-10R1 determines cellular responsiveness. However, the current knowledge about the expression and regulation of IL-10R1 is still limited. Here, we analyzed the expression of IL-10R1 on monocytic cell populations and demonstrated that human blood monocytes carry about 720 IL-10 binding sites on their surface. Compared to lymphocytes and various tissue cells as well as tissues, blood monocytes expressed highest IL-10R1 levels. The *in vitro* differentiation of these cells into macrophages provoked a further increase of IL-10R1 surface expression. In contrast, their differentiation into myeloid dendritic cells (mDCs) resulted in reduced mRNA and surface levels of IL-10R1. The different IL-10R1 levels expressed by the monocyte-derived antigenpresenting cell populations were reflected in their different responsiveness towards IL-10. Importantly, also *in vivo* developed immature macrophages and mDCs showed different IL-10 sensitivity. These data suggest that, compared to monocytes and macrophages, mDCs partially escape from IL-10 inhibitory mechanisms by downregulating IL-10R1. Given the high numbers of DCs in the skin of atopic dermatitis patients, the high IL-10 levels and the strong inflammation do not represent a contradiction. Our data also propose the use of different monocytic cells in different clinical situations. The application of IL-10-treated macrophages may be effective for tolerance induction. On the other hand, DCs with reduced IL-10 sensitivity may be helpful for cancer vaccination.

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Defective removal of ribonucleotides from DNA promotes systemic lupus erythematosus

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Systemic lupus erythematosus (SLE) is a heterogeneous autoimmune disease in which environmental exposures like virus infection and UV-irradiation trigger activation of the innate and adaptive immune system in genetically predisposed individuals. Heterozygous mutations of the human 3‘ repair exonuclease 1 are associated with SLE. Biallelic mutations in this enzyme cause Aicardi-Goutires syndrom, a rare encephalopathy with clinical manifestations similar to SLE that can also be based on biallelic mutations in ribonuclease H2 (RNaseH2). We therefore asked for associations of mutations in RNaseH2 and SLE. RNaseH2 is responsible for the removal of misincorporated ribonucleotides from DNA and is indispensable for genome surveillance. Here we demonstrate a genetic association for rare RNase H2 sequence variants with SLE. Such mutations impair RNase H2 function and result in accumulation of ribonucleotides in genomic DNA in AGS and SLE patient fibroblasts. The ensuing chronic low level DNA damage triggers a DNA damage response characterized by constitutive p53 phosphorylation and senescence. Patient fibroblasts proliferated slower than fibroblasts from healthy individuals and showed impairment of cell cycle progression. In addition, these primary cells show constitutive up-regulation of interferon-stimulated genes and an enhanced type I interferon response to the nucleic acid poly(I:C) and UV light-irradiation. Moreover, UV-induced cyclobutane pyrimidine dimer formation is significantly enhanced in ribonucleotide-containing DNA, providing a mechanism for photosensitivity in RNase H2-associated SLE. Collectively, our findings implicate RNase H2 in the pathogenesis of SLE, and suggest a role of DNA damage-associated pathways in increased basal and UV induced type I IFN expression for the initiation of autoimmunity.

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Secukinumab decreases inflammation as measured by a biomarker hsCRP in subjects with moderate-to-severe plaque psoriasis and concomitant psoriatic arthritis: subanalyses from two phase 3 studies

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Introduction and Objectives: Secukinumab, a fully human anti-interleukin-17A monoclonal antibody, demonstrated rapid, robust, and durable efficacy as well as acceptable safety versus placebo in the ERASURE study and versus placebo and etanercept in the FIXTURE study in moderateto- severe plaque psoriasis (Langley et al., 2014). This subgroup analysis evaluated the effects of secukinumab on levels of high-sensitivity C-reactive protein (hsCRP), a biomarker of skin and joint inflammation, in subjects with psoriasis and concomitant psoriatic arthritis (PsA).

Materials and Methods: In the 52-week, double-blind, placebo-controlled ERASURE study, subjects aged 18 years ($N = 738$) were randomized 1:1:1 to secukinumab sc 300 or 150 mg (1×/week for 4 weeks, then q4 wk starting at Week 4) or placebo. FIXTURE ($N = 1306$) had similar design with an additional treatment arm: etanercept sc 50 mg (2×/week for 12 weeks, then 1×/week). This subanalysis evaluated change in hsCRP levels from Baseline to Week 52 in subjects with concomitant PsA.

Results: Among subjects with concomitant PsA in ERASURE ($n = 171$; 23.2%), median hsCRP levels (in mg/l) at Baseline and Week 12 (primary endpoint), respectively, were 4.0 and 2.0 (secukinumab 300 mg), 5.2 and 2.2 (secukinumab 150 mg), and 4.4 and 4.7 (placebo). In subjects with concomitant PsA in FIXTURE ($n = 192$; 14.7%), median hsCRP levels at Baseline and Week 12, respectively, were 4.1 and 2.7 (secukinumab 300 mg), 3.4 and 2.1 (secukinumab 150 mg), 4.3 and 2.0 (etanercept), and 3.2 and 3.1 (placebo). Reductions in hsCRP at Week 12 were sustained to Week 52 in both studies. Secukinumab also reduced median hsCRP in subjects with the greatest impairment in physical function, defined as a baseline Health Assessment Questionnaire-Disability Index (HAQ-DI) score >0.5. In this group, median hsCRP levels at Baseline and Week 12, respectively, were 9.9 and 3.0 (secukinumab 300 mg), 7.4 and 2.3 (secukinumab 150 mg), 5.0 and 5.4 (placebo) in ERASURE and 5.3 and 3.5 (secukinumab 300 mg), 6.6 and 3.3 (secukinumab 150 mg), 4.3 and 2.0 (etanercept), and 6.0 and 9.4 (placebo) in FIXTURE. Reductions were sustained in both studies to Week 52. Secukinumab and etanercept were well tolerated with no unexpected clinically significant safety findings.

Conclusions: In subjects with psoriasis and concomitant PsA, secukinumab and etanercept were associated with pronounced and sustained reductions in hsCRP levels, indicating a decrease in inflammatory burden.

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The skin secretome of ectodermal dysplasia patients contains a reduced number of immune defense mediators

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In healthy human skin host defense molecules such as antimicrobial peptides (AMPs) contribute to skin immune homeostasis. In patients with the congenital disease ectodermal dysplasia (ED) skin integrity is disturbed and as a result patients have recurrent skin infections. The disease is characterized by developmental abnormalities of ectodermal derivatives and absent or reduced sweating. We hypothesized that ED patients have a reduced skin immune defense due to the reduced ability to sweat. Therefore, we performed a label-free quantitative proteome analysis of wash solution of human skin from ED patients or healthy individuals. A clear cut difference between both cohorts could be observed in cellular processes related to immunity and host defense. In line with the extensive underrepresentation of proteins of the immune system, dermcidin (DCD), a sweat derived AMP, was reduced in its abundance in the skin secretome of ED patients. Since ED patients frequently suffer from bacterial skin infections mainly caused by *Staphylococcus aureus*, we investigate if treatment of mice with a dermcidin containing ointment reduces *S. aureus* colonization. A significant reduction of colony forming units (CFU's) could be observed 24 h after the beginning of treatment with a DCD-1 containing ointment compared to mice treated with a placebo. This effect could be observed either for bacteria which were loosely attached to the epidermal surface, as well as for bacteria which were invaded into the skin. 72 h after the beginning of treatment again a reduction of CFU could be observed. Thus, dermcidin in the form of an ointment lead to a reduction of *S. aureus* load, which might be a new therapeutic option to prevent skin infections in ED patients. In summary, our proteome profiling provides insights into the actual situation of healthy versus diseased skin. The systematic reduction of immune system and defense related proteins may contribute to the high susceptibility of ED patients to skin infections and altered skin colonization.

P155 (O05/05)

Deficiency of mannose-binding lectin (MBL) negatively affects cutaneous leishmaniasis in naturally resistant mice

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The complement system is a powerful effector mechanism of the innate immune system in helping immunocompetent organisms to fight infection. The activation of complement on pathogen surfaces is initiated via the molecule C1 for the classical pathway, whereby the alternative pathway is initiated by activation of C3. Mannosebinding lectin (MBL), a member of the collectin family, mediates the activation of the antibody-independent lectin complement pathway via complexation with MBLassociated serine proteases. Additionally, it plays a decisive role in the induction of proinflammatory responses at an early phase of infection and maintenance of tissue integrity and homeostasis. Beside several murine studies focussing on bacterial or virus infections, little is known about the influence of MBL on defence mechanisms against protozoan, obligate intracellular Leishmania parasites. Thus, we investigated the role of various complement factors in C57BL/6 mice infected intradermally with 1000 metacyclic *Leishmania* (L.) *major* promastigotes. Interestingly, MBLA and -C gene-knockout (MBL-AC−/−, ko) mice showed significantly stronger ear lesion progression compared to resistant C57BL/6 wild types, C1q−/− or C2−/− mice accentuating the importance of both the MBL protein and the lectin complement pathway during *Leishmania* infections in contrast to other complement-related molecules. In parallel, significantly higher numbers of live parasites were detected in ears and spleen of MBL-AC−/− mice in comparison to wild type mice. Lower interferon-gamma (IFNγ) levels (~0.7-fold) were detected in supernatants of activated T cells in antigen-specifically stimulated draining lymph node (dLN) cells of infected MBL-ko mice supporting our findings on worsening of disease as this cytokine is a key player for mediating protection by inducing parasite killing inside of macrophages (MΦ). No differences were detectable in the levels of T cell-derived interleukin (IL)-4, IL-10, IL-17A, and dendritic cell (DC)-derived IL-12p40. In our experiments, the ko mice finally healed their infection. Next, we will focus on the parasite uptake by bone marrow-derived DC and tissue MΦ from both mouse strains after *in vitro* infection with different life-forms of *L. major*, while parasites are opsonized with normal or MBL-deficient mouse serum. We aim at further investigating how the strong phenotype in these mice after infection with *Leishmania* parasites is influenced by the MBL protein and which mechanisms finally lead to survival of these knockout animals.

P156

Post-septic immune-suppression following Gram positive sepsis is mediated by TLR dependent induction of myeloid derived suppressor cells

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Systemic Gram+ bacterial infection (sepsis) is a leading cause of death among critically ill patients. Treatment of acute sepsis has been improved, but secondary infections due to post-septic immune suppression are still associated with high mortality and underlying mechanisms are poorly understood. Therefore, we established a mouse model of Gram+ sepsis. Mice were iv. infected with Staphylococcus aureus SA113. Weight and bacterial CFU (kidneys) indicating sepsis severity, immune cell populations and cytokines were determined at different time points. Post-septic immune status was assessed by determining a cutaneous Tcell mediated recall response to FITC (FITC-contact hypersensitivity; CHS). Indeed, immune suppression in these mice was evident as FITC-CHS was significantly reduced. Strikingly, we found a massive expansion of the Gr1 + CD11b + so called myeloid derived suppressor cells (MDSC). We found induction of Ly6GCD11b+ granulocytic MDSC (grMDSC) but stronger and longer upregulation of Ly6C+Ly6G-CD11b+ monocytic MDSC (mMDSC) paralleled by reduced numbers of plasmacytoid, CD8− and activated dendritic cells (DC), suggesting inhibition of DC differentiation to be associated with MDSC accumulation. Importantly, mMDSC and grMDSC differed in their functional properties. Compared to grMDSC, mMDSC showed strong and sustained proliferative activity and a more immature phenotype during early infection with increased differentiation capacity later (expression of CD115, MHCII and F4/80). Only mMDSC, but not grMDSC completely blocked T-cell activation *ex vivo*, depending on NO and oxygen radicals. Elimination of MDSC by anti-Gr1 depleting antibody or blocking their differentiation by vitamin A abrogated post-septic immune suppression.

MDSC induction after sepsis *in vivo* was clearly reduced in IL-6-ko mice and when mice deficient in TLR-adaptor protein MyD88 or in TLR2/3/4/7/9 were infected.

In summary, we show for the first time post-septic immune suppression after Gram + sepsis to be mediated by mMDSC induced via MyD88 and TLR signaling and dependent on IL-6.

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Isolation of parasitophorous vacuoles from *L. major*-infected phagocytes

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Upon their internalization, the protozoan *Leishmania major* parasitizes in PMN, macrophages (MΦ and dendritic cells (DC) and causes a broad spectrum of human diseases. MΦ and DC are phagocytic cells, belong to the myeloid lineage and gain access to parasites early in the onset of the disease. Despite these similarities, their behavior during infection and the intracellular fate for Leishmania differs dramatically. MΦ are the primary host cells for Leishmania. After CR3-mediated internalization, *L. major* efficiently transforms into amastigotes and replicates within MΦ without inducing apparent inflammation. We have shown previously that in contrast infection of DC by FcγRI/III-mediated uptake leads to DC activation, parasite antigen processing and migration to draining lymph nodes. Infection also induces release of proinflammatory mediators such as IL-12, IL-23 or IL-1α/β DC initiate Tcell- dependent immunity against infection. Causal for the different behavior upon infection could be the molecular composition of parasitophorous vacuoles (PV) in MΦ and DC. With the intention to better understand phago(lyso)somal function, we plan to identify proteins associated with *L. major*-containing PV in DC compared to MΦ. To this aim, we first established a method to isolate *L. major*-containing PV from the murine MΦ-like BALB/c cell line J774A.1. For the isolation of PVs, MΦ were coincubated with RFP+ *L. major* (MOI 8 at ~7*105 cells/ml). Cells were harvested after 18 hrs and an overall infection rate of ~60% was obtained. Mechanical disruption by shear forces in a sinuous flow of infected MΦ resulted in lysis of 70–80%. Next, enrichment of PV was achieved by flow cytometric sorting with a gating strategy based on FSC/SCC separation of debris from intact PV and cells followed by positive selection of *L. major*-containing RFP+ PV. Isolated PVs were subsequently stained against LAMP2, EEA1 and Rab7 and showed expression of phagosomal maturation markers comparable to within infected intact cells. Thereby, we were able to demonstrate that parasites were still surrounded by the membrane after isolation and enrichment indicating isolation of intact PVs. EM-based verifications of the structural integrity of PVs are currently performed. We will next perform proteome analyses using label-free mass spectrometry with the aim to identify the protein content of PV from primary MΦ and DC. This approach will allow us to assess the specific components of PVs of different myeloid cells and contribute to our understanding of the molecular mechanisms of their different behavior in infection with *Leishmania*.

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Role of mast cells during *Leishmania* (L.) *major* infections depends on genetic background

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Mast cells (MC) play an important role during type I hypersensitivity reactions and responses against intestinal parasites. In parasitic infections – such as cutaneous leishmaniasis – the role of MC is still less clear. Leishmaniasis is a parasitic skin disease transmitted by the bite of a sand fly. Infections with *L. major* in C57BL/6 mice – as well as in humans – are associated with Th1/Tc1 immunity with self-healing lesions within weeks and long-lasting immunity. In contrast, susceptible BALB/c mice predominantly develop Th2/Th17/Treg immune responses associated with progressive disease. Previously, we showed that MC-deficient KitW/KitW-v as well as KitW-sh/KitW-sh mice (both on C57BL/6 background) developed significantly increased lesion volumes after physiological low-dose infection with 1000 metacyclic parasites – in line with increased parasite burdens in ear and spleen. Additionally, lymph node (LN) cells from infected mice showed reduced levels of IFN-gamma, IL-12p40 and IL-17A, whereas levels of IL-4 and IL-10 were strongly elevated after restimulation with soluble Leishmania antigen (SLA) compared to wild-type mice. Taken together, these findings suggested that MC-deficiency in mice on a C57BL/6 background leads to worsening of disease outcome. Next, we assessed the phenotype of congenic C.B6-KitW-sh mice, bearing the KitW-sh allele on BALB/ c background. Control BALB/c and C.B6-KitW-sh mice were inoculated intradermally in both ears with physiological doses of *L. major*. Lesion volumes were measured weekly. Unexpectedly and in contrast to the findings in MC-deficient C57BL/6 mice, C.B6-KitW-sh mice developed dramatically smaller lesion volumes compared to BALB/c control mice. In line, lesional and splenic parasite burdens were strongly decreased in C.B6-KitW-sh in week 9 post infection. Additionally, LN cells from infected mice restimulated with SLA released significantly increased levels of antigen-specific IFN-gamma in C.B6-KitW-sh mice (~1.2 fold), whereas IL-17A and IL-4 release were reduced

compared to BALB/c wild-type mice. In summary, depending on the genetic background of experimental mice – C57BL/6 or BALB/c – MC deficiency leads to different disease outcomes. Both MC-deficient C57BL/6 and BALB/c mice develop altered T cell-dependent immunity against *L. major* leading either to worsening of disease outcome in KitW-sh/KitW-sh or improvement in C.B6-KitW-sh mice. However, strain-dependent differences in the function and contribution of MC contribute significantly to disease outcome against this human pathogen. Further studies will have to investigate if similar effects may be observed in other MC-controlled diseases.

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Singlet oxygen eliminates *Leishmania major* parasites generated by a new class of photosensitizer

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The tropical disease leishmaniasis is initiated by flagellated parasites of the genus *Leishmania* (L.), which are inoculated into the skin during the blood meal of a sandfly vector. A broad spectrum of clinical manifestations in humans, ranging from a self-limiting cutaneous infection to disseminating and life threatening visceral leishmaniasis, are described with respect to the transmitted *Leishmania* species. During the last decades the experimental model of leishmaniasis, in which mice are infected with stationary phase promastigote parasites, allowed the examination of many immunological details of the host-parasite interaction. For instance, it is shown that the obligatory intracellular *Leishmania* parasites need myeloid cells for replication as soon as the parasites are located in the dermal compartment. Additionally it could be shown that *Leishmania* parasites have evolved a smart strategy to hide from the host immune response once they have entered the dermal compartment. Thus massive chemotherapeutic therapies are necessary to eliminate the parasites. Unfortunately, the drugs used to treat the patients such as Glucantime, liposomal Amphotericin B, Miltefosine, and Paromomycin are known to induce side effects. Moreover, an efficient vaccination and prophylactic therapies are not available at the moment. Therefore, alternative approaches that are capable of killing *Leishmania* parasites are needed to supplement standard treatment modalities. One alternative might be the photodynamic inactivation of pathogens by photosensitizers that release leishmanicidal oxygen species. SAPYR represents a new generation of photosensitizers that shows a singlet oxygen quantum yield ΦΔ of 99% and is water soluble and photostable. Additionally, it contains a positive charge for good adherence to cell walls of pathogens. In this study, we investigated both (i) the leishmanicidal potential of SAPYR and (ii) the side effects of SAPYR on cellular components of the innate and adaptive immune system. We showed for the first time that SAPYR induces a striking and high efficient elimination of *L. major* parasites *in vitro*. Furthermore our data demonstrate that T and B cells are more resistant against the photosensitizer SAPYR compared to myeloid cells. We propose that locally administration of SAPYR might be used to treat cutaneous leishmaniasis by elimination of parasites and myeloid host cells leaving the T cell mediated adaptive immunity unaffected.

P160 (O06/02)

Lack of IL-10 signaling in dendritic cells enhances anti-*Leishmania major* immunity

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Cutaneous leishmaniasis is a parasitic disease transmitted by the bite of a sand fly that causes skin sores characterized by ulcerating, sometimes painful nodules of the skin. In humans and mice, the infection is resolved by a T helper (Th)1/ cytotoxic T cell (Tc)1 response, which correlates with disappearance of the lesions. Persistence of small numbers of parasites in the skin and lymphoid tissues is crucial for resistance to re-infection. Thus, protective immunity requires a tight balance to contain, but not eradicate the parasites, which depends on Interleukin (IL)-10. Mice lacking IL-10 eliminate the parasites and are susceptible to re-infection. Dendritic cells (DC) have the unique capacity to balance skin immunity and tolerance and are targets of IL-10 both *in vitro* and in the skin. Moreover, we recently discovered that Langerhans cells are negative regulators of the anti-Leishmania response, which is in part mediated by regulatory T cell (Treg)-derived IL-10. To what extent IL-10 control of DC contributes to the phenotype of IL-10−/− animals and shapes the anti-Leishmania immune response remains elusive.

To this aim, we analyzed mice with a DC-specific deletion of the IL-10 receptor α (DC-IL10R−/−). Following inoculation with a physiological low dose of *L. major* (1000 parasites), DC-IL10R−/− mice more efficiently cleared the parasites both locally (ears) and systemically (spleen) as compared to wild type controls. To further analyze the antigen-specific T cell response at 6 weeks post infection, skin-draining lymph node (sdLN) cells were restimulated *in vitro* with soluble *L. major* antigen. While Th1/ Tc1- derived Interferon (IFN)γ was moderately increased in cultures of DC-IL10R−/− mice as compared to controls, Th2-associated IL-4 was significantly reduced resulting in a higher IFNγ/IL-4 ratio in DC-IL10R−/−. Additionally, we detected significantly more IL-12, IL-6, IL-17A and Tumor necrosis factor (TNF)α in restimulated sdLN cells of DC-IL10R−/− animals, whereas IL-10 and IL-23 levels were comparable to control mice.

Interestingly, despite faster parasite disappearance, infected DC-IL10R−/− and control mice developed similar ear lesions. In a following experiment, we investigated *L. major* reinfection in healed DC-IL10R−/− mice. Six months after the first infection, mice were inoculated with 1000 parasites per ear. The resulting lesions were comparable in DC-IL10R−/− and control mice, suggesting that the antigen-specific memory response remained intact in DC-IL10R−/−. In line, parasite burdens in the ears and spleens of both groups and cytokine production (IFNγ, IL-10, IL-4) were similar after complete healing. Thus, in contrast to IL10−/− mice, there is no sterile cure when IL-10 signaling is eliminated exclusively in DC.

In conclusion, our data establish that lack of IL-10 signaling in DC during leishmaniasis enables more effective clearance of the parasites, but is dispensable during the latent phase of infection, when IL-10 signaling in T cells and Treg-derived IL-10 are sufficient to promote residual parasite survival for the maintenance of T cell memory.

Both first and senior authors contributed equally to this work.

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Positive and negative influence of *Staphylococcus aureus* infection on Tight Junction functionality

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The most important function of the skin is to build a barrier and protect the body from environmental impacts, such as allergens, physical disruption and bacterial infection. The Tight Junction (TJ)barrier was shown to play an important role for the paracellular pathway for small molecules in the skin. We are interested in the role of Tight Junctions in the course of bacterial skin infection.

Previously, we showed temporarily increased transepithelial electrical resistance (TER) in HaCaT cells infected with *Staphylococcus epidermidis* followed by a decrease of TER, while there was only a decrease after infection with *Staphylococcus aureus*. In primary human keratinocytes, which exhibit higher TER levels than HaCaT cells, we now could confirm the increased/decreased TER after infection with *Staphylococcus epidermidis*, but interestingly also observed a significant and dose dependent temporarily increase in TER after *Staphylococcus aureus* infection during the first 12 h post infection. Western Blot and qPCR analyses showed that the increase is not due to a raise of TJ mRNA and protein levels, but increased levels of phospho-Occludin which already was shown to play a role in tightening of TJs can be observed. Immunohistochemical stainings revealed that localisation patterns of Occludin, ZO-1, Claudin-1 and Claudin-4 are changed.

When investigating barrier function in human skin equivalents and epidermis equivalents as well as normal human skin by using a 557 Da tracer and various combinations of double and triple immunofluorescence we demonstrate that a tracer stop which marks functional TJs, is found exclusively at sites positive for Occludin, ZO-1, JAM-1, Claudin-1 and Claudin-4 staining. Long term infection results in an impairment of the TJ barrier.

In conclusion, we show for the first time, that infection of primary keratinocytes not only with the commensal *S. epidermidis*, but also with the pathogenous strain *S. aureus* results in a transient upregulation of TJ functionality, hinting for a rescue mechanism of keratinocytes against invasion of pathogens. Long term infection impairs TJ functionality.

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Leishmania major induces distinct gene expression patterns in neutrophil granulocytes from resistant and susceptible mice

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Experimental leishmaniasis is an excellent model system for analyzing genetic host factors which contribute to the outcome of infection. Resistance to Leishmania (L.) major as seen in C57BL/6 mice depends on the development of a L. major specific Th1 response, while Th2 differentiation in BALB/c mice results in susceptibility. There is growing evidence that the early microenvironment of the infected tissue delivers initial triggers for Th-cell differentiation. Neutrophil granulocytes are among the first leukocytes which arrive in the infected tissue and take up parasites. They have long been regarded as short-lived effector cells but are now recognized to also influence the development of adaptive Th-cell immunity. Thus, we analyzed genetic differences in the interaction of granulocytes and L. major between susceptible and resistant mice.

We isolated granulocytes from polyacrylamide gel pellets which were implanted subcutaneously into C57BL/6 and BALB/c mice. Applying adherence to plastic surfaces and negative MACS selection using the macrophage cell surface marker F4/80 we depleted contaminating macrophages from granulocyte cultures. We added infectious metacyclic L. major promastigotes at a ratio of 5:1 per granulocyte. Phagocytosis of L. major was similar between both mice strains. Employing microarray technology, real-time PCR and protein assays (cytometric bead assay) we found that interaction between L. major and granulocytes resulted in altered gene expression in granulocytes from both strains of mice. While many genes were down regulated we also found considerable induction of gene expression including chemokines like MIP-1-alpha and beta in resistant and susceptible mice. Gene induction was observed on the RNA and also on the protein level. Of special interest we found 24 genes which were differentially regulated between BALB/c and C57BL/6 mice. Bioinformatical analysis revealed a cluster of genes, which are known to be regulated by type I interferon, which was more strongly induced in BALB/c mice.

Thus, our data indicate genetic differences in L. major induced gene expression in granulocytes between C57BL/6 and BALB/c mice. This could contribute to the early local microenvironment and thereby influence Th1/2 immunity and ultimately the outcome of infection.

P163

Laminin-derived peptides are involved in inflammation, chronic wounds and skin infections

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Laminins play a fundamental role in basement membrane (BM) architecture and function in human skin. The C-terminal laminin G domain-like (LG) modules of laminin α chains are modified by proteolysis to generate LG1-3 and secreted LG4-5 tandem modules.

Invasive pathogens utilize host extracellular matrix proteins like laminin and collagen for adhesion and invasion of the host.

Thus, pathogens breach the BM and can degrade extracellular matrix proteins. Our group has previously reported that peptides fragments from the LG4 module exhibit a dose-dependent antimicrobial activity against several pathogens.

Furthermore, we demonstrated that processing of the LG4-5 module generates bioactive peptides, which plays a key role in inflammation and wound healing.

We suggest, that after wounding macrophages or keratinocytes at the site of tissue damage release proinflammatory cytokines and growth factors such as TNF or TGF-β, which lead to increased synthesis of laminin α chains and also to increased release of its processed LG4-5 tandem modules. We propose that bacterial and/ or endogenous proteases can cleave LG4-5 module to further generate smaller fragments. Thus, our data strongly suggest a novel multifunctional role for lamininderived peptides in human skin and its involvement in physiological processes and pathological conditions such as inflammation, chronic wounds and skin infection.

Pharmacology

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Dexpanthenol modulates skin regeneration and gene regulation in a novel standardized human three-dimensional skin wound healing model using non-sequential fractional ultrapulsed CO_2 laser treatments

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Dexpanthenol, the stable alcoholic analog of pantothenic acid, is widely used in dermatological therapy. It shows good skin penetration and high local concentrations, helps to stabilize skin barrier function, prevents skin irritation, stimulates skin regeneration and promotes wound healing. Previous data reveals dexpanthenol has stimulatory effects on cellular migration, proliferation, and influences molecular gene regulatory mechanisms in human fibroblasts.

In this study, we established a novel human three-dimensional (3D) skin wound healing model using scaffold and collagen 3D organotypic skin equivalents irradiated with a non-sequential fractional

ultrapulsed CO_2 laser. The laser irradiated skin models showed clearly defined lesions of the epidermis and dermis directly after injury. These standardized injured skin equivalents enable microarray, qRT-PCR, and histological studies analyzing the effect of various topically applied dexpanhenol containing ointments or systemically applied calciumpantothenat on skin wound healing and gene regulation.
The human laser-irradiated skin models were found to be appropriate for *in vitro* wound healing analysis. Topical treatment of skin wounds with a 5% dexpanthenol water-in-oil emulsion or two different 5% dexpanthenol oil-in-water emulsions clearly enhanced wound closure compared to laser-irradiated untreated control models. To find out whether this positive effect is caused by the active substance dexpanthenol, laser-irradiated skin models were cultured in calciumpantothenate containing medium (20 μg/ml) compared to skin equivalents cultured without calciumpantothenate. 3D models cultured in calciumpantothenate revealed considerably faster wound closure compared to the control models. In the skin model cultured with calciumpantothenate the re-epithelisation was nearly completed whereas the control model still displayed a large skin leason. Furthermore, immunofluorescence staining revealed that Ki67 protein expression is upregulated in laser irradiated 3D skin equivalents cultured in calciumpantothenate compared to control models cultured without calciumpanotenate.
To confirm these dexpanthenol-mediated stimulatory effects on wound closure, we investigated the influence of calciumpantothenate on gene expression in laser irradiated 3D skin models cultured with calciumpantothenate using an Affymetix gene array and quantitative real-time PCR. These gene expression studies showed enhanced mRNA expression of MMP3, IL1α, keratin-associated protein 4-12 (KRTAP4-12), and decreased expression of S100A7 in laser-irradiated skin models cultured in medium containing calciumpantothenate.
In conclusion, this novel standardized human 3D skin wound healing model proves useful for topical pharmacological studies on wound healing and reveals new insights into molecular mechanisms of dexpanthenol-mediated effects on wound healing. In addition, these novel 3D model systems can be used to monitor ex vivo effects of various laser systems on gene expression and morphology of human skin.

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Induction of the progeroid/cancer prone XP-like phenotype by a medical drug is mediated via reversible downregulation of DNA repair

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Prophylactic protection of patients with severe immunosuppression is of vital importance to shield the patient from opportunistic fungal infections.
It has been reported, that patients treated with a broad spectrum antimycotic drug develop adverse effects such as phototoxicity followed by pigmentary changes and the development of ultraviolet radiation (UV) associated non melanoma skin tumors. Thus, patients closely resemble the phenotype of the progeroid disorder xeroderma pigmentosum (XP), known to be caused by a defect in the DNA repair mechanism nucleotide excision repair (NER). So far the underlying molecular mechanisms by which this drug leads to the XP-like clinical phenotype have not been clarified. Therefore, we investigated if the antimycotic drug leads to a reduction of DNA repair and increases DNA damage. We found that long term treatment lead to suppression of unscheduled DNA synthesis as well as increased comet formation while double strand breaks were not induced. Importantly repair suppressive effects were transient since removal lead to normalization of all repair associated parameters. Furthermore, compound treatment did not cause significant transcriptional regulation of mRNA levels of NER proteins such as XPA – G, ERCC1 and RAD23 A/B and of DNA damage signaling factors (ATM and ATR). Furthermore, we found a higher level of Mdm2, XPB and XPD proteins in complex with p53 upon AD treatment and it is known that p53 is involved in chromatin remodeling during damage processing. Interestingly electronmicroscopy revealed AD induced changes in Chromatin density.
When exposed to the compound cells also did not show cell cycle arrest even in the presence of DNA damage but proliferated similar to untreated controls.
Taken together these results indicate that the broad spectrum antimycotic could suppress NER, increase DNA damage and thus, within months lead to photosensitivity, pigmentary changes and ultimately skin tumors.

Photobiology

P166

UVA irradiation of senescent fibroblasts epigenetically unlock anti-apoptotic GDF15 expression via interleukin 6-dependent promoter demethylation in melanoma cells enhancing their survival

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Over the past few years, strong evidence has accumulated that p53 engages also in transcriptionally regulating powerful pro-survival pathways by active transcription of genes counteracting apoptosis. The transfer of phospho-p53 into the nucleus and space for binding to anti-apoptotic gene promoters in their methylated state may be critical parameter regulating gene function. Among many p53 transcriptionally regulated genes, GDF15 coding for the Growth Differentiation Factor 15 (GDF15) plays a role in anti-apoptotic pathways in melanoma progression. However, so far the underlying mechanisms and the conditions under which phosphorylation site of p53; critical for p53 binding and transactivation of the GDF-15 promoter in melanoma cells are not fully understood. Interestingly, p53S392 was identified to translocate to the nucleus of melanoma cells after UVA irradiation, however, does not protect from apoptosis. By contrast, UVA irradiation of melanoma cells in the presence of supernatants from senescent cells and to a much higher extent from senescent UVA irradiated fibroblasts enhance melanoma cell survival and suppress apoptosis. Enhanced IL-6 released from UVA irradiated senescent but not from young fibroblasts enhanced GDF15 expression by demethylation of its promoter, thus providing topological configuration space for p53S932 binding. Demethylation was verified by pyrosequencing and by methylation sensitive and insensitive restriction enzymes. Neutralizing antibodies against IL-6, silencing of IL-6 by lentivirally transduced IL-6 shRNA in senescent fibroblasts or the use of methyl methanesulphonate, a demethylation inhibitor, almost completely abrogated demethylation of the GDF15 promoter and reduced GDF15 expression in melanoma cells exposed to supernatants of UVA treated senescent fibroblasts. Using a reporter Luciferase construct under the control of the GDF15 promoter we found that increasing concentrations of rhIL-6 enhanced luciferase activity in melanoma cells indicative of GDF15 promoter activity. In addition, IL-6 or supernatants from UVA irradiated senescent fibroblasts significantly enhanced the survival of melanoma cells. Silencing IL-6 or using chemicals suppressing demethylation abrogates the survival advantage of melanoma cells. Chromatin immunoprecipitation revealed enhanced physical binding of p53S293 to the GDF15 promoter in melanoma cells treated with increasing IL-6 concentrations. Our study for the first time sheds light on a novel paracrine mechanism of IL-6 epigenetically regulating the expression of GDF15 in melanoma cells presumably serving as a rescue mechanism against apoptosis. These findings provide mechanistic insight into the critical role of UVA irradiation on epigenetic regulation of anti-apoptotic genes and fibroblast senescence on melanoma progression.

P167

Lipid oxidation patterns and -kinetics under senescence-promoting stress in keratinocytes

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Oxidation of lipids and proteins is not only a manifestation of aged skin but also potentially causative for age-related aesthetic decline and pathologic damage. Extrinsic oxidative stress promotes the accumulation of reactive lipid oxidation products. Polyunsaturated fatty acids of phospholipids (PL) are easily oxidized by extrinsic stressors that promote skin aging, and the resulting lipid mediators elicit stress responses.
To study in keratinocytes, which oxidation products are generated upon environmental UV stress and to study the kinetics of intrinsically generated and extrinsically added oxidized PL, we performed lipidomic analysis. We applied a HPLC-tandem-MS method recently developed by us and quantified over 500 PUFAPL oxidation products in Keratinocytes immediately and 24 h after irradiation with 40 J/cm^2 UVA-1. We also performed analysis of global mRNA expression and of selected cyto/chemokines and stress response enzymes on protein level.
Studying the PL kinetics, we found,unexpectedly, that immediately after UVA-1 radiation PL containing esterified dicarboxylic acids show higher accumulation than PL hydroperoxides and -hydoxides. Levels of dicarboxylic acid containing PL returned to baseline after 24 h, while significantly increased PL-hydroxide levels persisted. Exogenously added UV-oxidized PL initially underwent rapid oxidation and chain shortening, whereas after 24 h a massive increase of F and E,I,D class PL-Isoprostanes was detected. The transcriptomic and proteomic profiling of the stressed KC performed in parallel indicated that part of the observed changes in the lipid profile after 24 h would be compatible with the transcriptional modulation of enzymes regulating prostanoid metabolism, like prostaglandin F synthase. As isoprostanes and isoprostane containing PL are correlated to aging (also of the skin), the modulation of isoprostane levels by UVA may be a novel mechanism contributing to photoageing.

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Autophagy deficient mouse melanocytes have a senescence associated secretory phenotype (SASP) and enhanced ER stress after UV exposure

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Autophagy deficient mouse melanocytes are susceptible to premature senescence and have a dysregulated antioxidant response, as we have recently reported. While it does not profoundly affect pigmentation in homeostasis *in vivo*, this unexpected phenotype may give insights into the molecular basis of pigmentation disorders connected to melanocyte stress and senescence, like vitiligo. We studied the transcriptome, secreted factors and lipid oxdiation in autophagy deficient cells versus autophagy competent cells in homeostasis and after UVA exposure to elucidate underlying gene regulation and potential effector molecules of the senescent phenotype.
Melanocytes were isolated from pups (Atg7 f/f or Atg7 f/f Tyr::Cre, in which Atg7 was knocked out under control of the tyrosinase promoter) younger than 5 days and cultured for 23 days. Then cells were either exposed to 20 J/cm^2 UVA-1 or sham exposed. Six hours later total RNA was isolated and global gene expression was assayed with Mouse Gene 2.0 ST Array (Affymetrix). Alternatively, supernatants were collected 24 h after irradiation for proteomic analyis with the 'Mouse Cytokine Array Panel A' (RnD). Lipidomics of oxidized phospholipids was performed using HPLC-MS/MS analysis.
A total of 151 genes was significantly increased in autophagy deficient cells compared to autophagy competent cells in basal condition. After UVA exposure, 41 genes were induced in autophagy deficient cells, of which 22 were not UVA inducible in autophagy competent cells. Conversely, 60 genes were significantly reduced in knockouts, and 55 were reduced in these cells by UVA, of which 45 were specific for the knockout. A bioinformatic gene ontology analysis revealed that the functional annotation clusters for the terms 'secreted', 'ECM/collagen' and 'chemokine' were significantly enriched in the autophagy deficient samples. In samples that were additionally UVA exposed, the terms 'transcriptional repression' and 'unfolded protein response' were significantly enriched. Stress inducible oxidized lipid mediators were increased in autophagy deficient melanocytes. Analysis of factors secreted by the melanocytes verified significant increase in chemokines Cxcl1, Cxcl10, Cxcl2 and Cxcl12. Together with the observed increase in matrix metalloproteinases Mmp13, Mmp1b, Mmp3 and Mmp19, these data demonstrate that in the absence of stress autophagy deficient melanocytes have a senescence associated secretory phenotype SASP, whereas they show enhanced ER stress and an unfolded protein response when exposed to UVA.

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Infrared radiation reduces UVB-induced apoptosis in normal human melanocytes via modification of apoptosis-related proteins

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While it is widely accepted that Ultraviolet Radiation (UVR) is a main risk factor for non-melanoma skin cancer, the link between solar radiation and melanoma is still a matter of debate. Infrared Radiation (IR) accounts for more than 50% of total radiation energy reaching the earth's surface. Until recently this kind of radiation has been considered to have only warming effects. However, evidence is increasing that IR has waveband specific effects and changes the expression pattern of several molecules. From a previous study there is evidence that IR might enhance the development of non-epithelial skin cancer and possibly also of melanoma. The aim of the present study is to evaluate the impact of IR on UVR-induced apoptosis and DNA repair in normal human melanocytes since malignant transformation depends on the balance between these two effects.
Melanocytes of different donors were exposed to 250 J/cm^2 of IR-A (780–1400 nm), 0.4 J/cm^2 of UVB (290–320 nm) or both simultaneously. Apoptosis was determined using cell death ELISA and Annexin V staining 24 h after exposure. UVBinduced DNA damage was detected applying South-Western dot blot analysis using antibodies against cyclobutane pyrimidine dimers 6 h after exposure. To investigate different pathways of apoptosis we determined activity of caspase-8 and 9 and the expression of the apoptosis-related proteins BAX, BID, CD95, FLIP(long). FACS analysis was performed by intra- or extra-cellular staining using the respective antibodies.
Apoptosis rate was significantly reduced in melanocytes exposed to IR-A and UVB compared to cells exposed only to UVB. In contrast IR-A did not accelerate the repair of UVB-induced DNA damage. Since DNA damage is a trigger of the UV-induced intrinsic pathway of apoptosis, we analysed the activity of caspase-9 and realised that IR-A didn't have any effects. With regard to the extrinsic apoptotic pathway, we observed activation of caspase-8 and up-regulation of the anti-apoptotic protein

FLIP(long) upon combined treatment of IR-A and UVB. On the other hand, the expression of pro-apoptotic BAX, BID and CD95 was downregulated suggesting that IR-A reduces UVB-induced apoptosis via inhibition of the extrinsic pathway. Since the repair of UVB-induced DNA-damage is not altered by IR-A, IR-A probably enhances the survival of severely UV-damaged melanocytes and thus might contribute to an increased risk of malignant transformation of melanocytes.

P170

Leukocyte-derived S100-'alarmins' are UVB-dependent therapeutic markers in psoriasis

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Psoriasis is a common, chronic immune-mediated inflammatory disease where certain antimicrobial proteins (AMP) are important players in cutaneous inflammation. The AMPs koebnerisin (S100A15) and psoriasin (S100A7) are induced in the epidermis of psoriatic skin lesions and mediate inflammation by acting as chemoattractants for immune cells. Circulating leukocytes contribute to the skin and systemic inflammation in psoriasis. In the current study, we identified circulating leukocytes as a novel source of koebnerisin and psoriasin. Moreover, peripheral blood mononuclear cells (PBMCs) from patients with psoriasis produced increased levels of koebnerisin and psoriasin. Both S100 proteins further acted as danger signals (alarmins) inducing PBMCs to produce proinflammatory cytokines that are implicated in the pathogenesis of psoriasis, such as IL-1 beta, IL-6, TNFalpha, and IL-8. The expression of koebnerisin and psoriasin was suppressed in circulating PBMCs in psoriatic patients when effectively treated with narrow-band UVB. Thus, data propose the antimicrobial proteins koebnerisin and psoriasin as multifunctional mediators of inflammation in psoriasis and promising targets for therapeutic intervention.

Pruritus

P171

4-1BB signaling in the skin spontaneously induces severe inflammation and chronic itch

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The costimulatory receptor 4-1BB (CD137), a member of the tumor necrosis factor family, is expressed by effector and regulatory T cells, mast cells, eosinophils or neutrophils, and is up regulated upon activation. Its ligand 4-1BBL can be found on dendritic cells, B cells and macrophages, indicating a widespread immunomodulatory function of the 4-1BB/4-1BBL signaling pathway. To analyze the effects of 4-1BB/4-1BBL interactions on cutaneous immunity *in vivo*, we generated transgenic (tg) mice overexpressing 4-1BB under control of the keratin 14 (K14)- promoter in basal keratinocytes. Interestingly, at the age of 3 months K14-4-1BB tg mice spontaneously developed inflammatory skin lesions at the ears, snouts and neck, which were histologically characterized by epidermal irregular hyperplasia, dermal fibrosis with thickened collagen fibers and the infiltration of T cells as well as mast cells into the dermis, thus pointing to a prurigo-like skin disease. In support of this, we observed an increased scratching frequency in tg mice compared to wildtype (wt) controls. Of note, the phenotype was mediated by 4-1BB/4-1BBL signaling since blocking this pathway using specific antibodies resulted in an amelioration of disease in tg mice. As human pruritus has been associated with a reduced density of intraepidermal sensory neurons in the skin we quantified nerve fibers. By performing PGP 9.5 immunofluorescence staining we detected markedly decreased numbers of C fibers in lesional and non-lesional skin from tg mice compared to wt controls. Beside a reduced nerve fiber density human pruritus has been linked to an upregulated histamine release by infiltrating mast cells. To characterize the role of mast cells for the development of prurigo-like skin inflammation in tg mice we depleted mast cells by breeding K14-4-1BB tg mice to KitW-sh mutants. However, mast cell deficient K14-4-1BB tg mice developed skin inflammation and itch to a similar extend as compared to mast cell competent controls pointing to a minor role of mast cells or histamine for the 4-1BB-mediated induction of itch. Accordingly, systemic treatment of K14-4-1BB tg mice with antihistamines did not result in the healing of disease. To analyze the cellular and molecular mechanism underlying 4-1BB-induced itch and inflammation in more detail we treated K14-4-1BB tg mice with aprepitant, a neurokinin-1 receptor antagonist, or naloxone, a mu-opioid receptor antagonist, before onset of disease since in clinical trials blocking neurokinin 1 and mu-opioid interactions has been demonstrated to relief itch and scratch lesions. Notably, in contrast to naloxone, treatment with aprepitant completely prevented scratching and skin lesion development in tg mice indicating a role of substance P/neurokin-1 receptor signaling during the induction of 4-1BB-mediated skin inflammation and itch. Besides opioid- or neurokinin receptor engagement IL-31 producing T cells are known to contribute to pruritus development. Hence, we quantified T cells in lesional and non-lesional skin from tg mice using immunofluorescence staining and flow cytometry. Interestingly, the numbers of total CD8+ and CD4+ T cells were markedly increased in lesional skin from K14-4-1BB tg mice versus non-lesional skin of the same animals or wt controls. Of note, these T cells expressed high concentrations of IL-31 as assessed on mRNA and protein level, possibly pointing to an effect of T cell-secreted IL-31 on the activation of sensory neurons during the development of 4-1BB-mediated itch and inflammation – similar to observations made in atopic dermatitis recently.

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Differences in neurosensory reactions in chronic pruritus with cutaneous and non-cutaneous causes after stimulation with cowhage, capsaicin or histamine

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Chronic pruritus (CP) is a symptom of many different diseases with high impact on quality of life. Treatment of CP is extremely difficult mainly because the neuroimmunological cutaneous mechanisms are not yet elucidated. One mechanism could be the sensitization of cutaneous nerve fibers by inflammatory mediators. Generally known in this processes are the mechano-insensitive (CMi-) fibers which are excitable by histamine or capsaicin. Recent studies pointed to an important role of mechano- and heat-sensitive C-fibers (CMH). CMH fibers are histamine-insensitive and can be activated by cowhage (proteinase of the cowitch – Mucuna pruriens). We assume that different mediators are relevant for sensitizing cutaneous CMi and CMH fibers in different disease associated with CP. In order to investigate this in an experimental setting, patients with atopic dermatitis (AD), brachioradial pruritus (BRP), prurigo nodularis (PN) and healthy controls (HC) have been treated with cowhage-containing spicules or inactivated spicules loaded with histamine, capsaicin or NaCl. Assessment of itch intensity, duration, wheal and erythema were performed for 10 min. Skin biopsies were taken for quantification of the intraepidermal nerve fiber density (IENF; PGP 9.5 staining). In all subjects a quantitative sensory testing (QST) was performed in order to identify functional abnormalities related to skin sensitization. In total 60 participants were included (AD $n = 10$; BRP $n = 16$; PN $n = 24$; HC $n = 10$). Mean age was 54.2 ± 15.8 years; the majority were women ($n = 38$). IENF density at the forearm was 10.7 ± 5.4 fibers/mm without showing differences between pruritic and non-pruritic individuals. Cowhage induced a higher itch intensity compared to histamine in all patients (measured by visual analog scale), being more prominent in AD (VAS 5) compared to BRP and PN (VAS 3-4). HC showed the strongest response after capsaicin administration (VAS 4). Time latency to maximal itch intensity after stimulation was 1 to 4 min, with the longest latency in the cowhage-treated group. Here, a trend between patient groups (AD 1.5 min < BRP 2.0 min < PN 2.5 min) and HC (4 min) was observed. In general, PN showed the longest latency independent of the experimental stimulus. Concerning the itch duration after stimulation, cowhage induced the longest reaction (6–8 min) compared to histamine or capsaicin (3–4 min). Decreased detection thresholds (loss of function) for various QST parameters e. g. heat and cold detection and pain threshold indicate a peripheral fiber dysfunction. In sum, we have found differences in the neuronal response to different experimental stimuli in the three pruritic diseases. While the neuronal anatomy seems to be unchanged in all diseases, the functional response suggests a peripheral sensitization of CMH fibers in AD. In contrast, in PN, a long latency and low reaction suggests a loss of function.

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Pruritogen Response in single and combinatorial TRP-channel KO mice indicates Interactions of TRP-V1, -V4, -A1, and -M8

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The pathophysiology of itch is not exactly understood. Many receptors are known to modulate and integrate pruritic stimuli, among them Transient Receptor Potential (TRP) ion channels. Here, we asked how the knockout of different TRP-channels (TRPV1, TRPV4, TRPA1, TRPM8) in mice modulates the scratching behavior after injections of different pruritogens. Moreover, to investigate potential crosstalks between TRP-channels, TRPV1/TRPA1/TRPM8-knockout (triple-KO) mice were studied. Cheek injections of different pruritogens (histamine, IL-31, endothelin-1 and chloroquine) in TRPV1-KO, TRPV4-KO, TRPA1-KO, TRPM8-KO and triple- KO mice were performed and the scratching behavior was counted. Histamineinduced scratching behavior was reduced in triple-KO mice and interestingly, we found an additive effect of TRPV1 and TRPA1 in IL-31-induced scratching behavior. Moreover, our data suggests a rather protective and inhibitory effect of TRPM8 after endothelin-1-cheek injections. After chloroquine-injection, triple-knockout mice demonstrated a gender difference with higher scratching bouts in triple-KO females as compared to triple-KO males. In sum, our results suggest that TRP-channels integrate und modulate pruritic stimuli differently and moreover that crosstalks between TRPV1, TRPA1 and TRPM8 exist, which underlines the important role of TRP ion channels as potential targets for pruritic skin diseases.

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Characterization of itch inducers in skin of healthy subjects and atopic dermatitis patients

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Itch is the main driver of quality of life impairment in many dermatological diseases such as atopic dermatitis, psoriasis or cutaneous T cell lymphoma as well as in systemic diseases. While histamine is the best characterized itch-inducing substance in humans, its role in most of these diseases is negligible. Here, we aimed at characterizing the potential of other clinically relevant substances to induce itch in humans.

As we have speculated that the reaction to inflammatory substances may be different in patients with chronic itch and/or chronically inflamed skin, we have included both healthy subjects ($n = 22$) and patients with atopic dermatitis (AD, $n = 23$). After obtaining informed consent, all subjects were challenged by skin prick testing in a random order in an eczema-free area on the volar aspect of the forearm with saline (negative control), substance P, bradykinin, endothelin-1 (ET-1), platelet activating factor (PAF), histamine and codeine and cowhage spicules (which are known to induce itch via PAR-2) were rubbed on the skin. Itch was then measured every minute over 30 min using the visual analogue scale (VAS ranging from 0 to 100), and skin reactions (wheal and flare) were assessed 20, 40, 60, 90, and 120 min after provocation by planimetric morphometry and digital volumetric analyses.

With the exception of saline, all tested substances induced reproducible itch responses in all healthy subjects with highest ratings for maximum itch (VAS) after challenge with cowhage (43 ± 6), followed by histamine- (37 ± 5) and codeine-challenge (195). Substance P, ET-1, bradykinin and PAF also induced itch responses, albeit to a lower extent (12 ± 4, 7 ± 2, 6 ± 2, 3 ± 1, respectively). Reproducible wheal development was observed after challenge with histamine, substance P, bradykinin and codeine and flare developed after challenge with histamine, substance P, PAF and codeine. Overall, the itch response in healthy subjects was similar to the ratings in AD patients with the exception of cowhage-induced itch. Here, maximum itch ratings peaked after 5 min in healthy subjects and then dropped rapidly with an overall itch duration of 16 min. In contrast, AD patients showed a plateau for maximum itch ratings from 5 to 8 min followed by a slow decrease in itch intensity with many patients still reporting about itch after 30 min.

Taken together, our data show that factors other than histamine can contribute to clinically significant itch in humans. Furthermore, the substantially longer PAR-2- mediated itch sensation induced by cowhage in AD patients indicates that PAR-2 might represent an ideal target for anti-itch therapy in AD patients.

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Itch induced by cowhage is increased and altered at sites of skin inflammation

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Background: Atopic dermatitis (AD), a common inflammatory skin condition comediated by mast cells, is associated with itch and represents a challenge for physicians and their patients. The physiopathology of itch in AD is still unclear. New models of inducible eczema and itch have been developed in recent years. As of yet, these models have not been used to assess itch in inflamed skin in detail.

Methods: We induced AD-like dermatitis in 31 healthy volunteers by repeated topical application of sodium lauryl sulfate (SLS, 2% SLS on both volar forearms for up to 6 days). Itch was induced by cowhage (40 spicules rubbed for 45 s on 2 cm^2 skin before and after eczema induction on volar forearms). Itch intensity was measured by Itch-VAS every minute for 30 min, and overall itch (area under the curve), maximum itch intensity, and itch duration in inflamed and non-inflamed skin were compared. Itch quality was assessed with a modified Eppendorf Itch Questionnaire.

Results: Eczema induction resulted in skin inflammation and AD-like lesions. Itch induction resulted in pruritus in inflamed skin and non-inflamed skin, i.e. after and before eczema induction. Overall

itch and maximum itch intensity were significantly increased in inflamed skin as compared to non-inflamed skin (overall itch: +20%, $P < 0.03$; maximum itch: +28%, $P < 0.0001$). In addition, itch quality was altered in inflamed skin, showing a more painful sensation as compared to the itch in noninflamed skin. There was no statistical difference in itch duration.
Conclusion: Responses to itch induction are different in inflamed and non-inflamed skin, and models of itch and eczema induction may help to identify and characterize the reasons for these differences.

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Anxiety about body symptoms increases perception of itch

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Pruritus is defined as an unpleasant sensation triggering a desire to scratch. This definition reflects subjective nature of itch and implies difficulties in measurement of its intensity, both clinically in patients with pruritus and in laboratory models of itch. Subjective, psychological factors modifying perception of pruritus are largely unknown.
The aim of the study was to investigate psychological variables potentially modifying perception of pruritus in induced itch models in atopic dermatitis (AD) patients and healthy controls.
Twenty-three AD patients (11 females), mean age 30.1 years 4.6, and 22 healthy controls, age and sex matched (mean age 30.3 years 5.5, 12 females) after obtaining informed consent were challenged in randomized order by skin prick-testing on eczema-free volar surface areas of their forearms with saline, histamine, codeine and cowhage spicules applied by rubbing the spicules on the skin. Itch intensity was assessed every minute over 30 min after application. Patients were asked to fill out battery of psychological tests, assessing: depressive mood (Beck's Depression Inventory – BDI), perceived stress (Perceived Stress Questionnaire – PSQ), anxiety (State-Trait Anxiety Inventory – STAI), fear of bodily symptoms (Body Sensations Questionnaire – BSQ) and dysfunctional cognitions concerning the perception and interpretation of bodily sensations (Fragebogen zu Körper und Gesundheit – FKG).
From all psychological questionnaires tested only BSQ was linked to the intensity of pruritus. BSQ correlated with intensity of histamine-induced itch (for maximum itch: $r = 0.456$; $P < 0.05$ in atopic dermatitis patients; $r = 0.472$; $P < 0.05$ in healthy volunteers; for area under the curve for itch intensity: $r = 0.538$; $P < 0.01$ in atopic dermatitis patients; $r = 0.372$; $P = 0.088$ in healthy volunteers) and codeine-induced itch (for maximum itch: $r = 0.509$; $P < 0.05$ in atopic dermatitis patients; $r = 0.472$; $P < 0.05$ in healthy volunteers; for area under the curve for itch intensity: $r = 0.549$; $P < 0.01$ in atopic dermatitis; $r = 0.472$; $P < 0.05$ in healthy volunteers). There was no correlation observed between BSQ and cowhage-induced itch intensity. There were no difference in BSQ levels between atopic dermatitis patients and healthy volunteers.
Our results suggest that subjects, both healthy controls and AD patients, presenting with a higher level of anxiety related to their bodily symptoms, may be sensitized to itch or may tend to evaluate the same itch intensity as being higher. Interestingly, cognitive factors related to perception and interpretation of bodily symptoms appeared to do not play any role in this process. Here, only healthy controls and AD patients were tested, but this mechanism may play a role also in the other conditions with pruritus. Our results are in support of use of relaxation techniques in reducing itch and suggest that cognitive psychotherapy may play rather marginal role in this condition.

Tumor Biology

P177 (O01/04)

Dual immune checkpoint blockage delays GNAQ-driven tumor growth in an autologous murine uveal melanoma model.

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Introduction: Uveal melanoma is the most common intraocular malignancy in adults with a disease specific mortality rate of ~40%. Oncogenic mutations in GNAQ and GNA11 were recently identified as driver mutations in ~90% of uveal melanoma. While localized disease can be effectively treated by surgery or radiotherapy, treatment options for metastatic uveal melanoma are limited. To investigate the interplay between uveal melanoma and the hosts' immune system and to test immunotherapeutic approaches, we have established an autologous mouse model of GNAQ oncogenedriven melanoma.
Materials and Methods: Melan-a cells were transduced with retroviruses expressing an activating Q209L mutation of GNAQ or a corresponding wild-type (wt) plasmid. Tumor formation was measured with calipers after subcutaneous inoculation of the cell line in C57BL/6 or RAG2−/− mice. Splenocytes were harvested at the time of sacrifice and analyzed by flow cytometry. For NK cells depletion, an anti-NK1.1 antibody was administered weekly. Murine anti-CTLA-4 antibodies (100 μg), anti-PD-1 antibodies (250 μg) or the combination of both were given weekly in a therapeutic setting. Peripheral blood mononuclear cells (PBMC) from uveal melanoma patients were obtained after informed consent and analyzed by flow cytometry.
Results: Melan-a cells with mutant but not with wt GNAQ form tumors when injected into mice, resulting in a model of G-protein-driven experimental murine melanoma (GEM). No significant difference in tumor growth was observed between C57BL/6 or RAG2−/− mouse strains ($P = 0.15$). Elimination of NK cells in RAG2−/− mice led to increased tumor growth compared to non-depleted controls ($P < 0.05$). Flow cytometry revealed a significant increase of CD11b+Gr-1int myeloid-derived suppressor cells (MDSC) as well as regulatory T cells (Treg) in tumor-bearing animals as compared to nontumor- bearing animals. In PBMC from patients with metastatic uveal melanoma, an increased frequency of monocytic MDSC and Treg was found as compared to healthy controls and patients with localized disease. In the GEM model, concurrent administration of an anti-CTLA-4 antibody and an anti-PD-1-antibody does delay tumor growth significantly while monotherapy with immune checkpoint blocking antibodies did not affect tumor growth. In mice treated with dual immune-checkpoint blockage, no reduction in the accumulation of Treg and MDSC was found when comparing treated and untreated mice.
Conclusion: In the autologous GEM model, tumor growth seems to be controlled by NK cells and can be delayed by dual immune checkpoint blockage. In this model, tumor formation is accompanied by an immunosuppressive leukocyte network also found in patients with metastatic uveal melanoma. Specific elimination of these cells might therefore synergize with immune checkpoint blocking antibodies. This hypothesis needs to be tested in future studies to better define its treatment potential for patients with advanced uveal melanoma.

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Alpha-melanocyte-stimulating hormone reduces the expansion of tumorpromoting myeloid-derived suppressor cells in mice and humans

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The neuropeptide alpha-melanocyte-stimulating hormone (α-MSH) is a potent immunomodulator and previously we could show that α-MSH up-regulates the numbers of cytotoxic T lymphocytes (CTL) in tumor-bearing mice as well as in peripheral blood from skin cancer patients. To characterize the underlying cellular and molecular mechanism in more detail we investigated the effect of α-MSH on tumor development and progression using a two-stage chemo-carcinogenesis model. Upon epicutaneous DMBA/TPA application mice injected with α-MSH developed significantly fewer skin tumors compared to PBS-treated controls. This effect correlated with increased numbers of tumor-specific CD8+ CTL and NK cells in regional lymph nodes as well as in tumor tissue from α-MSH-injected mice versus controls. Importantly, both cell subsets showed an up-regulated cytolytic activity when compared to cells isolated from PBS-treated mice. Since in mice as well as in patients the expansion and function of anti-tumoral effector cells can be controlled by myeloid-derived suppressor cells (MDSC) we quantified the numbers of MDSC in peripheral blood, bone marrow and spleens from α-MSH-treated mice and controls. Surprisingly, we detected significantly reduced levels of MDSC in DMBA/TPA treated α MSH-injected mice versus PBS-injected controls indicating that α-MSH up-regulated anti-tumoral immunity by preventing the generation of suppressive MDSC. Of note this effect was clearly dependent on binding of α-MSH to the melanocortin-1 receptor (MC-1R) since C57BL/Je/e mice lacking a functional MC-1R did neither show reduced tumor-development nor decreased levels of MDSC after DMBA/TPA treatment and injection of α-MSH. Interestingly, the inhibitory effect of α-MSH on MDSC expansion was restricted to an inflammatory tumor environment as α-MSH injection into Grm1EPv mice, which spontaneously develop melanomas, failed to reduce MDSC numbers and tumor incidence or to increase CTL levels. To investigate, whether α-MSH might be able to reduce the numbers of suppressive MDSC in humans as well we isolated MDSC from peripheral blood of skin cancer patients and treated them with PBS or α-MSH. Notably, α-MSH efficiently down-regulated MDSC levels in samples from basal cell (BCC) and squamous cell carcinoma (SCC) patients whereas we did not observe an effect of α-MSH on MDSC numbers in samples from melanoma patients suggesting that similar to the mouse model α-MSH inhibited the expansion of MDSC in an inflammatory tumor environment whereas the neuropeptide had minor effects on MDSC proliferation in melanoma. Moreover, in a mixed lymphocyte reaction we could demonstrate that α- MSH-treated MDSC from BCC and SCC patients were less efficient in suppressing the proliferation of CD8+ T cells as compared to PBS-treated cells from the same individuals. Next, we analyzed whether α-MSH might be able to modulate the *in vitro* generation of MDSC in peripheral blood from healthy donors as well. However, whereas α-MSH down-regulated the numbers and suppressive activity of MDSC in PBMC from BCC and SCC patients we detected only slightly reduced MDSC levels in PBMC from healthy donors that have been stimulated with an MDSC-inducing cytokine/antibody cocktail in the presence of α-MSH. These data might again point to the importance of the inflammatory tumor environment for the inhibitory effects of α-MSH on MDSC expansion. Together, our data demonstrate that in inflammationmediated skin cancer of mice and humans α-MSH, by binding to MC-1R, prevents the induction of suppressive MDSC resulting in up-regulated numbers of anti-tumoral effector cells. Thus, our data might suggest α-MSH as potential therapeutic option for non-melanoma skin cancer.

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Dimethylfumarate inhibits colon carcinona cell proliferation: evidence for cell cycle arrest, apoptosis and autophagy

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Background: Colorectal cancer is the second most common malignant neoplasm in women and the third most common in men with it forming the fourth most common cause of cancer death. Dimethylfumarate (DMF) is employed successfully as a drug for the treatment of inflammatory skin diseases, e.g. psoriasis, and lately also for immunmodulatory therapy of autoimmune disease multiple sclerosis. Furthermore recent studies have proven that DMF has a marked anti-proliferative impact on diverse cancer entities in both *in vitro* and *in vivo* trials, e.g. on malignant melanoma or head and neck cancer. In addition, anti-angiogenic properties by suppresssion of VEGFR-2 expression could be demonstrated. With the intention of exploring its antitumorigenic potential, we examined the effects of DMF on human colon carcinoma cell lines and the underlying mechanisms of action.
Methods: Colon carcinoma cell lines Caco-2, HT-29 and T-84 were cultured *in vitro* and treated with or without DMF. Effects of DMF on proliferation, cell cycle progression and apoptosis were analyzed mainly by BrdU- and LDH-assays, flow cytometry, and immunoblotting.
Results: The proliferation assays showed that DMF inhibits cell proliferation in a time- and dose-dependent manner in each of the three cell lines. However, it does not act cytotoxic on the cells. In order to find the causal mechanisms we studied the cell cycle via FACS analysis and the apoptotic pathways. In HT-29 it was shown that DMF induces a cell cycle arrest in G0/G1 phase, which is accompanied by upregulation of p21 and down-regulation of cyclin D1 and CDK4. Besides, up-regulation of LC3 I/II suggests that autophagy is involved in the inhibition of proliferation in HT-29. In T-84, the up-regulation of both LC3 I/II and p62 (SQSTM1) and the activation of caspase 8 indicates autophagy to be a major mechanism of action of growth inhibition prompted by DMF. In addition, the detection of an increased number of mono- and oligonucleosomes provides evidence that apoptosis is induced in T-84. For all cell lines, cellular senescence could be excluded as a mechanism of action by negative SA-β-Gal staining. Furthermore, we explored the anti-proliferative effect of DMF in combination with oxaliplatin which is used as an antineoplastic drug in colorectal cancer chemotherapy. Our results show that DMF supports the action of oxaliplatin in a synergetic manner.
Conclusion: Taken together, our results demonstrate that DMF has distinct anti-tumorigenic, cell line dependent effects on colon cancer cells by arresting cell cycle in G0/G1 phase as well as activating both the autophagic pathway and apoptosis.

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Induction of vascular adhesion molecules in melanoma metastases results in significantly increased infiltration of activated cytotoxic T lymphocytes

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Lymphocyte infiltration into melanoma tissue is an important prerequisite for effective anti-tumoral immunity. However, analysis of human metastatic melanoma has shown that tumor blood vessels hardly express leukocyte adhesion receptors thereby impairing the entry of cytotoxic lymphocytes into tumor tissue. To investigate whether endothelial activation and thus, modulation of human melanoma vessels *in vivo* might be able to induce adhesion molecules and to up-regulate lymphocyte infiltration we developed a melanoma xenograft mouse model. Therefore, biopsies from human melanoma metastases were engrafted subcutaneously onto immunodeficient NSG (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ) mice and interestingly, by immunofluorescence staining using antibodies specific for mouse or human CD31, respectively we could show that human blood vessels were preserved inside the melanoma grafts and connected to mouse circulation within 2 weeks after transplantation. Additionally, murine vessels infiltrated melanoma tissue and by assessing the proliferative activity of the transplanted tumors using BrdU and Ki67 staining we confirmed the connection of the grafts to the circulation. After having proven the blood supply and proliferation of the transplants recipient mice were intravenously injected with lymphocytes from the same melanoma patients that donated the grafts. Subsequently, melanoma vasculature was activated by intra-tumoral injection of TNF-α in combination with the chemokine TARC (CCL17) since we have shown previously in *in vitro* studies that TNF-α, in contrast to interferon-γ or histamine, resulted in a 4-fold increased expression of ICAM-1 and a 72-fold increased expression of E-selectin on tumor vasculature as quantified by realtime PCR. While the expression of adhesion molecules like ICAM-1, E-selectin or VCAM was moderate on tumor vessels from recipient mice that were injected intra-tumorally with PBS + TARC injection or TNF-α + TARC into the grafts significantly upregulated the expression of ICAM-1 (9-fold) and E-selectin (51-fold) as compared to controls. To analyze whether the TNF-α-mediated increased expression of adhesion molecules indeed improved lymphocyte homing to melanoma tissue we quantified the numbers of T cells expressing characteristic cytotoxic markers like Fas-ligand, granzyme or perforin in the grafts and interestingly, detected significantly increased numbers of cells in TNF-α-treated melanoma metastases (8.25 5.7 per viewing field) compared to PBS-treated controls (4.95 2.8 cells per viewing field) or tumor tissue before engraftment onto NSG mice (1.1 1.9 cells per

viewing field). Therefore, our data demonstrate that endothelial adhesion molecules can be induced on human melanoma vasculature *in vivo* by TNF-α treatment resulting in a significantly improved homing of activated autologous cytotoxic T cells to melanoma tissue. Hence, these observations potentially need to be taken into account when designing protocols for immunotherapy of malignant melanoma.

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The BRAF inhibitor LGX818 (encorafenib) induces endoplasmic reticulum stress and sensitizes NRAS-mutant melanoma cells to the MEK inhibitor binimetinib

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15–25% of all melanomas harbor activating NRAS mutations. Activated NRAS stimulates a number of intracellular signaling pathways including the RAF/MEK/ ERK pathway. Overall survival for NRAS-mutant melanoma patients is worse than their wild-type counterparts. In a phase 2 trial, the MEK inhibitor binimetinib showed activity in patients with NRAS-mutant melanoma with overall response rates of >20% and a median progression-free survival of 4 months. In a previous study, we showed that vemurafenib induces apoptosis in BRAFV600-mutant melanoma cells through a mechanism involving induction of endoplasmic reticulum (ER) stress. ER stress induction appeared to be an off-target effect of vemurafenib that remarkably enhances its pro-apoptotic activity in BRAFV600-mutant melanoma. In this study, we investigated whether it is possible to take advantage of ER stress induction to enhance the antitumor activity of MEK inhibitors in patients with NRAS-mutant melanoma.

BRAF-mutant and NRAS-mutant metastatic melanoma cell lines were treated with the BRAF inhibitors vemurafenib, dabrafenib and LGX818, and were subjected to electron microscopy. In particular, LGX818 induced morphological features of ER stress, including a significant dilation of the ER in both BRAF-mutant and NRASmutant melanoma cell lines. As expected, LGX818 inhibited phosphorylation of ERK and growth and induced apoptosis in BRAF-mutant but not in NRAS-mutant melanoma cells. However, LGX818 significantly enhanced growth inhibition and apoptosis induced by the MEK inhibitor binimetinib in NRAS-mutant melanoma cells in monolayer, spheroid and organotypic culture. Moreover, LGX818 in combination with binimetinib induced the expression of the ER stress-related factors p8, ATF4, ATF3 and CHOP in NRAS-mutant melanoma cells. siRNA inhibition of ATF4 reduced melanoma cell apoptosis induced by LGX818 combined with binimetinib.

These data suggest that the BRAF inhibitor LGX818 induces endoplasmic reticulum stress and potentiates the antitumor activity of MEK inhibitors in NRAS-mutant melanoma.

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Inhibition of the PI3K pathway with buparlisib (BKM120) is a suitable strategy to overcome therapy resistance in melanoma-derived brain metastasis *in vitro* and *in vivo*

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In melanoma, the RAF-MEK-ERK and PI3K-AKT signaling pathways play a major role in melanoma progression and drug resistance. On the basis of significant improvement in overall survival, the BRAF inhibitor vemurafenib gained FDA approval for the treatment of patients with metastatic BRAFV600 mutated melanoma. However, vemurafenib appears to be less effective in melanoma brain metastases, and brain metastases are the most common cause of death in patients with metastatic melanoma. In our previous study we reported that the AKT survival pathway is hyperactivated in melanoma brain metastases. The current study aims to investigate the mechanisms of AKT hyperactivation and the antitumor activity of the PI3K inhibitor BKM120 in melanoma brain metastases *in vitro* and *in vivo*. To simulate the tumor environment of brain metastases and extracerebral metastases, brain and matched extracerebral metastatic melanoma cells were stimulated by astrocyte- and fibroblast-conditioned medium, respectively. Both brain and extracerebral metastatic melanoma cells stimulated by astrocyte-conditioned medium showed higher AKT activation and invasiveness in a transwell matrigel invasion assay than cells stimulated by fibroblast-conditioned medium. The PI3K inhibitor BKM120 inhibited the phosphorylation of AKT and the growth of >10 newly isolated cell lines derived from melanoma brain metastases achieving growth inhibition rates of up to 80%. These effects did not depend on BRAF, NRAS or KIT mutation status. Furthermore, BKM120 potently induced apoptosis in brain metastatic melanoma cells and significantly inhibited the tumor growth of human BRAF- and NRAS-mutant brain metastatic melanoma cells in the brain of nude mice as shown by MRI scans.

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The chemosensitizing activity of the mTOR inhibitor temsirolimus in metastatic melanoma involves DKK1

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The BRAFV600E inhibitor vemurafenib achieves remarkable clinical responses in patients with mutant BRAF melanoma. Its effects are limited by the onset of drug resistance. In the case of resistance, chemotherapy is often applied as second line therapy. However, it yields low response rates and strategies are urgently needed to potentiate its effects. In a previous study, we showed that the inhibition of the PI3K-AKT-mTOR pathway significantly increased sensitivity of melanoma cells to chemotherapeutic drugs.

Here, we show that the combination of the mTOR inhibitor temsirolimus with the chemotherapeutic agent temozolomide significantly increased growth inhibition and apoptosis in melanoma cells compared to temsirolimus or temozolomide alone. The combination of temozolomide with temsirolimus was also effective in established and newly isolated vemurafenib-resistant metastatic melanoma cells. These effects were associated with the down-regulation of the anti-apoptotic protein Mcl-1 and the up-regulation of the Wnt antagonist Dickkopf homolog 1 (DKK1). Knockdown of DKK1 suppressed apoptosis induction by the combination of temsirolimus and temozolomide.

These data suggest that the inhibition of the mTOR pathway increases sensitivity of melanoma cells towards temozolomide. Chemosensitization is associated with increased expression of the Wnt antagonist DKK1.

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Epigenetic impacts of ascorbate on human metastatic melanoma cells

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In recent years, increasing evidence has emerged demonstrating that high-dose ascorbate bears cytotoxic effects on cancer cells *in vitro* and *in vivo*, making ascorbate a pro-oxidative drug that catalyzes hydrogen peroxide production in tissues instead of acting as a radical scavenger. This anticancer effect of ascorbate is HIF-1α- and O2-dependent. However, whether the intracellular mechanisms governing this effect are modulated by epigenetic phenomena remains unknown.

We treated human melanoma cells with physiological (200 μM) or pharmacological (8 mM) ascorbate for 1 h to record the impact on DNA methyltransferase (DNMT)- activity, histone deacetylases (HDACs) and microRNA expression after 12 h. The results were analyzed with the MIRUMIR online tool that estimates the power of microRNA to serve as potential biomarkers to predict survival of cancer patients.

FACS cell cycle analyses showed that 8 mM ascorbate shifted BLM melanoma cells towards the sub-G1 fraction starting at 12 h after an initial primary G2/M arrest, indicative for secondary apoptosis induction. In pharmacological doses ascorbate inhibited the DNMT-activity in nuclear extracts of MeWo and BLM melanoma cells, but did not inhibit human HDAC enzymes of classes I, II and IV. The expression of 151 microRNAs was altered 12 h after ascorbate treatment of BLM cells in physiological or pharmacological doses. Pharmacological doses up-regulated 32 microRNAs (≥4-fold) mainly involved in tumor suppression and drug resistance in our preliminary microRNA screening array. The most prominently up-regulated microRNAs correlated with a significantly increased overall survival of breast cancer- or nasopharyngeal carcinoma patients of the MIRUMIR database with high expression of the respective microRNA.

Our results suggest a possible epigenetic signature of pharmacological doses of ascorbate in human melanoma cells and support further pre-clinical and possibly even clinical evaluation of ascorbate for melanoma therapy.

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Processing and nuclear translocation of NF-κB2 in human melanoma cells

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Dysregulation of signaling cascades such as the NF-κB pathway can support the survival of melanoma cells. Of note, some chemotherapeutics may activate these pathways, thus inducing chemoresistance. In turn, inhibition of NF-κB signaling may increase the susceptibility of melanoma cells to chemotherapy. We examined whether the alternative NF-κB pathway, in addition to the well-known classical pathway, is relevant for melanoma progression. The alternative NF-κB pathway is regulated by a homodimer of IKKα which catalyzes the processing of NF-κB2 from its inactive precursor, p100, to the transcriptionally active form, p52. Western blot analysis of 8 melanoma cell lines revealed a clear shift from p100 to p52 in LOX cells and a moderate shift in Brown cells. In murine xenograft tumors of LOX cells, p52 localized to the nucleus, indicating transcriptional activity. A nuclear signal of p52 was shown in 2 of 21 human primary melanomas and melanoma metastases. Functional analysis of LOX melanoma cells revealed a slightly reduced NF-κB activity upon transient (knockdown) transfection with IKKα-specific siRNA constructs. Additionally, downregulation of IKKα caused a slight reduction of melanoma cell migration and CXCL8 transcription. Other NF-κB-regulated genes involved in tumor progression were not affected by IKKα reduction. The apoptosis rate of melanoma cells was not altered following IKKα knockdown, even when the cells were treated with the NF-κB-activating chemotherapeutic, doxorubicin. Furthermore, overexpression of IKKα in human melanoma cells led to an increase of constitutive as well as doxorubicin- or TNFα-induced NF-κB activity. This enhanced NF-κB signaling, however, affected neither NF-κB-dependent gene expression nor apoptosis induction by doxorubicin.

In summary, our data indicate that the alternative IKKα-dependent pathway of NF-κB is active in some melanoma cells. However, the restriction of IKKα kinase activity to a minority of melanomas, the lacking effect on doxorubicin toxicity and the only moderate influence on progression-related cell functions seem to limit its usefulness in melanoma therapy.

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Tumor Protein P53 Inducible Nuclear Protein 2 is a tumour suppressor in melanoma

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Melanoma is a malignant tumor with high metastatic potential and low therapeutic response due to development of drug resistance. Therefore, the new therapeutic strategies must focus on identification and reactivation of the inactivated tumour suppressor genes by targeting their upstream repressors. In our study, we identified Tumor Protein P53 Inducible Nuclear Protein 2 (TP53INP2) as a direct primary target for oncogenic microRNA miR-638. To determine the role of TP53INP2 in melanoma, TaqMan® gene expression analysis was performed which showed reduced TP53INP2 mRNA levels in melanoma cells as compared with primary fibroblasts and benign melanocytes. While siRNA mediated knockdown of this protein significantly enhanced the proliferative, migratory and invasive properties of melanoma cells, TP53INP2 overexpressing melanoma cells demonstrated significantly reduced proliferation and invasion *in vitro*. These results indicated towards a tumour- suppressive role of TP53INP2 in melanoma. Next, we addressed the question, whether reduced TP53INP2 expression can also promote development of therapeutic resistance in melanoma. Indeed, in TP53INP2-depleted BRAF(V600E) mutant melanoma cells significantly higher concentrations of BRAF(V600E)-inhibitor vemurafenib were necessary to reduce proliferation as compared with control cells. Interestingly, TP53INP2-depleted cells secreted higher levels of cytokines IL-6 and IL-8 which are known direct mediators of STAT-3-induced therapeutic resistance. Taken together, TP53INP2 was identified as a new tumour suppressor in melanoma. Reduced expression of TP53INP2 promotes development of therapeutic resistance probably mediated by IL-6 and IL-8 cytokines and via STAT-3 signalling.

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Immune-cell poor Hgf-Cdk4 mouse melanomas benefit from antibody mediated PD-1 blockade after targeted activation of the type I IFN system

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Infiltration of human primary melanomas with cytotoxic immune cells correlates with the spontaneous activation of the type I interferon (IFN) system and a favorable prognosis. Therapeutic antibody mediated blockade of immune inhibitory receptors in patients with pre-existing lymphocytic infiltrates prolongs survival. However, new complementary strategies are needed to efficiently activate innate and adaptive anti-tumor immunity in immune cell-poor human melanomas. In this study we experimentally show that primary cutaneous melanomas in Hgf-Cdk4(R24C) mice, which imitate a

sub-group of human immune cell-poor melanomas with a low type I IFN response signature, escape type I IFN-induced immune surveillance as well as immunoediting. Peritumoral injections of the immunostimulatory RNA polyinosinic:polycytidylic acid (polyI:C) initiated a cytotoxic inflammatory response in the tumor microenvironment and significantly impaired tumor growth of primary and transplanted Hgf-Cdk4 melanomas. This critically required the coordinated activation of the type I IFN system by dendritic, myeloid, NK and T cells. However, targeted activation of the type I IFN system led to an up regulation of PD-L1 on melanoma cells and increased the number of PD1+CD8+ circulating T cells. Importantly, antibody-mediated blockade of the IFN-induced immune-inhibitory interaction between PD-L1 and PD-1 receptors significantly prolonged survival of melanoma bearing mice. These results highlight important interconnections between the type I IFN system and immune-inhibitory receptor signaling in melanoma pathogenesis which serve as targets for combination immunotherapies.

P188 (O05/04)

Contribution of mast cell-derived VEGF to tumor growth

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Mast cells actively participate in growth of tumors, either by directly affecting proliferation and invasiveness of tumor cells or by indirectly regulating the tumor microenvironment. Mast cell infiltrates have been correlated with tumor progression, prognosis and microvessel density in various neoplastic diseases, for example in primary cutaneous lymphoma, melanoma, and lung cancer. During tumor progression, an angiogenic switch causes formation of new vessels from existing vasculature. The best-studied inducer of angiogenesis is vascular endothelial growth factor (VEGF).

To address the role of mast cells in tumor angiogenesis, we generated transgenic mice lacking mast cell-derived VEGF by crossing VEGF fl/fl mice to the Mcpt5Cre strain, which expresses Cre recombinase specifically in connective tissue type mast cells. Tumor growth of subcutaneously injected Lewis lung carcinoma cells (LLC) was significantly decreased in Mcpt5Cre/VEGF fl/fl mice as well as in mast cell-deficient Mcpt5Cre/iDTR mice. Moreover, induced depletion of mast cells in Mcpt5Cre/ iDTR mice during tumor growth resulted in smaller LLC tumors. Of note, tumor growth was also reduced by treating growing tumors with the anti-VEGF antibody bevacizumab. To explore the interaction between mast cells and tumor cells in more detail, LLC cells were incubated with mast cell supernatant. Here, we observed enhanced proliferation of LLC cells upon stimulation with mast cell supernatant. The anti-VEGF antibody bevacizumab inhibited mast cell-mediated proliferation of LLC cells. We next investigated mast cells in different histological subtypes of human lung adenocarcinoma by immunohistochemistry and found significantly increased numbers of mast cells. Patients with metastatic lung adenocarcinoma showed higher mast cell counts than non-metastatic patients. Furthermore, increased numbers of extensively degranulated mast cells and decreased numbers of not degranulated mast cells were associated with metastatic patients, suggesting also enhanced activation of mast cells in metastatic lung adenocarcinoma.

Taken together, our findings demonstrate that mast cells promote growth of murine LLC tumors and the metastatic potential of human lung adenocarcinoma. Mast cellderived VEGF may account for part of this effect. Hence, anti-VEGF antibodies may be a promising therapeutic approach in mast cell-associated tumors.

P189

Nrf2 activation promotes survival of pre-malignant keratinocytes through induction of metabolic pathways

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The transcription factor Nrf2 is a master regulator of the cellular antioxidant defense system through the regulation of antioxidant enzymes, cytoprotective proteins and transporters. Therefore, compounds that activate Nrf2 are in pre-clinical and clinical trials as anti-cancer drugs.

We tested the consequences of Nrf2 activation on skin tumorigenesis using transgenic mice expressing a constitutively active (ca) Nrf2 mutant in keratinocytes, which we treated with DMBA/TPA. In these mice detoxification of DMBA and of TPAinduced reactive oxygen species (ROS) was increased, resulting in a reduction of DNA mutations. However, surprisingly, only a marginal decrease in tumorigenesis was observed, suggesting a counteracting pro-tumorigenic activity of activated Nrf2. To investigate this, we tested the consequences of Nrf2 activation in keratinocytes in a virus-induced skin carcinogenesis model, which does not require treatment with chemical compounds. Surprisingly, in these mice skin tumorigenesis was remarkably enhanced. Mass spectrometry identified increased levels of glutathione, purines and NADPH in the epidermis of mice subjected to both cancer models. These metabolic changes had no influence on proliferation and differentiation of keratinocytes, but they promoted the survival of pre-malignant keratinocytes.

A similar Nrf2-protective mechanism was observed in cultured human keratinocytes. Furthermore, increased expression of classical Nrf2 targets and of genes involved in glutathione, purine and NADPH synthesis was observed in human pre-cancerous skin lesions.

Taken together, our results demonstrate that NRF2 activation can have a protumorigenic activity during early tumorigenesis. Therefore, the use of NRF2 activating compounds for the prevention of skin cancer should be reconsidered.

P190 (O02/02)

Distinct functions of epidermis- and myeloid cell-specific VEGF-A in human papillomavirus type 8-mediated tumorigenesis

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The genus beta human papillomaviruses (HPV) are involved in nonmelanoma skin cancer development in patients with epidermodysplasia verruciformis. However, the mechanism of action remains a challenge. To gain insight into the molecular mechanisms underlying HPV-mediated skin tumor development, we previously developed a transgenic mouse line expressing the complete early genome region (CER) of HPV8 under the control of human keratin14 (K14) promoter. HPV8 mice recapitulate the HPV-induced SCC pathology and have been proven to be a valuable *in vivo* model to unravel the molecular pathology of HPV-induced skin cancer. Recent evidence suggests a central role of Vascular endothelial growth factor-A (VEGFA) in regulating tumor development both through induction of tumor angiogenesis but also via angiogenesis-independent mechanisms. Up to date, the role of VEGFA in HPV-induced NMSC is not resolved, neither the question whether diverse cellular sources of VEGF-A may impact this process. In this study we dissected the contribution of epidermis- versus myeloid cell-derived VEGF-A in HPV8-mediated skin cancer using a combination of HPV8 transgenic mice and conditional gene targeting for VEGF-A. Here we show, that epidermis-specific deletion of VEGF-A results in complete abrogation of tumor initiation in HPV8 mice both spontaneous and in the presence of diverse tumor promoting conditions. In contrast, myeloid cellderived VEGF-A is only critical in regeneration-induced tumorigenesis triggered by full thickness excision skin injury. Mechanistically, we show that blocking VEGFR2 inhibited injury-induced papilloma formation in HPV8 transgenic mice, indicating an important paracrine function of VEGF-A on tumor angiogenesis. Furthermore, our findings provide evidence that epidermal HPV8 proteins can deviate a primarily beneficial and healing-promoting acute inflammatory response into a sustained inflammatory response leading to hyperplastic growth, and that myeloid cell-derived VEGF-A plays a critical role in this process. Interestingly, reduced clonal growth of VEGF-A depleted keratinocytes *in vitro* could not be rescued by external rVEGFA, suggesting an additional cell-autonomous activity of VEGF-A in keratinocytes, independent from angiogenesis. Gene expression analysis and IHC staining suggest an autocrine mechanism mediated by VEGFR1 and Nrp1. Taken together, here we provide novel mechanistic insights in distinct functions of epidermal- versus myeloid cell-derived VEGF-A in HPV8-mediated tumor development, which may have important implications for the prevention and treatment of HPV-mediated skin cancer.

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Insulin resistance as a pathomechanism in malignant melanoma?

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Malignant melanoma is one of the most aggressive cancers and despite a growing number of promising therapeutic approaches, the prognosis remains poor for most patients. There is evidence that the risk for several cancer types like pancreatic, hepatic, colorectal and breast cancer is increased in diabetic patients and that molecular insulin resistance may represent a pathomechanisms in carcinogenesis. This association can be explained at least in part by the 'paradoxon of insulin resistance': While target tissues in norminsulinemic subjects respond to insulin mainly with metabolic effects via the activation of the PI3-K/Akt pathway, insulin receptor signaling in hyperinsulinemic subjects may be attenuated for the metabolic branch, but not for the mitogenic, MAPK-dependant branch, thus favoring the development of malignomas.

In malignant melanoma this correlation is still relatively unclear. Nevertheless first indications of a potential association between obesity and insulin resistance as an independent risk factor have been pointed out. Thus we investigated, whether molecular insulin resistance contributes to carcinogenic alterations in different melanoma cells.

We could show that healthy melanocytes respond to short-term insulin treatment with activation of PI3-K/ Akt and MAPK signaling, while hardly any mTOR activity could be detected. In contrast, melanoma cell lines with hyperactivated BRAF (V600E) show constitutive mTOR and MAPK activity that can not be further enhanced by insulin treatment, while Akt is sensitive to insulin stimulation. However, after chronic exposure to insulin, Akt activity can not be induced by short term insulin treatment, which is characteristic of molecular insulin resistance. Blocking mTOR or MAPK activity with either rapamycin or U0126 restores insulin sensitivity suggesting that BRAF mediated hyperactivation of these kinases contributes to molecular insulin resistance and could represent a carcinogenic pathomechanism.

Measuring cell proliferation we found that insulin seems to have a slightly supporting, but not significant effect on cell growth, suggesting that insulin can not further activate the mitogenic branch. The permanent activation of the MAPK pathway due to BRAF-mutations may be a reason for the low influence of additional external growth signals. This is supported by the finding that blockade of MAPK signaling using U-0126 strongly suppressed cell proliferation. However, the PI3K/Akt pathway also seems to play an important role in melanoma cell growth, as inhibition of components of this pathway blocked proliferation.

In summary we could show that melanoma cells show signs of molecular insulin resistance under conditions of hyperinsulinemia, but the cellular outcome of this state remains to be determined. It can not be excluded that insulin resistance either contributes to early melanoma development before mutation dependant pathway hyperactivation occurs or affects cellular changes contributing to metastasis. In addition a contribution of insulin signaling to drug resistance is also discussed in literature.

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ATR-Chk1-Wee1 pathway controls melanoma cell cycle in G2-M and G0-G1

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Cell cycle checkpoints are critical for cell cycle progression of benign and malignant cells and are controlled by ATR-Chk1-Wee1 and ATM-Chk2-p53-p21 pathways. Here, both pathways were analysed in cell cycle control of melanoma cells. Treatment of p53 wild type melanoma cells with the genotoxic agent doxorubicin induced inhibitory phosphorylation of cell cycle kinase Cdc2 (CDK1), enhanced expression of p53/p21 and G2-M arrest. Chk1 and Wee1 inhibition under this treatment significantly reduced G2-M arrest and induced apoptosis. Interestingly, Chk1 inhibition alone even showed further enhanced apoptosis (more than 50% of cells). This may be due to the fact that Chk1 inhibition alone almost completely abrogated both G2-M and G0-G1 arrest, while combined treatment with doxorubicin maintained a significant G0-G1 arrest and may thereby rescue many cells from apoptosis. Chk1 inhibition alone induced only a slight p53/p21 induction, while a strong induction of both proteins was observed by the combination with doxorubicin. These findings are suggestive for a particular role of p53/p21 in G0-G1 arrest. In line with this, p53-mutant SK-Mel-28 melanoma cells did not mount a significant G0- G1 arrest under combined doxorubicin and Chk1 inhibitor treatment and showed extensive apoptosis. Knockdown of p53 and p21 in p53 wild type melanoma cells dramatically reduced stress-induced G0-G1 arrest under doxorubicin and Chk1 inhibition, followed by massive DNA damage and apoptosis induction. Treatment of p53 wild type melanoma cells with an inhibitor of Chk2 upstream kinase ATM and doxorubicin almost completely abrogated G0-G1 arrest. Taken together, it is shown that the ATR-Chk1-Wee1 pathway acts as a mediator of G2-M arrest, while both ATR-Chk1-Wee1 and ATM-Chk2-p53-p21 pathways are mediators of G0-G1 arrest in melanoma cells. Checkpoint targeting substances combined with chemotherapeutic agents such as doxorubicin may help to overcome apoptosis resistance of p53 wild type melanoma cells. However, one should be aware of the fact that chemotherapeutic agents such as doxorubicin induce p53 expression and may thereby rescue many cells from apoptosis.

P193

Senescent-associated secretion of Chemerin from human dermal fibroblasts enhances skin squamous cell carcinoma progression

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Squamous cell carcinoma (SCC) represents the second most common type of skin cancer worldwide and the incidence rate has been reported to increase with age. Recent evidence suggests that both accumulation of mutations as well as senescence-associated pro-oncogenic changes in tissue

microenvironment contribute to the age-related increase in cancers. DNA damage responses with activation of p53 and p16INK4a have been identified to induce senescence in skin fibroblasts. Accumulation of senescent fibroblasts releasing the senescentassociated secretory phenotype (SASP) is proposed to be involved in tumor progression and metastasis. SASP consists of various inflammatory cytokines, chemokines or matrix remodeling factors that, depending on the biological condition, may promote either beneficial or deleterious effects. The mechanisms underlying SASP-induced progression of squamous cell carcinoma are not fully understood in molecular details. Therefore, we aimed to identify secreted factors specifically involved in SCC progression. We identified transcripts and their corresponding secreted factors in senescent fibroblasts using RNA profiling and antibody array analysis of supernatants derived from senescent fibroblasts and young fibroblasts. The chemokines CXCL6/ GCP-2, CXCL5/ENA-78, MCP-1/CCL2 and RANTES/ CCL5 were detected to be upregulated in senescent fibroblasts compared to young fibroblasts. Both the transcript and protein level of Chemerin/ RARRES 2 were augmented in senescent fibroblasts compared to young fibroblasts. The analysis of chemokine receptor expression demonstrated that the Chemerin receptors CCRL2 and GPR-1, but not CMKLR1, were highly expressed in squamous cell carcinoma (SCC) lines compared to normal keratinocytes. Enhanced concentrations of Chemerin were detected in dermal fibroblasts of human skin sections derived from old compared to young individuals as shown by immunofluorescence staining. Immunostaining of SCC biopsies revealed that Chemerin was highly expressed in cancer-associated fibroblasts, while CCRL2 was detected in cytokeratin-positive SCC cells.

We further aimed to investigate the paracrine SASP effect of senescent fibroblasts and the selected chemokines on the motility of SCC lines using a Transwell migration assay. Conditioned media of senescent fibroblasts significantly increased the migration of SCC lines in comparison to media conditioned by young fibroblasts. Interestingly, Chemerin enhanced the migration of SCC lines *in vitro*, which was mediated through activation of the mitogen-activated protein kinase (MAPK) signaling pathway. Taken together, these data suggest that the contribution of senescent fibroblast in ageing of the stroma assists in tumor progression, of which Chemerin is one of the prime mediators relevant for SCC progression.

P194

Voriconazole does not affect the viability of human keratinocytes treated with UV-A, -B or retinoids *in vitro* and in organotypic skin models

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Voriconazole (Vfend®) is a triazole antifungal medication that is generally used to treat serious, invasive fungal infections. It is approved for the treatment of invasive aspergillosis, candidemia in non-neutropenic patients, fluconazole-resistant serious invasive Candida infections (including C. krusei) and serious fungal infections caused by Scedosporium spp. and Fusarium spp.

The primary mode of action of voriconazole is the inhibition of fungal cytochrome P-450-mediated 14 alpha-lanosterol demethylation, an essential step in fungal ergosterol biosynthesis.

Among the dermatological adverse events associated with voriconazole treatment, induction and/or rapid progression of cutaneous squamous cell carcinoma (SCCs), especially in Organ Transplant Recipient (OTR) patients, is of great interest.

Here we demonstrate that *in vitro* voriconazole treatment of primary human keratinocytes, HaCat cells as well as human cutaneous SCC cell lines with or without exposure to UV-A, UV-B and/or retinoids (short and long term cultures) neither affect the cell viability nor induces apoptosis. Furthermore, we observed similar results in a human organotypic skin model.

Our findings are in accordance with and extend a recent publication by Angeles JGC et al. (J Clin Exp Dermatol Res 2013, 4:173).

We conclude that other factors, such as the immunosuppressive regimen, rather than a direct effect on keratinocytes, might play a role in induction and/or rapid progression of SCC development by voriconazole in OTRs and this warrants further investigations.

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The Ratio of Mcl-1 and Noxa determines resistance to the BH3 agonist ABT737 in squamous cell carcinoma of the skin

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Tumor progression and therapy resistance in squamous cell carcinoma of the skin (SCC) is strongly associated with resistance to intrinsic mitochondrial apoptosis. We thus investigated the role of various anti-apoptotic Bcl-2 proteins for apoptosis protection in SCC using the BH3 agonist ABT737 that can overcome multidomain Bcl-2 protein protection. Sensitive SCC cells underwent rapid loss of mitochondrial membrane potential (MMP), subsequent apoptosis concomitant with caspase-3 activation and an early release of mitochondria-derived cytochrome c and smac/ DIABLO. In contrast, ABT737 resistance in subsets of SCC cells was not explained by XIAP, important for protection from DR-induced apoptosis in SCC. Of note, ABT737 did not prime SCC cells to DR-induced apoptosis. Interestingly, the ratio of Mcl-1 and Noxa determined sensitivity to ABT737: Loss of Mcl-1 rendered resistant cells sensitive to ABT737, whereas loss of Noxa promoted resistance in sensitive cells. In line, suppression of Mcl-1 by the pan-Bcl-2 inhibitor Obatoclax or overexpression of Noxa rendered resistant SCC cells sensitive to BH3 mimetics. Our data indicate that targeting of the Mcl-1/Noxa axis is important to overcome resistance to mitochondrial apoptosis in SCC. Therefore combination treatment of ABT737 or derivatives with Mcl-1 inhibitors, or inducers of Noxa, may represent a novel option of targeted therapy in metastatic SCC of the skin.

P196 (O06/03)

RIPK3 promoter methylation confers necroptosis resistance to malignant melanoma

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Melanoma cells have or acquire resistance to apoptotic and necroptotic stimuli which is considered a major hindrance to therapeutic success. Inhibitor-of-apoptosis proteins (IAPs) are important inhibitors of apoptotic and necroptotic cell death. Necroptosis is activated by Ripoptosome or Necrosome assembly and downstream signalling leading to mixed lineage kinase like protein (MLKL) phosphorylation and activation. These molecular signalling events are critically required for efficient necroptosis execution. When we investigated the impact of IAPs for cell death regulation in melanomas, suppression of IAPs strongly sensitized a panel of melanoma cells to death ligand-induced cell death which, surprisingly, was largely mediated by apoptosis as suggested by the complete rescue of cell death by addition of caspase inhibitors. Interestingly, the absence of necroptosis signalling correlated with fully repressed RIPK3 mRNA and protein expression in melanomas cell lines, while primary melanocytes and cultured nevus cells expressed RIPK3 mRNA and protein. Reconstitution of RIPK3, but not a RIPK3-kinase dead (KD) mutant in a set of melanoma cell lines overcame CD95L/ IAP antagonist-induced necroptosis resistance independent of autocrine TNF secretion. Using specific inhibitors, functional studies revealed that RIPK3-mediated MLKL phosphorylation and necroptosis induction critically required RIPK1 signalling. The absence of RIPK3 at the mRNA level suggested the possibility that loss of RIPK3 is mediated by transcriptional repression. When we investigated the importance of promoter methylation as a cause of RIP3 mRNA repression, demethylation studies using 5- aza-2′-deoxycytidine dramatically promoted reexpression of RIP3 at mRNA and protein level that, importantly, promoted CD95L/IAP antagonist-mediated necroptosis in melanoma. Our data suggest that the loss of RIPK3 in melanoma is critical to protect from necroptosis. Moreover treatment combinations of DNA demethylating agents together with drugs that inhibit IAPs may allow unmasking the necroptotic signalling machinery in melanoma as a potential innovative treatment for metastatic melanoma.

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Targeting Rab27a to suppress melanoma proliferation and invasion

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Metastatic melanoma is notoriously difficult to treat. Although treatment options have improved with the introduction of new targeted therapies and immunotherapies, more therapeutic strategies are needed. The Rab GTPase family of trafficking proteins is being increasingly implicated in cancer cell biology. Rab27a has been identified as a potential driver gene in a study of chromosomal copy number and gene expression in melanoma. While the role of Rab27a in melanosome trafficking in melanocytes is well known – its role in melanoma cell biology is poorly understood.

Consistent with Rab27a's role as a driver gene for melanoma, we found that Rab27a expression was significantly increased in melanoma samples compared to benign nevi. Rab27a was also highly expressed in many melanoma cell lines. In order to investigate the function of Rab27a in melanoma cells, shRNA knockdown of Rab27a in Rab27a-high and overexpression of Rab27a in Rab27a-low melanoma cell lines were performed. Loss of Rab27a inhibited proliferation and invasion in a 3D spheroid assay. This decrease in invasion was accompanied by a loss of invadopodia activity. Moreover, Rab27a-GFP, ectopically expressed in Rab27a-low melanoma lines, localized to both melanosomes and non-melanosomal structures, including actin rich foci. Taken together, these findings indicate that Rab27a regulates invadopodia formation in melanoma cells.

Treatment of melanoma cells with simvastatin, which is a non-specific inhibitor of Rab function, also inhibited invasion at low concentrations, while high concentrations caused cell death. Invasion is more substantially inhibited in Rab27a-high cell lines, suggesting inhibition of Rab27a may partially explain the effect of statins on melanoma invasion.

Our data indicate that Rab27a plays a central role in proliferation and invasion in Rab27a-high melanoma cells, although the molecular mechanisms underlying the effect on proliferation are still unclear. Rab27a expression is confined to melanocytic cells as well as some other specialized cell types making Rab27a a novel potential therapeutic target. Although no specific Rab inhibitors are available, statins, which have minimal side effects and are commonly used to treat hypercholesterolemia, are known to inhibit the function of small GTPases such as Rabs. At clinically relevant low concentrations, statins were able to reduce melanoma invasion. Statins may thus be a safe preventative therapy in high-risk individuals or early stage melanoma for preventing melanoma metastasis, and future development of more specific Rab27a inhibitors may also have therapeutic potential.

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Melanoma tumor sub-populations are defined by MITF expression, and exhibit enhanced proliferation and characteristics of an EMT

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Background: Melanoma drug resistance may be, in part, due to tumor heterogeneity. Heterogeneity is the occurrence of different sub-populations of cancer cells within a tumor, resulting in multiple cellular phenotypes within a single site. These populations can be proliferating or arrested, invading or quiescent. As these cancer cells exhibit variable behaviors, they also respond to therapies uniquely. Understanding the molecular signature influencing cancer cell activity within tumors is therefore crucial to design the most effective therapeutic regimen.

Methods: To better understand tumor heterogeneity within melanoma, cutting edge imaging technology and the fluorescence ubiquitination cell cycle indicator (FUCCI) system were employed to observe different phases of the cell cycle in real-time.

Results: Interestingly, we found that tumor xenografts grown from melanoma cells produced two cohorts. One that contained distinct clusters of arrested or proliferating cells, and another that displayed a more homogenous dispersion. It was then determined these two phenotypes could be separated by microphthalmiaassociated transcription factor (MITF) expression, with high MITF levels correlating with uniform cellular distribution. Furthermore, in WM164 cells, which normally give rise to homogenous tumor xenografts, knockdown of MITF by shRNA converted the phenotype to become clustered. Cells that express MITF were grown into 3D tumor spheroids embedded in collagen, and stained for MITF. MITF expression was found predominantly in the periphery of the spheroid, which also had high Slug and Vimentin expression, with a decrease in E-cadherin, indicative of an epithelial to mesenchymal transition (EMT). This area also corresponds with the region of highly proliferative cells. Additionally, serum starvation, resulting in cell cycle arrest, resulted in decreased MITF levels, and knockdown of MITF by shRNA gave rise to more cells arrested in G1.

Conclusion: These data outline how MITF and tumor heterogeneity are tightly intertwined within tumor architecture, making it an important marker for therapy design.

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Melanoma cells in G1 phase escape proteasome inhibitor cytotoxicity

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Background: Using the fluorescent ubiquitination-based cell cycle indicator (FUCCI), which facilitates real-time cell cycle tracking, we have demonstrated *in vitro* and *in vivo* that melanomas are composed of differentially cycling tumour cells in a subcompartment-specific distribution. Further, we have shown that targeting the endoplasmic reticulum with fenretinide (synthetic retinoid) or bortezomib (26S proteasome inhibitor) induces cell cycle arrest and apoptosis of metastatic melanoma cells *in vitro* and *in vivo*. This study aims to investigate the effect of ER stress-inducing agents on the dynamics of cell division and cell death of individual melanoma cells within the complex tumor microenvironment, and to develop combination strategies that increase the efficacy of ER stress-inducing agents for melanoma therapy.

Methods & Results: 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. Utilising the F-XBP1ΔDBD-venus reporter construct, which labels the cytoplasm in response to ER stress, we found that bortezomib induced ER stress, delayed cell cycle progression, and combination with fenretinide increased cell death in 2D and 3D culture. Flow cytometry and confocal microscopy indicated that treatment of FUCCI-melanoma cells with bortezomib induced G2 accumulation in 2D and 3D culture over the course of 24 h. In contrast, by 72 h the majority of cells were in G1 phase. Interestingly, bortezomib induced both G1- and G2 arrest, but preferentially killed G2-phase cells. While temozolomide enhanced the cytotoxic effect of bortezomib, MEK inhibitors blocked it in all melanoma cells, as did selective BRAF inhibitors in BRAF mutant cells.
Conclusion: Our data suggest that bortezomib combined with fenretinide or temozolamide is a strategy worth exploring for the treatment of BRAF-inhibitor insensitive or resistant melanoma. Importantly, melanoma cells arrested in G1 are protected from bortezomib cytotoxicity, which excludes MAPK inhibitors as combination partners.

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Impact of neurotrophin receptor signalling on melanoma cell migration in human and murine melanoma cell lines

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Adaptation of cancer cells to changes in the tumormicroenvironment plays a crucial role in tumor cell survival and therapy resistance. Over the recent years it has become clear that not only hardwired genetic changes in single tumor cell clones but also reversible adaptive processes contribute considerably to survival, outgrowth and metastasation even in unfavourable conditions resulting in tumors consisting of heterogenous cells.
In our previous work we could show that melanoma cells exist in a dynamic, interconvertible equilibrium between differentiated and dedifferentiated subpopulations that rapidly adapts to inflammatory signals in the environment. We identified TNF-alpha as an important modulator of this phenomenon and demonstrated that it potently upregulates the neurotrophin receptor Ngfr (CD271) on melanoma cells.
We therefore hypothesized that neurotrophin signalling via Ngfr may play an important role for survival and progression of tumor cells under proinflammatory conditions.
We analyzed the expression of the neurotrophins Ngf, Bdnf, NT-3, NT-4/5 and their receptors Ngfr, Trk-A, -B, and -C on a large panel of human melanoma cell lines by PCR and FACS or ELISA under normal and proinflammatory conditions. We could confirm our previous findings that TNF-alpha treatment leads to a reversible phenotype shift to less differentiated Ngfrhigh melanoma cell subpopulations in a large panel of human melanoma cell lines. We could also demonstrate robust expression of Trk-A irrespective of TNF-stimulation.
In trans-well chamber migration assays we could show that the neurotrophin Ngf leads to increased migration of melanoma cells and pre-treatment with TNF-alpha further enhances these migratory capacities. In a next step we tried to determine which of the two receptors for Ngf – Ngfr or Trk-A – is important for migration of the melanoma cells. Interestingly blockade of Ngfr by a monoclonal antibody did not result in signficanty changed migration compared to untreated controls under normal or proinflammatory conditions. However pharmacological blockade with a specific Trk-A inhibitor resulted in significantly reduced migration compared to untreated cell lines under normal and proinflammatory culture conditions. We could confirm these results in murine melanoma cell lines as well.
Taken together we could show that the neurotrophin Ngf is a chemo-attractant for human melanoma cells and TNF-a pre-treatment endows melanoma cells with enhanced migratory capacities. Additionally, the Ngf receptor Trk-A seems to play an important role in migration of melanoma cells.
We are currently trying to delete either Ngfr or Trk-A by the CRISPR-Cas9 system in selected murine melanoma cell lines to dissect these pathways further and eventually evaluate the physiological role in a mouse model of transplantable melanoma.

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Lecithin retinol acyltransferase (LRAT) affects retinoid sensitivity in malignant melanoma

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Retinoids such as all-trans retinoic acid (ATRA) influence cell growth, differentiation and apoptosis and may play decisive roles in tumor development and progression. While murine melanoma cells are highly sensitive to retinoid treatment, human melanoma cells have developed still unidentified mechanisms that mediate cellular retinoid resistance. One of the key retinoid metabolizing enzymes is lecithin retinol acyltransferase (LRAT), which catalyzes the transformation of all-trans retinol (vitamin A; ATRol) into inactive retinyl esters. LRAT is highly expressed in human melanoma cells. The aim of this study was to identify the mechanisms in retinol metabolism that are responsible for cellular retinoid sensitivity in the murine melanoma cell line B16F10 and for retinoid resistance in the human melanoma cell line SkMel23.
We found that the murine retinoid-sensitive B16F10 cell line does not express the enzyme LRAT whereas human melanoma cells like SkMel23 does. LRAT overexpression in B16F10 cells decreased the anti-proliferative effects of retinoid treatment in these murine melanoma cells. HPLC analysis revealed that the level of inactive retinyl ester increased after ATRol treatment and levels of the substrate ATRol and biologically active ATRA significantly decreased in LRAT-overexpressing murine melanoma. Further, we showed that a stable LRAT knockdown (KD) in the human melanoma cell line SkMel23 leads to significantly increased levels of the substrate ATRol and biologically active ATRA. LRAT KD restored cellular sensitivity to retinoids analysed in cell culture assays and melanoma 3D skin models. Furthermore, ATRA-induced gene regulatory mechanisms drive depletion of added ATRol in LRAT KD cells possibly via a significant upregulation of retinoid-regulated genes like CYP26A1 and STRA6 in LRAT KD cells suggesting their possible involvement in mediating retinoid resistance in melanoma cells.
In conclusion, LRAT seems to be important for melanoma progression. We propose that reduction of ATRol levels in human melanoma cells by LRAT leads to a disturbance in cellular retinoid level. Thus, our data suggest that LRAT overexpression represents a novel mechanism by which tumor cells can escape high supplementary ATRA levels that mediate tumor-suppressive RAR signaling. Balanced LRAT expression and activity may provide protection against melanoma development and progression. Pharmacological inhibition of LRAT activity could be a promising strategy for overcoming retinoid insensitivity in human melanoma cells.

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Notch4 drives mesenchymal-epithelial transition in melanoma

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Notch signaling is fundamental in regulating development and adult tissue homeostasis. In cancer, the effects of Notch signaling are highly context dependent and both, oncogenic and tumor suppressive functions of Notch have been described. Notch4 recently has been reported to regulate the embryonic morphogen Nodal, thus contributing indirectly to melanoma progression. Here we show that the overexpression of the constitutively active intracellular domain of N4 (N4ICD) caused a mesenchymal-epithelial switch in melanoma cell lines. The N4ICD overexpressing cell lines showed strongly reduced invasion, migration and proliferation properties. On the molecular level this switch was accomplished by downregulation of the epithelial-mesenchymal transition regulators Slug and Twist1. By EMSAs we found that N4ICD induced transcription factors Hey-1 and Hey-2 bind directly to the promoter regions of Slug and Twist1 thereby suppressing gene transcription as determnined by luciferase assays. Slug and Twist1 have been reported to activate Vimentin and MMP2, both of which were down regulated in N4ICD overexpressing cells. N4ICD overexpressing cells showed increased E-cadherin expression. Therefore N4ICD overexpression indirectly leads to the formation of a less aggressive epithelial phenotype suggesting a role for Notch4 as a tumor suppressor in melanoma.

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A stress induced early innate response causes multi-drug tolerance in melanoma

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Acquired drug resistance constitutes a major challenge for effective cancer therapies with melanoma being no exception. The dynamics leading to permanent resistance are poorly understood but are important to design better treatments. Here we show that drug exposure, hypoxia or nutrient starvation leads to an early innate cell response in melanoma cells resulting in multi-drug resistance, termed induced drug tolerant cells (IDTC). Transition into the IDTC state seems to be an inherent stress reaction for survival towards unfavorable environmental conditions or drug exposure. The response comprises chromatin remodeling, activation of signaling cascades, and markers implicated in cancer stemness with higher angiogenic potential and tumorigenicity. These changes are characterized by a common increase in CD271 expression concomitantly with loss of differentiation markers such as melan-A and tyrosinase, enhanced ALDH activity and upregulation of histone demethylases. Accordingly, IDTCs show a loss of H3K4me3, H3K27me3 and gain of H3K9me3 suggesting activation and repression of differential genes. Drug holidays at the IDTC state allow for reversion into parent cells re-sensitizing them to the drug they were primarily exposed to. However, upon continuous drug exposure IDTCs eventually transform into permanent and irreversible drug resistant cells. Knockdown of CD271 or KDM5B decreases transition into the IDTC state substantially but does not prevent it. Targeting IDTCs would be crucial for sustainable disease management and prevention of acquired drug resistance.

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A chemokine expression signature correlates with melanoma metastasis

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Interconnecting processes in the tumor microenvironment is decisive to promote and sustain metastasis. Molecules linking processes like lymphangiogenesis with tumor/ host immunology could be key players. In a SCID xenotransplantation melanoma mouse model, the chemokine profile of primary tumors and lymph node metastasis was compared to controls. Expression of twelve chemokines in mRNA level proved to be differently regulated. These chemokines were subsequently analyzed in FFPE samples of human stage T1 to T4 melanomas using RT-PCR. Interestingly, a chemokine pattern primarily reflecting differences in biological behavior and not only the different tumor stages could be identified. From the upregulated chemokines we aim to identify the ones involved in the metastatic process. An increased mRNA level could be observed even in metastasizing T1 melanomas. To proof the biological relevance *in vivo*, overexpression was carried out in cell lines with diverse metastatic behaviors including two primarly isolated from the same melanoma tumor patient and studied in a xenotransplantation SCID mouse model. Animals bearing Chemokineoverexpressing tumor cells displayed a significant increase in lymph node and lung metastasis. Characteristic macroscopic tumor morphology along with microscopic findings revealed a more invasive phenotype in the overexpressing cell lines. A marked increase in neutrophil infiltration and tumor lymphangiogenesis in the overexpressing tumors compared to controls could explain the increment.

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The chemokines MIF (macrophage migration inhibitory factor) and MIF-2/DDT (D-dopachrome tautomerase) as key mediators in the pathogenesis of cutaneous squamous cell carcinoma

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MIF activates the MAP kinase cascade, modulates cell migration and acts as an important anti-apoptotic factor. Recent evidences suggest a key role of MIF in the pathogenesis of SCC. Previous studies identified MIF as a promoter of UV-induced skin tumorigenesis. Moreover, the newly described structural homolog of MIF, DDT, bind to the MIF surface receptor complex CD74/CD44 and shares certain cell signalling and effector functions. Recent findings suggest a pro-tumorigenic role for D-DT, but its function in skin cancer has not been studied yet. Here, we aim to scrutinize the role of MIF and D-DT in the development of SCC, as chemotactic factors and their exocrine and/or intracrine effects on skin keratinocytes. To further understand the pathophysiological function of MIF and D-DT, we performed immunohistochemical studies in skin lesions of patients with SCC. MIF and DDT expression was clearly enhanced in SCC compared to normal human skin. These findings might indicate that keratinocytes are a main source of MIF and DDT. To elucidate a potential role of MIF and D-DT in skin photocarcinogenesis, we studied the capacity of NHEK, HaCaT cells, and human SCC cell lines, to release MIF and D-DT protein after stimulation with UVB light and observed a timedependent, UVB-induced increased MIF and D-DT release. MIF and D-DT are known to interact with CD74, which signals via regulated intramembrane cleavage or by co-activating CD44 and by complexing with the chemokine receptors CXCR2/4. Using immunohistological staining, we observed an enhanced CD74 expression in skin lesions of actinic keratosis and SCC compared to normal skin. To find out whether skin keratinocytes themselves are not only a major source of cutaneous MIF release but also a putative target, expression of the different MIF receptors was studied in NHEK and HaCaT cells using FACS analysis. NHEK and HaCaT cells essentially lacked any constitutive expression of CXCR2/4 and CD74. However, we found that keratinocytes stimulated with IFNγ in an inflammatory environment upregulated CD74 surface expression enabling responsiveness to MIF. Accordingly, we observed that untreated keratinocytes lacking MIF expression (Mif−/−) and thus

autocrine or intracrine activation by MIF showed a significant alteration in their gene expression profile compared to wildtype keratinocytes using gene expression arrays. Recent evidence suggests that MIF plays a crucial role in leukocyte recruitment. MIF promotes pro-migratory processes both indirectly through stimulating the release of other migratory factors and directly through an interaction with CXCR2/4. To get more insight in the effect of MIF and D-DT on leukocyte migration in the context of skin inflammation and SCC, we performed a chemotaxis assay with monocytederived dendritic cells (DC) and showed that both MIF and D-DT promoted DC migration. Furthermore, we observed maintaining of DC in 3D equivalents after incubation with MIF compared to control models cultured with MIF/anti-MIF where DC migrated from the epidermal in the dermal layer and disappeared from the model. Together, our findings suggest that MIF and D-DT are important factors enhancing the promotion of SCC tumorigenesis and progression in an inflammatory environment. Further studies to get a more complex understanding of the possibly synergistic, additive, or neutralising interaction of MIF and D-DT in the pathogenesis of SCC are required.

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Type I interferon signaling in myeloid cells controls the balance between immunity and inflammation in melanoma

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Background: We hypothesized that activation of the type I IFN system promotes antitumor immunity and limits pro-tumorigenic inflammation in melanoma.
Methods: To test this hypothesis we treated established Hcmel3 melanomas in C57BL/6 or global/conditional Ifnar1 knockout mice with adoptively transferred melanoma-specific CD8 T cells and IL-2. Tumor growth kinetics, T cell expansion and effector function as well as tumor-infiltrating immune cells were analyzed.
Results: Transplanted melanomas escaped adoptive T cell treatment rapidly in Ifnar1-deficient mice which leads to decreased overall survival compared to Ifnar1- competent mice. Surprisingly we observed significantly elevated numbers of T cells in Ifnar1-deficient compared to Ifnar1-competent mice. Histological analysis showed prominent loss of gp100 in melanoma cells that escaped immunesurveillance in Ifnar1-deficient mice along with a proinflammatory tumor microenvironment mostly composed of myeloid cells. Interestingly, increased T cell expansion and early escape due to inflammation-induced dedifferentiation were recapitulated in conditional knockout mice lacking a functional type I IFN system only in myeloid cells.
Conclusions: Our results show that a functional type I IFN system in myeloid immune cells is required for effective treatment of melanoma with adoptively transferred T cells and IL-2.

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Exposure of melanoma cell lines to TRAIL induces an ameboid-like phenotype

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By stimulation of its death receptors TRAILR1 and TRAILR2 TRAIL can induce caspase-mediated apoptosis, but also non-apoptotic signalling via caspase-8. The outcome of receptor triggering depends on the activation of caspase-8 target proteins, e.g. caspase-3, but also CYLD and ROCK-1. Melanoma cell lines are known to be relatively resistant towards TRAIL-induced cell death. It has been shown that death receptor-resistant tumor cells show an enhanced metastatic potential upon TRAIL triggering.
Here, we analyzed the response of primary melanocytes and 6 different melanoma cell lines towards TRAIL. Although TRAIL death receptors were consistently expressed in melanocytic cells, we confirmed that primary melanocytes and the melanoma cell lines are relatively resistant towards TRAIL-induced cell death. Interestingly, some of the melanoma cell lines responded to TRAIL by acquiring a rounded, ameboid-like morphology, while primary melanocytes did not change their shape. Time-lapse microscopy revealed that the rounded cells did neither divide nor die. Some of them stretched out to move and rounded up again, while others returned to their normal shape after a couple of hours. Strikingly, the TRAIL-induced ameboid-like phenotype is caspase-mediated. Analysis of caspase-8 target proteins showed a TRAIL-dependent cleavage of ROCK-1, caspase-3 and PARP as well as degradation of CYLD. Moreover, the pan-caspase inhibitor zVAD-fmk as well as the ROCK-1 inhibitor Y-27632 repressed the TRAIL-induced morphologic alterations.
Taken together, TRAIL, apart from inducing cell death, can promote other caspasedependent functions that potentially lead to an altered cellular shape and eventually an altered metastatic and/or migratory capacity. These preliminary findings deserve further studies to evaluate a possible use of ROCK-1 inhibitors for future therapeutic approaches.

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Large T antigen truncating mutations occur before Merkel cell poyomavirus integration in Merkel cell carcinoma cells

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Merkel cell carcinoma (MCC) is an aggressive neuroendocrine tumor of the skin whose etiology has long been unsettled. Since the discovery of the Merkel cell polyomavirus (MCPyV) in 2008, however, this virus has been established as a possible causative factor in at least most of the tumors. Indeed, MCC is currently the best model of a polyomavirus-associated/induced tumor. Thus, well characterized MCC cell lines are needed to study this causal relationship in details. Consequently, we established and characterized six MCPyV-positive MCC cell lines in the current study. One, PeTA, was generated from a primary tumor, WaGa from ascites, BroLi and LoKe from pleural effusion and WoWe and AlDo from skin metastases. Comparative genomic hybridization revealed a rather stable genome carrying only a limited number of chromosomal amplifications and deletions typically for MCC. All cell lines were CK20 and NSE positive, and all expressed MCC-characteristic truncated MCPyV-encoded large T antigen (LT). For BroLi this truncation was caused by insertion, while the others carried stop codon mutations. For five of the cell lines we were able to identify the insertion sites in introns of different genes. Stop codon mutations and insertion sites could be confirmed in respective clinical samples of the patients for which we generated the cell lines. Inverse PCR suggested that three of the cell lines, i.e. WaGa, WoWe and LoKe, contained MCPyV genomes as concatemers, which could be confirmed for the two cell lines WaGa and LoKe with known integration sites. To evaluate MCPyV copy numbers we performed real time PCR and calculated the relative presence revealing that the highest relative presence was present in WoWe. Indeed, in those cell lines, which supposedly contain multiple copies of MCPyV, generally the highest relative presences were measured. Surprisingly, a high relative presence was also measured for PeTa suggesting that chromosomal amplification of the integration site occurred after viral integration. Importantly, our observation of distinct stop codon mutations in cell lines with concatemeric MCPyV integration suggest that stop codon mutation occurs before integration. In summary, we provide the detailed characterization of six established MCPyV-positive MCC cell lines, which are likely to serve as a valuable tools in future MCC research.

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Phosphorylation of the Merkel cell polyoma virus Large T antigen on serine 220 is essential for its ability to support growth of Merkel cell carcinoma cells

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Accumulating evidence suggests that the Merkel cell polyomavirus (MCPyV) is a major causal factor in carcinogenesis of the aggressive skin cancer Merkel cell carcinoma (MCC). Tumor cell growth of MCPyV-positive MCC cells is dependent on the expression of a truncated viral Large T antigen (LT) with an intact retinoblastoma protein (RB)-binding site. In order to determine phosphorylation of MCC-characteristic truncated MCPyV-LT, we performed mass spectrometry revealing that it is a multi-phospho-protein phosphorylated on several serine and threonine residues. Notably, most point mutations altering the different putative phosphorylation sites did not affect the ability to rescue knockdown of endogenous T antigens indicating that phosphorylation of the respective amino acids is not essential for the growth promoting function of MCPyV-LT in MCC cells. In contrast, however, alteration of serine 220 to alanine completely abolished the ability of MCPyV-LT to support proliferation of MCC cells. Accordingly, a mutation of the same site to glutamic acid, mimicking phosphorylation, was fully functional. Moreover, MCPyVLTS220A demonstrates reduced binding to the Retinoblastoma protein (RB) in coimmunoprecipitation experiments suggesting that phosphorylation of serine 220 is required for efficient RB inactivation. Thus, phosphorylation of serine 220 by a yet not identified cellular kinase seems to be a critical step for MCPyV-LT function in tumor cells and may, therefore, be a potential target for future therapeutic approaches.

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Ultraviolet (UV)-A irradiation induces melanoma invasion via enhanced Warburg effect

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Melanoma is a malignant tumor with high mortality and increasing prevalence for which exposure to ultraviolet (UV) radiation is considered to be an important risk factor. Especially UVA (320–400 nm) radiation induces the formation of reactive oxygen species (ROS) which oxidatively damage cellular molecules. It was recently shown that UVA radiation is capable to induce murine melanoma, but the role of UVA in the progression of melanoma is still not investigated. During early progression of melanomas before metastasising, most melanomas show initial proliferation of melanoma cells and a metabolic characteristic of most proliferating tumor cells is the preference of aerobic glycolysis instead of oxidative phosphorylation (Warburg effect). Here we investigated the role of UVA radiation in progression of melanoma, especially induction of progression markers, changes in Warburg effect and invasive potential. Upon UVA radiation, initial melanoma cells show increased Warburg effect with increased glucose consumption and increased lactate production. The tumor marker transketolase and phosphorylated Akt kinase, which are involved in metabolic changes and associated with proliferation, are also elevated upon UVA radiation. With *in vitro* invasion assays we show, that lactate, which is produced via UVA enhanced Warburg effect, increases invasiveness of initial melanoma cells. This effect is mediated by reactive oxygen species which are induced by UVA radiation as treatment with ROS scavengers impairs UVA induced lactate production and invasion. Furthermore transcription of tumor relevant matrix metalloproteinases and not TIMP1 are highly upregulated upon treatment with lactate. Therefore we could show in melanoma cells, derived from melanomas of early progression that production of lactate, induced by UVA radiation, increases invasiveness of initial melanoma cells via expression of MMPs.

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Mutual enhancement of the antiproliferative and proapoptotic effects of imiquimod and diclofenac in cutaneous SCC cells

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Actinic keratoses (AK) occur on sun-exposed skin and are characterized by high prevalence as well as by the risk to proceed to cutaneous squamous cell carcinoma (SCC). Imiquimod (Aldara®) and diclofenac/HA (Solaraze® 3% Gel) have been approved for AK treatment. The toll-like receptor (TLR) agonist and nucleoside analogue imiquimod mediates pronounced stimulation of an antitumor immune response, and also direct effects on tumor cells have been reported. On the other hand, diclofenac functions as inhibitor of cyclooxygenase-2 (COX-2) and thus interferes with prostaglandin E2 (PGE2)-mediated induction of cell proliferation and inhibition of apoptosis. Previously, we have shown that diclofenac enhances apoptosis induction by death ligands as CD95L/FasL, TRAIL (TNF-related apoptosisinducing ligand) and TNF-alpha.
Here, we prove the direct induction of apoptosis and inhibition of cell proliferation by both treatments in cutaneous SCC cell lines. Inhibition of cell proliferation was partly accompanied by an upregulation of the cell cycle inhibitory protein p21. Importantly, the combination of both drugs resulted in a strong mutual enhancement of inhibition of cell proliferation, the induction of apoptosis and reduction of cell viability. These effects were accompanied by a characteristic loss of the mitochondrial membrane potential, indicative for an enhancement of proapoptotic mitochondrial pathways. Interestingly, the effects appeared as completely independent of toll-like receptors TLR7 and TLR8. As both drugs are approved, combination therapies may be considered for treatment of patients.

P212

Stabilizing the cancer genome by Th1 cytokine-mediated immunotherapy

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Immunotherapy with tumor-associated antigen (TAA) specific T-helper-1 (Th1) cells can reduce the tumor burden in patients with skin cancers, such as melanoma or squamous cell carcinoma. We recently showed that such Th1 cells can arrest cancers by inducing a strictly interferon-γ (IFN-γ)- and tumor necrosis factor (TNF)- dependent growth arrest in cancers. The combined action of the Th1 cytokines IFN-γ and TNF induces senescence in a large panel of cancers by activating the tumor suppressor p16INK4a. To determine the underlying mechanisms, we studied carcinomas in mice, where the transgenic expression of SV40 large T antigen 2 (Tag2) under the rat insulin promoter 1 (RIP) results in loss of the cell cycle control through inhibition of the tumor suppressors p53 and Rb. *In vitro* treatment of isolated β-cancer cells with IFN-γ and TNF stabilized the hypophosphorylated state of Rb and suppressed the transcription factor E2F2. As Rb hypophosphorylation and E2F suppression both should arrest cycle progression, we studied the effect of immunotherapy with TAA-specific Th1 cells of the accumulation of genomic alterations in β-cancers *in vitro* and *in vivo*. To analyze this question, we first performed array comparative genomic hybridization (CGH) of tumors. As expected, we detected multiple quantitative chromosomal aberrations in cancers of sham-treated RIP-Tag2 mice. In clear contrast, β-cancers from mice treated with TAA-specific Th1 cells remained genetically stable without developing chromosomal aberrations. This genome-stabilization was strictly cytokine-dependent, as cancers from mice with a disruption of the IFN-γ- and TNF-signaling pathway showed a similar chaotic genome with multiple CGH-aberrations as sham-treated mice. In line with this, functional data showed that *in vitro* cultured β-cancers from Th1-treated mice remained growth

arrested, whereas β-cancer cells from sham-treated mice showed the exponential growth pattern of cancer cells. Again, the growth arrest was abrogated in mice with defective IFN-signaling. Moreover, flow cytometry confirmed the abnormal DNA content in cancers of STAT1-deficient or sham-treated mice, while islets from cancer prone mice treated with Th1 cells preserved a normal DNA content. Thus, Th1 immunity can protect from cancer progression by stabilizing the genome, probably through restoration of the Rb-signaling pathway.

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JunB and its role in cytokine induced tumor senescence

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Tumor immunotherapy has recently become a more and more relevant field in treatment of melanoma and other cancers. Interestingly, immunotherapy does not always lead to cancer cell apoptosis or killer-cell-mediated killing but to a stable growth arrest. This stable growth arrest, also known as senescence, can be induced by the Th1 cytokines interferon gamma (IFNγ) and the tumor necrosis factor alpha (TNFα) *in vitro* and *in vivo*. Previous data showed an upregulation of the tumor suppressor gene p16INK4a, but the pathway, by which cellular senescence is induced, is still unknown. A possible candidate is JunB. This protein is known as a target of the TNFα signaling pathway, but an interaction between JunB and the p16INK4a promoter is yet unknown. Furthermore, it is unclear whether JunB can bind directly to p16INK4a or if it might be associated in a protein complex, for example AP-1.

In order to investigate this signaling pathway in detail, we performed Chromatin Immunoprecipitation (ChIP) assays using cell lines isolated from the pancreas of RIPTag2 mice. In this mouse model, the SV40 large T antigen 2 (Tag2) is expressed under the rat insulin promoter (RIP). This causes an inhibition of p53 and Rb1 exclusively in the Langerhans islets cells leading to a multistage carcinogenesis.

First data show a translocation of JunB from the cytoplasma into the nucleus after cytokine treatment. In addition, a binding of JunB on the JunB promoter was observed after 72 h of stimulation, consistent with a higher JunB concentration on protein level. Thus, this finding indicates an autocrine loop of JunB. A high relative enrichment of JunB on the cyclin-dependent kinase 4 (CDK4) promoter in control cells compared to cytokine treated cells indicates a dissociation of JunB from this promoter in senescence cell. A direct binding of JunB to the p16INK4a promoter could not be observed.

In sum, our findings suggest a role of JunB in senescence cells, but it remains to be investigated if JunB, probably as a part of a protein complex, might interact with the promoter of p16INK4a.

P214

RET-melanoma recruit CD19+ CD24+ IL10+ cells to lymph nodes by a Pselectin- mediated mechanism

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Melanoma escapes the control of the immune system at least partially through the recruitment of suppressive leukocytes. To analyze the melanoma infiltrating leukocytes we used the RET melanoma model. C57BL/6 mice were injected s.c. with RET-melanoma cells into the flanks. One to two weeks later FACS analysis of the tumor draining inguinal lymph nodes revealed a significant increase of CD19+CD24+ cells in tumor bearing mice (20% control vs 30% RET tumor). Furthermore, 7 days after C57BL/6 mice had been challenged with RET-melanoma cells we also recorded increased numbers of CD19+CD24+ cells in the peritoneum, which serves as reservoir for regulatory B cells (Breg), and the detailed analysis of the CD19+CD24+ cells showed a high IL-10 expression. Thus, these data suggest that RET melanoma may induce Breg. Lately, it has been shown that P-selectin is a ligand for CD24. We reasoned whether melanoma-affected endothelial cells may recruit Bregs by CD24:P-selectin interactions. Therefore, we cocultured RET cells with the endothelial cell line bEnd.3 and detected a substantial increase of P-selectin on bEnd.3 cells after coculture with RET melanoma. In contrast, unstimulated control bEnd.3 had only modest P-selectin expression. Moreover, in boyden chamber assays RET melanomas were able to stimulate migration CD19+CD24+ B cells towards the RET cells. In summary, our findings suggest that melanoma may recruit Breg through the up-regulation of P-selectin on endothelial cells, resulting in suppression of anti-tumor immunity.

P215

The oxidative stress inducer elesclomol targets drug resistance in melanoma

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Melanoma therapy with cytotoxic agents is commonly accompanied by drug resistance and tumor relapse. Therapeutic resistance can be induced by intrinsic, adaptive, and/or acquired mechanisms. Recently, we have identified a subpopulation of intrinsic multi-drug-resistant cells, which are characterized by a slow-cycling phenotype, long-term tumor-maintaining properties, and an elevated oxidative bioenergy supply in mitochondria. In this study, we have attempted to target these multi-drug-resistant cells by inducing mitochondrial oxidative stress. For this purpose, we have applied the first-in-class copper-chelator elesclomol. Elesclomol-copper complexes lead to oxidative stress by interference with the mitochondrial respiration chain or by indirect non-mitochondrial induction of reactive oxygen species. We could demonstrate *in vitro* that elesclomol leads to increased mitochondrial ROS levels in melanoma cells, eliminates the slow-cycling subpopulation, and attenuates longterm growth. Moreover, enrichment for drug-resistant slow cycling cells upon drug treatment with common melanoma therapeutics such as cisplatin was prevented by co-treatment with elesclomol. In conclusion, our data indicate that mitochondrial stress inducers, like elesclomol, can eliminate the multi-drug-resistant slow-cycling subpopulation of melanoma cells. Thus, a two-tiered therapeutic approach might be suitable in future to prevent tumor relapse, i.e. the combination of classical cytotoxic agents eliminating rapidly proliferating melanoma cells plus a mitochondrial stress inducer targeting the slow-cycling melanoma subpopulation.

P216

Role of the BH3-only pro-apoptotic BIK/NBK in Vemurafenib/Trametinib induced apoptosis in melanoma cell lines

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Melanoma is a highly aggressive skin cancer in which the MAPK pathway (RAF/MEK/ERK) is frequently activated. Specific inhibitors of BRAFV600E (e.g. Vemurafenib) and MEK (e.g. Trametinib) have demonstrated impressive reduction of tumor burden in patients, which, however, is frequently followed by relapse.

The biological response of cultured melanoma cells to Vemurafenib and Trametinib is characterized by a fraction of cells that undergo apoptosis while the remaining survive and acquire a senescence-like cell cycle arrest. To find out what determines the difference between these two responses we analyzed a series of single cell clones derived from the M14 melanoma cell line. We identified two single cell clones which responded with a high rate of apoptosis and two sub-lines which predominantly arrested upon treatment with Vemurafenib and Trametinib.

Screening of these clones for differentially expressed apoptosis-related mRNAs revealed loss of BCL2-interacting killer (BIK) mRNA in the arresting sub-lines. Ectopic expression of BIK was associated with increased apoptosis rates following Vemurafenib/Trametinib treatment, suggesting that BIK is a critical mediator of melanoma cell death induced by MAPK pathway inhibition. This is further supported by the finding that BIK levels are decreased in the surviving cells following Vemurafenib/Trametinib treatment.

Moreover, we demonstrate that BIK protein expression is low, even in melanoma cells with high mRNA levels, due to proteasomal degradation, as BIK strongly increases upon treatment with the proteasome inhibitor Bortezomib. Our current work aims to analyze whether RAF/MEK inhibition interferes with the proteasomal degradation of BIK and whether BIK can be derepressed in melanoma cells in order to enforce the apoptotic response.

In conclusion, our results suggest that BIK is a critical mediator of melanoma cell fate determination in response to MAPK pathway inhibition.

P217

Inhibition of oncogenic signalling increases the efficacy of adoptive cell transfer (ACT) immunotherapy

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Inhibition of oncogenic signalling and immunotherapeutic approaches prolong survival in metastatic melanoma patients, but most tumors relapse. We hypothesize that targeted inhibition of oncogenic signalling increases the efficacy of adoptive cell transfer (ACT) immunotherapy directed against melanocytic antigens. Indeed, clinical studies with signalling inhibitors have implicated the induction of melanocytic antigens as potential trigger of an accompanying immunological response.

To test this hypothesis we exploited MET tyrosine kinase oncogene addiction in our Hgf-Cdk4R24C murine melanoma model. We analyzed the effect of selective MET inhibitors (METi) on Hgf-Cdk4R24C derived melanoma cells and gp100 (pmel-1) transgenic T cells *in vitro* and *in vivo* and on transplanted melanomas. Melanoma bearing mice were treated with ACT immunotherapy alone, combined with METi or METi alone. METi efficiently blocked cell proliferation of Hgf-Cdk4 derived murine melanoma cells consistent with abrogation of MET downstream signalling *in vitro* and induced an increased expression of the melanocytic differentiation antigens gp100 and tyrosinase. *In vivo* METi treatment inhibited the growth of transplanted melanomas in a dose-dependent manner and strongly enhanced pigmentation and gp100 expression. Melanoma bearing mice treated with the combination protocol (METi+ACT) showed a significantly prolonged survival and elevated numbers of circulating gp100 (pmel-1) T cells in the blood together with a pronounced vitiligo like fur depigmentation that is consistent with an intensified systemic anti-melanocytic immune response.

Conclusion: Our results strongly support the rational that inhibtion of oncogenic signalling increases the efficacy of adoptive cell transfer (ACT) immunotherapy targeting melanocytic antigens. We currently address the contribution of increased antigen load following METi treatment versus the modulation of the tumor microenvironment.

P218

Y-box binding protein 1 – a prospective target in melanoma cell cycle regulation and vemurafenib resistance

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Previously, we showed that the oncogenic transcription and translation factor YB-1 is upregulated and translocated to the nucleus during melanoma progression and that YB-1 plays an important role in the regulation of proliferation, survival and invasive growth of melanoma cells. Furthermore, our previous data indicated that in patient samples also phosphorylation at Ser102 in the nucleic acid binding domain of YB-1 is increased during melanoma progression. Interestingly, nuclear translocation and transcriptional activation of YB-1 was reported to be mediated by this Ser102- phosphorylation.

Aim of this study was to investigate the functional effects of Ser102-phosphorylated YB-1 in malignant melanoma and a possible role of enhanced YB-1 transcriptional activity in mediating resistance of melanoma cells towards the BRAF(V600E) inhibitor vemurafenib (PLX4032).

Interestingly, mitotic melanoma cells exhibit strikingly enhanced levels of P(Ser102)-YB-1 compared to cells in the interphase. Co-localization of the Ser102- phosphorylated YB-1 with the mitotic spindle apparatus further hints at a direct involvement in cell cycle regulation.

Moreover, our data show that in melanoma cell lines with acquired resistance to vemurafenib Ser102-phosphorylation of YB-1 as well as its nuclear occurrence and transcriptional activity is significantly enhanced in comparison to their sensitive counterparts. This increased YB-1 activation is based on elevated MAPK signalling and interestingly seems to be mediated by the active p90 ribosomal S6 kinase (RSK) signalling. Intriguingly, RSK inhibition can increase the sensitivity of vemurafenib resistant melanoma cell lines to PLX4032 treatment. These data suggest that active RSK signalling mediates YB-1 Ser102-phosphorylation and nuclear activity, which might be involved in mediating vemurafenib resistance and consequently an attractive therapeutical strategy to overcome secondary resistance to the BRAF(V600E) inhibitor.

P219

Immune surveillance by Th1 cells controls the differentiation of cancers during malignant transformation

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RIP1-Tag2 mice expressing the T antigen (Tag) under control of the rat-insulinpromotor (RIP) develop malignant β-cell carcinomas. Due to the unrestricted proliferation of β-cancer cells and extensive tumor load with a non-regulated secretion of insulin, the mice fail to control their blood glucose levels and die from severe hypogycemia at the age of 13–15 weeks. As shown previously, adoptive transfer of Tag-specific T helper 1 (Th1) cells significantly prolongs the survival (up to two-fold) of these mice. In depth analysis of the Th1 cell-induced anti-cancer effect revealed that the Th1 cytokines interferon-γ (IFN-γ) and tumor necrosis factor (TNF) drive the β-cancer cells into premature senescence leading to a complete arrest of the tumor cell growth. Surprisingly, the Th1 cell-treated mice developed neither hypoglycemia nor a diabetic phenotype. When treated with cancer-specific Th1 cells the mice gained the capacity to normally control their blood glucose levels. These data indicate that Th1 cell-mediated immune surveillance does not only stop the unrestricted proliferation of the tumor cells but also influences the differentiation status during malignant transformation.

To investigate whether senescence induction affects the differentiation status of β-cancer cells, we analyzed the expression of 3 key differentiation markers that faithfully characterize β-cell differentiation during development. For this, we stained cryosections of tumors or isolated tumor cells from RIP1-Tag2 mice of different age (i. e. at well-defined steps of carcinogenesis) with antibodies against the 3 β-cell differentiation markers synaptophysin, insulin and the glucose transporter 2

(Glut2). To functionally characterize the isolated β-cancer cells, we measured their response to high glucose concentrations in a colorimetric mitochondrial activity assay *in vitro*. We found that the β-cells almost completely lose Glut2 and partially insulin during carcinogenesis, whereas the primitive neuroendocrine marker protein synaptophysin remains long time expressed by the β-cancer cells. Loss of Glut2 concurred with the inability of the β-cancer cells to adequately respond to glucose stress. Importantly, Th1 cell-based immunotherapy prevented the phenotypical loss of the differentiation markers insulin and Glut2 as well as the functional dedifferentiation of the β-cancer cells.
In conclusion, senescence surveillance by tumor-specific Th1 cells arrests both, unrestricted tumor growth and malignant transformation by keeping the tumor cells in a differentiated state. This non-toxic mechanism may underly the newly introduced antibody-based immunotherapies that induce stable disease and prolonged survival in patients with advanced malignant melanoma.

P220

Argonaute protein Ago2 in cytokine-induced senescence of human cancer

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Serrano et al. originally described that overexpression of an oncogenic version of HRAS (HRASG12V) in normal cells did not increase their proliferation but arrested cell division in these cells permanently. As the cells showed functional, biochemical and molecular changes that were indistinguishable from senescence, the phenomenon was named oncogene-induced senescence. This description established the concept of senescence as tumor suppressive mechanism. Previous studies by us showed that adaptive immunity and the T helper cell 1 (TH1) cytokines interferon-gamma (IFN-γ) and tumor necrosis factor (TNF) can induce tumor dormancy in a mouse model of endogenous pancreatic cancer. Subsequently we showed that these dormant cancer cells were arrested owing to senescence. The induction of this cytokine-induced senescence was dependent on the activation of the CDK inhibitor p16INK4a and consecutive inactivation of the E2F family of transcription factors. As some argonaute (Ago) proteins, namely Ago2 suppress E2F target genes and induce heterochromatic foci in doxorubicin-induced senescent cancers, we analyzed the role of Ago2 in senescence induction by TH1 cytokines.
Treatment of the human breast cancer line MCF-7 and the human rhabdomyosarcoma line A204 with IFN-γ and TNF stably reduced cancer cell proliferation, as compared with sham-treated cells. To study whether the TH1 cytokines induced senescence in the human cancer cell lines, we performed senescence-associated β-galactosidase and growth arrest assays. Both analyses confirmed the induction of senescence after cytokine treatment. Trypan-blue assays excluded that the cells had undergone apoptosis or necrosis. As the involvement of Ago proteins in cytokine-induced senescence is unknown, we first tested the expression and localization of Ago2 protein after treatment with TH1 cytokines by immunofluorescence. All cancer cells showed Ago2 expression in the cytoplasm independent of the senescence induction. Yet, following treatment with TH1 cytokines Ago2 trans-located from the cytoplasm into the nucleus. We found Ago2 translocation only in non-proliferating senescent cells; the data were confirmed by double-staining with the proliferation marker Ki67. Again, only senescent cells showed a prominent Ago2 translocation from the cytoplasm into the nucleus but not the Ki67+ proliferating cancer cells. Translocation started at 24 h and continued up to 72 h.
Thus, cytokine-induced senescence is an important tumor suppressive mechanism also in a variety of human cancers. As Ago2 trans-located into the nucleus, where it binds to the DNA, probably as a co-repressor of the E2F/Rb repressor complex, cytokine-induced Ago2 contributes significantly to senescence induction in human cancers.

P221

Integrative differential drug affinity-based proteomics identifies AURKA as a therapeutic target in human melanoma

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Drug discovery effort has paradoxically ignored the critical role of MITF in melanoma cell biology, including intrinsic and acquired drug resistance. To search the kinase(s) crucial to MITF-engendered properties, we used multi-kinase inhibitor-directed chemical proteomics-based differential target affinity screen in immortalized human melanocytes carrying ectopic MITF overexpression. Through subsequent 'gene expression-disease stage' associations and detailed functional interrogation in molecular-genetically diverse melanoma cellular systems, we identify Aurora Kinase A (AURKA) as a potential target in human melanoma, irrespective of underlying alterations including high MITF expression levels. The pre-emptive illustration of a MITF/Cyclin D1-mediated prospective resistance to AURKA inhibition, further informed dual AURKA/MITF and/or AURKA/BRAF targeting as intelligent synergistic combination regimens with potentially more robust and sustained responses. Furthermore, offering insights into high AURKA expression levels, our data suggests profusely active MAPK signaling in melanoma as an indirect regulator of AURKA expression in a cell cycle-dependent manner.

P222 (O02/01)

Tumor-derived VEGF-A mediates Hypercoagulation via von Willebrand factor fiber generation in Tumor vessels of Malignant Melanoma

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Human malignant melanoma is a cancer of the skin with poor prognosis due to high metastatic potential with metastasis to liver, lungs and brain. Moreover, it is well documented that melanoma patients hold a high risk of venous thromboembolism, even though the underlying mechanisms are not well understood so far. To metastasize, a tumor cell needs to interact with the vascular endothelium of blood vessels to extravasate through the vessel wall. However, how this interaction of tumor cells and endothelial cells (ECs) drives cancer-associated hypercoagulation remains unknown. Our previous *in vitro* studies demonstrate that human melanoma cells are able to activate ECs via different pathways. On the one hand, we identified an indirect mechanism mediated by tissue factor of tumor cells promoting thrombin generation. On the other hand, we recently could show that VEGF-A secreted by tumor cells induces a direct activation of ECs. The consequence of both pathways is the exocytosis of Weibel palade bodies (WPBs) followed by the luminal release of the procoagulatory von Willebrand factor (VWF). This glycoprotein forms ultralarge VWF (ULVWF) fibers highly adhesive for platelets. This effect may contribute to cancer-associated thrombosis and to the formation of metastatic lesions as platelets promote tumor cell extravasation. It is worthful to mention that under physiological conitions the enzyme a disintegrin-like and metalloproteinase with thrombospondin type I repeats 13 (ADAMTS13) cleaves ULVWF fibers into smaller fragments, thus down-regulating the hemostatic activity of VWF. Therefore, the objective of this study was to analyze the impact of EC activation on tumor progression and coagulation *in vivo* and to assess the effects of anticoagulants on this process.
Using two different animal models (ret transgenic mouse model of spontaneous melanoma and a xenograft mouse model) and tissue samples obtained from melanoma patients, we demonstrate EC activation reflected by intraluminal VWF fiber formation, platelet binding and thrombus formation in tumor microvessels. Evaluation of tumor tissue and blood samples of patients showed that a profound release of VWF and a local inhibition of ADAMTS13 is needed for the observed formation of ULVWF fibers within the tumor microvasculature. This effect was abolished by treatment with the low molecular weight heparin tinzaparin associated with a strong survival benefit and a reduced tumor weight in the ret transgenic mouse model. Finally, *in vitro* studies and ex vivo analysis of lymph nodes and tumor tissue implicated a mechanism wherein tinzaparin attenuates tumor-derived VEGF-A.
In conclusion, our data strongly indicate that melanoma microenvironment promotes EC activation, VWF secrection and attenuation of ADAMTS13. Therefore, our data do not only provide new aspects of VWF function and processing, but also envision a sound molecular explanation of tumor-triggered thrombosis in cancer patients. What is more, inhibition of EC activation or microthrombi formation may provide new therapeutical targets in cancer treatment using clinically approved heparins, such as tinzaparin, an anticoagulant recommended in cancer-associated thromboembolism.

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RanBP3 regulates melanoma cell proliferation via differential control of nucleocytoplasmic transport

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Deregulated protein sub-cellular localization, consequent to hyperactivity of key nuclear export proteins has frequently been described as a significant pathogenetic mechanism in cancer cells. Consistently, others and we have recently substantiated the candidacy of aberrant nucleocytoplasmic transport as a potential therapeutic target in human melanoma, with Chromosome Region Maintenance 1 (CRM1) being the forerunner. However, due to its central role in nuclear export, CRM1 targeting is invariably marred with extensive toxicity. In the current study, we investigated the suitability of Ran Binding Protein 3 (RanBP3), a CRM1 cofactor protein, as a target for potential therapeutic intervention in human melanoma. Utilizing a lossof- function experimental setting in genetically heterogeneous melanoma cellular systems, we witness the requirement of RanBP3 for melanoma cell proliferation and survival. Mechanistically, we demonstrate RanBP3-mediated negative regulation of TGF-β-Smad2/3-p21 tumor-suppressor axis through Smad2/3 nuclear export. Further employing extensive Nuclear Export Sequence (NES) alignment/analyses and immunofluorescence-based protein localization studies, we suggest a selective requirement of RanBP3 in the nuclear export of weak NES-harboring ERK1/2, while being fully dispensable for the nuclear exit of other CRM1 cargoes that harbor a strong NES. Our data predicts RanBP3 targeting as a viable strategy to selectively reset transformation-associated aberrant cellular protein localization and thus compromise melanoma cell proliferation.

P224

Efficient melanoma cell killing via shock-wave mediated cytolysis and secondary apoptosis induction

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Malignant melanoma is a highly metastatic cancer frequently resulting in multiple subcutaneous lesions and distant metastasis at later stages. Non-invasive and nonsystemic therapies could be of great value in order to remove local cancerous tissue without causing severe side effects in patients. Therefore, we evaluated whether the elastomechanical properties of melanoma cells can be exploited to kill them in a specific manner. In detail, we measured the elastic modulus of BLM melanoma cells in comparison with human fibroblasts using atomic force microscopy. Melanoma cells turned out to be three times less rigid than fibroblasts. Therefore, we used a shockwave system with different settings to evaluate primary and secondary effects in four different melanoma cell lines using fibroblasts as reference. We could identify an event dependent cytotoxicity using several instrument settings by measuring cellular viabilty directly after treating the cells in suspension. In order to screen for melanoma-specific settings we modulated the frequency and energy of the shockwaves. We identified settings that preferentially damaged the tumor cells and which were less harmful to fibroblasts. Secondary induction of cytotoxicity was analyzed 3 days after the treatment of the cell lines by cell cycle analysis, revealing apoptosis induction in the primary survivor melanoma cells but to a much lesser extent in fibroblasts. As proof of principle we initialized the treatment with shockwaves of excised tumor tissue from metastases and stained for primary cell death. Our results propose a putative Achilles heel of melanoma cells, caused by their altered cytoskeleton and mechano-structural properties, which could be used for local tumor cell destruction.

P225

Two distinct functions of Heparanse-1 during melanoma progression – shedding of the extracellular matrix and suppression of gene transcription through DNA binding

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Heparanase-1 (HPSE) is able to degrade heparan sulfate, thus playing a pivotal role in structural remodeling of the ECM and glycocalyx. The protumorigenic, proangiogenic and prometastatic properties of HPSE have been identified in many human cancer types. However, in addition to its extracellular function, recent studies suggest an intracellular activity of HPSE with a largely unknown significance during tumor progression. Therefore, we investigated the relevance of HPSE duality in malignant melanoma *in vitro* as well as in mouse melanoma models basing on the intra-dermal injection of B16F10 or ret transgenic melanoma cells. In line with its extracellular action, HPSE-deficiency led to a reduced shedding of the glycocalyx accompanied with retainment of VEGF at the cellular surface and an impaired tumor cell invasion. However, we also measured an increased nuclear translocalization of NF-κB followed by a strongly elevated expression of the protumorigenic factors pentraxin-3, tissue factor, TNF-α and most prominently MMP-9 upon HPSE knockdown. *In vivo*, HPSE-deficiency was related to increased lymph node metastasis as well as a reduced survival. While inhibition of its extracellular function heparin was unable to block the gene regulatory impact of HPSE we proposed an intracellular mechanism. Immunofluorescence stainings revealed a counterlocalization of HPSE and NF-κB in the nucleus. Accordingly, activation of NF-κB with recombinant TNF-α reduces not only the expression of HPSE but also its nuclear localization suggesting a competitive relationship between both proteins. This finding was further supported by the discovery of a direct charge-driven molecular interaction between HPSE and DNA using atomic force microscopy and a coprecipitation approach. Our findings are novel and point towards a dual function of HPSE in malignant melanoma with a protumorigenic extracellular activity and a tumor suppressive nuclear action. Identification of molecular strategies to shuttle extracellular HPSE into the nuclei of cancer cells may envisions new therapeutic options.

P226

The role of beta-catenin in the therapy resistance of malignant melanoma to BRAF inhibitors

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Many mechanisms are known for the development of resistance towards vemurafenib, the approved standard therapy for stage IV BRAFV600E melanomas. The majority of vemurafenib-treated melanoma patients rapidly develop therapy resistance within 7 months. A deep and accurate understanding of the underlying mechanisms is of great demand in order to overcome this fatal development. The importance of beta-catenin in these resistance mechanisms is unknown so far.

Here, we examined the beta-catenin expression levels of resistant melanoma cells *in vitro* and *in vivo* and found increased beta-catenin protein levels in some vemurafenib resistant cell lines and in tumor biopsies of vemurafenib resistant patients. Additionally, we found signs of nuclear translocation, a strong indicator for transcriptional activity of beta-catenin. Therefore, we investigated the activity of the canonical Wnt signalling pathway via a luciferase reporter which surprisingly showed no increased luciferase signal in resistant cell lines compared to the sensitive parental cells. Hence, we screened for novel protein interactants of betacatenin in resistant cells in order to elucidate the non-canonical, Wnt indepenent signalling cascades of beta-catenin in the resistant melanoma cells. We detected Stat3 as a novel candidate, critical for the beta-catenin signaling in the course of resistance to BRAF inhibitors. Further investigations based on inhibitor treatment and overexpression of Stat3 in combination with beta-catenin knockdown and vemurafenib treatment suppose an important role of the beta-catenin interactants for resistance formation. Functionally, knockdown of beta-catenin increased the effects of vemurafenib in two BRAFV600E melanoma cell lines via increased growth inhibition and apoptosis induction. Moreover, knockdown of beta-catenin significantly retarded tumor growth of the vemurafenib resistant tumors in a xenograft mouse model and re-sensitized the resistant melanoma cell lines to BRAF inhibition *in vitro*. Therefore, we propose a novel model for resistance of melanoma cells against BRAF inhibitors in which beta-catenin plays a central role as a regulator of Wnt independent, non-canonical signaling pathways.

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P53 mediated effects of BRAF inhibitors are essential for effective cytotoxicity and apoptosis induction in malignant melanoma

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BRAF inhibitors (BRAFi) specific for V600-mutated BRAF were successfully introduced in the standard regimens for patients suffering from advanced metastatic melanoma. Unfortunately, long-term efficacy and survival benefit for the patients are limited due to the evolution of different resistance mechanisms.

In this study we evaluated the effects of the inhibitor of the mutated BRAF vemurafenib on the activation of p53 and its relevance on cell cycle arrest and apoptosis induction in melanoma cells.

We could show that cells with wildtype p53 and BRAFV600E activate p53 upon BRAFi treatment. Additional p53 activation by co-treatment with nutlin-3 strongly enhanced the cytotoxic effects of vemurafenib in these cells. However, nutlin-3 did not show these effects in cells with mutated forms of p53 which confirms the p53 mediated effects of vemurafenib. Interestingly, co-treatment of BRAFi-resistant cell lines with nutlin-3 re-sensitized these cells to vemurafenib therapy to a remarkable extent. We further evaluated the expression of Hdm2 and Hdmx in the drug-naïve melanoma cell lines and the corresponding cell lines with resistance to BRAFi.

Our results propose a p53 dependent effect of BRAFi like vemurafenib or dabrafenib and postulate a putative mediation of resistance to BRAFi in chronically treated melanoma cells. Further experiments are needed in order to evaluate if p53 activity can be restored in melanoma cells with p53 mutations as well and whether these would enhance the effects of BRAFi in terms of efficacy and prevention of resistance development.

P228

Senescence induction in metastatic melanoma during interferonimmunotherapy

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Melanoma immunotherapy was shown to be therapeutically efficient, but it remains enigmatic whether the therapeutic success depends only on cytolysis or apoptosis, or whether it also involves growth inhibitory processes, e.g. senescence induction in the tumor cells. We have recently shown that proinflammatory cytokines are capable of stopping cancer growth through induction of senescence in a variety of malignant cells, including primary melanoma cells. To further analyze the effects of interferon (IFN) and tumor necrosis factor (TNF) on melanomas, we treated a panel of cell lines with these two cytokines. FACS analysis showed that the combined action of IFN and TNF can cause apoptosis and a senescence characterizing G0/G1 arrest. Furthermore, we could detect an upregulation of senescence-associated-β- galactosidase and a stable cell cycle arrest in the cytokine-treated melanoma cells that remained stable after withdrawal of IFN and TNF. Moreover, the cytokine-treated melanoma cells showed a senescence-associated secretory phenotype with the production of IL-6, IL-8, IP-10 and CCL-2. In line with this, we could also detect IFNalpha- induced senescence in primary cells from a patient with stage IV malignant melanoma *in vitro*, and, more importantly, during IFN-alpha immunotherapy of the same patient *in vivo*. The patient had an ECOG performance status of 4 due to a malignant ascites. The tumor board suggested a compassionate use treatment with IFN-alpha administered intraperitoneally. This treatment cleared the ascites completely. Ex vivo analyses with staining for Ki67, p16INK4a, senescenceassociated- β-galactosidase and growth-arrest-assays of the ascites-derived melanoma cells confirmed the induction of senescence during the treatment. Thus, interferon can drive human melanomas into senescence *in vitro* and *in vivo*, a discovery that is of great therapeutic relevance.

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Melanoma cells adapt flexibly to variation of N- and E-cadherin-density on the nanoscale

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Aim: The 'cadherin switch' characterized by loss of membranous E-cadherinexpression and parallel upregulation of N-cadherin has been associated with melanoma progression. However, analysis of *in-vivo* specimens show a very heterogenous expression of those cadherins in melanoma cells underlining the fact that the true function of N- and E-cadherin, respectively, is still unclear. To better understand N- and E-cadherin interactions we developed innovative matrices presenting tunable N-and E-cadherin on the nanoscale.

Method: Surfaces with precisely tunable densities of the N- and E-cadherinbiomolecule, respectively, were created using block-copolymer-nanolithography: Glass substrates were covered with nanopatterns of 6 nm gold nanoparticles by self-assembly of diblock copolymer micelles. With this method, the distance between gold nanoparticles could be precisely adjusted between 30 and 180 nm. The respective cadherin-dimer was then covalently bound in an ortho-directional orientation to the gold nanoparticles resulting in defined cadherin-site-densities in a physiologically relevant range (35/μm^2 to 1128/μm^2).

Results: Six different melanoma cells showed differential N- and E-cadherin expression with four of them expressing N-cadherin (A375, MeWo, LOX, Mel-2a), one of them expressing E-cadherin (Sk-mel 23) and one of them neither N- nor Ecadherin (MV-3).

A375-melanoma cells (N-cadherin positive) showed longitudinal cell spreading on nanoscopic N-cadherin. On four differential site densities of N-cadherin (30, 60, 90 and 180 nm) A375 melanoma cells showed linear attachment in a density dependent fashion in the range of 30–90 nm site densities (corresponding to a ligand density of 1128/μm^2–91/μm^2) whereas the 180 nm surface (35/μm^2) was not able to induce attachment. In contrast to that cell spreading was independent of variation of ligand density in a range of 30–100 nm (1128/μm^2–91/μm^2). In comparison, the melanoma cell line Sk-mel 23 (E-cadherin positive) showed similar behavior with regard on density-dependent attachment but density-independent cell spreading in a range of 30–100 nm (1128/μm^2–91/μm^2).

Specificity of our model system was confirmed by specific knock-down of N-cadherin in A375-melanoma which inhibited attachment to nanoscopic N-cadherin. A375 melanoma cells (E-cadherin negative) were not able to attach to or spread on nanoscopic E-cadherin. The melanoma cell line MV3 (N- and E-cadherin negative) was neither able to interact with nanoscopic E- nor with N-cadherin, respectively.

Conclusions: Our results show that E-and N-cadherin both specifically regulate attachment in a density-dependent fashion. In contrast to that a characteristic longitudinal cell spreading is completely induced regardless of N- or E-cadherindensity variation in a range of 91/μm^2–1128/μm^2 according to an 'all-or-nothing'- principle in a certain range of ligand-density. This feature clearly distinguishes the N/ E-cadherin ligand from previously analyzed ligands (RGD, VCAM-1) and therefore underlines a unique function and flexibility in the interaction of melanoma cells with the tumoral environment using N- or E-cadherin.

P230

The antihistamines Clemastine and Desloratidine cause cell death of ALCL and other lymphoma cell lines

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Introduction: We found recently in cutaneous T cell lymphoma cell lines that the antihistamines clemastine and desloratidine caused cell death by down regulation of constitutive DNA- binding activities of the transcription factors STAT3 and STAT5 and the c-Myc protein. To determine, whether also other skin and non-skin lymphoma and leukemia cell lines can be killed by these two antihistamines by the same mechanism, we treated anaplastic large cell lymphoma (ALCL), Burkitt Lymphoma, and CML cell lines with both anti-histamines and investigated their effects on STAT3, STAT5, and c-Myc DNA binding activities.

Methods: Cell culture, antihistamine treatment, trypan exclusion test, transcription factor DNA-binding ELISA.

Results: Cell death occurred at the same concentrations of clemastine (4 ug/ml) and desloratidiene (10 ug/ml) as for the CTCL cell lines HUT78 and MyLa 2059. The Mac2A cell line was more sensitive (2 ug/ml clemastine, 2.5 ug/ml desloratidine). Constitutive activities of the transcription factors STAT5a and STAT5b were detected in Mac2A and K562 cells, for STAT3 in Mac2A, K562 and Karpas 299 cells and for c- Myc in all 5 cell lines. All these constitutive DNA-binding activities were inhibited by clemastine and desloratidine. c-Myc was the only common factor that was detected in all 5 cell lines. To prove that the inhibition of c-Myc is indeed responsible for the cell death of the tested cell lines we treated them with the specific c-Myc inhibitor 10058F4. The addition of 100058F4 to the media killed the cells, indicating that inhibition of c-Myc by clemastine and desloratidine may be the way how these two antihistamines induce cell death.

Discussion: The results suggest that inhibition of c-Myc may be the way how clemastine and desloratidine kill lymphoma and leukemia cells. The c-Myc protein plays an important role in providing fast growing cells as cancer cells with building blocks for nucleotides and proteins. Thus, inhibiting c-Myc may be crucial to starve cancer cells selectively to death. The doses of clemastine and desloratidine used to treat allergic reactions are quite lower than those that are needed to kill cancer cells. However, they are by far lower than the corresponding LD50s, which are 35 times higher for desloratidine and 182 times for clemastine.

P231 (O06/01)

Melanoma-derived ADAM-9 modulates melanoma development and metastasis *in vivo*

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ADAM-9 is a proteolytic and adhesive protein belonging to the adamalysins family of metalloproteinases. Increased expression of ADAM-9 has been shown in several cancers including melanoma. In human melanoma ADAM-9 expression is localized at the areas of the tumor invading the dermis, particularly in tumor and adjacent fibroblastic cells. We have previously shown that deletion of ADAM-9 in stromal cells increases melanoma growth. However, the functional role of ADAM-9 expression in melanoma during tumor progression is unknown. To address the role of ADAM-9 in melanoma *in vivo*, we have crossed Adam-9−/− mice with Hgf/Cdk4 mutant mice known to spontaneously develop melanoma which closely resemble human tumors. The generated animals, deficient for ADAM-9 in melanocytes as well as in stromal cells, were either followed over time for formation of spontaneous melanomas or treated with DMBA to follow tumor formation with faster kinetic. Mice lacking ADAM-9, upon DMBA treatment, initially developed a higher number of tumors, which was significantly reduced at later time points when compared to controls. Analysis of proliferation, apoptosis and inflammation indicated that an altered proliferation of the tumor cells might be responsible for the observed differences in tumor development. At the age of ca. 1 year the number of spontaneous tumor developed in Adam-9−/−/ Hgf/Cdk4 mice were also significantly reduced as compared to controls, thus indicating that this effect was not solely dependent on the DMBA induction. Strikingly deletion of ADAM-9 resulted in a significantly reduced lung metastases formation post DMBA treatment. This effect, even though less prominent, was also detected in untreated Adam-9−/−/Hgf/Cdk4 mice as compared to controls at ca. 1 year of age.

Reduced lung metastatization may result from decreased extravasation of ADAM-9 deficient melanoma cells to the endothelium. In support of this, in *in vitro* studies we could observe that ADAM-9 deficient melanoma cells displayed reduced adhesion to and transmigration through an activated endothelial cell layer. Taken together, these data show that ADAM-9 *in vivo* modulates melanoma development and metastatization in an induced and spontaneous model of melanoma *in vivo*.

P232

Sensitization of melanoma cells for TRAIL-induced apoptosis by cellular stress conditions – identification of common principles based on proapoptotic Bcl-2 proteins

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Background: Melanoma only poorly responds to chemotherapy, and besides new therapeutic developments as BRAF inhibitors and immune modulators, the death ligand TRAIL (TNF-related apoptosis-inducing ligand) appears as a promising therapeutic strategy. This ligand triggers apoptosis via the two death receptors TRAIL-R1/R2 (DR4/DR5). However, prevalent and inducible resistance

may limit its clinical use. Thus in previous studies, we and others have demonstrated a number of therapeutic strategies to sensitize melanoma cells for TRAIL-induced apoptosis however, the common principles remained elusive. A variety of stress factors have been published to induce tumor cell apoptosis. Here, stress conditions as high cell density and serum starvation are shown to influence melanoma cell sensitivity to TRAIL, thus suggesting a common explanation for enhancement of TRAIL-induced apoptosis.
Methods: TRAIL-sensitive melanoma cell lines (A-375, SK-Mel-13, Mel-HO) were compared to permanently resistant cell lines (MeWo, Mel-2a) as well as to cell lines selected for death ligand resistance (A-375-TS, SK-Mel-13-TS; TS = TRAILselected).
Results: In TRAIL-sensitive cells and in selected cell lines with induced resistance, high cell density (confluence) resulted in a G1 cell cycle arrest, which was correlated to increased apoptosis sensitivity to TRAIL. In contrast, two permanently resistant melanoma cell lines were not sensitized by high cell density alone and also showed no G1 arrest. In these cells, additional stress conditions, namely high cell density and serum starvation, could induce both G1 arrest and TRAIL sensitivity.
Addressing the signaling pathways revealed an activation of proapoptotic caspases as well as an early disruption of the mitochondrial membrane potential and activation of the proapoptotic Bcl-2 protein Bax, thus clearly underlining the involvement of mitochondrial apoptosis pathways. Most pronounced was the upregulation of small proapoptotic Bcl-2 proteins as the BH3-only protein Puma and of Bcl-x (S), the proapoptotic splice variant of Bcl-x(L). The critical role of Puma was proven by its siRNA-mediated downregulation, and also constitutive Bcl-2 overexpression blocked stress and TRAIL-induced apoptosis.
Conclusions: Taking into account the physiological role of death ligands in immune surveillance, sensitization of melanoma cells for death ligands is a prerequisite for an anti-tumor immune response. The present data allow a more general understanding on the strategies of TRAIL sensitization, and strategies based on proapoptotic Bcl-2 proteins may indeed be translated into clinical approaches, as recently has been demonstrated by the use of BH3 mimetics.

P233

Malignant T cells inhibit anti-cancer and anti-infectious immunity in cutaneous T cell lymphoma

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In cutaneous T cell lymphoma (CTCL), the malignant T cells are a source of suppressive Th2 cytokines, such as IL-4, and progressive impairment of cellular immunity is a hallmark of the disease. IL-4 is known for its capacity to sustain Th2 cell differentiation, when acting directly on T cells, but can also initiate an IL-12 dependent negative regulatory feedback loop and initiate protective Th1 immune response when present during the initial activation of dendritic cells (DC). Interestingly, we found an association of increased IL-4 production and, at the same time decreased IL-12 levels, with advanced stage CTCL. Neutralization of IL-4 restored Th1 but not Th17 immune responses in CTCL, and DC activation was directly suppressed through co-inhibitory T cell surface molecules. This points out towards an abrogated DC-T cell regulatory loop in patients with CTCL and suggests an immune escape mechanism that allows cancer cells to evade recognition from the innate immune system, and subsequently abrogate the differentiation of a protective non-malignant effector CD4+ T cell population.

P234

Cell cycle arrest, induction of apoptosis and sensitization of melanoma cells for TRAIL-induced apoptosis by a selective aurora kinase-A inhibitor

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Background: Therapeutic strategies in metastatic melanoma are still a challenge because of its high resistance to traditional chemotherapeutics. New promising therapeutic approaches, as BRAF inhibitors and immune modulators, are in clinical trials. Besides these, strategies based on the death ligand TRAIL (TNF-related apoptosis-inducing ligand) or on inhibitors for other tumor-related kinases, as aurora kinase A (AURKA), appear of additional value. TRAIL was shown to trigger apoptosis in cancer cells by two death receptors TRAIL-R1/R2 (DR4/DR5), while normal cells were largely spared. However, prevalent and inducible TRAIL resistance is still limiting its clinical use. Several kinase inhibitors have recently been shown by our group to overcome TRAIL resistance in melanoma cells. The serine/threonine kinase AURKA is essential for cell division, mitotic entry and cytokinesis, and this kinase was shown to be overexpressed in a variety of tumor entities. Thus, its inhibition results in polyploidy, G2 arrest, inhibition of cell proliferation and induction of apoptosis in different tumor cells.
Methods: A selective inhibitor for aurora kinase A (Alisertib, MLN 8237) was applied in TRAIL-sensitive melanoma cell lines (A-375, Mel-HO), permanently TRAILresistant cell lines (MeWo, Mel-2a) as well as in cells selected for death ligand resistance (A375-TS; TS = TRAIL-selected).
Results: Alisertib treatment alone resulted in melanoma cells in polyploidy, G2 cell cycle arrest and inhibition of cell proliferation. Furthermore, it directly induced apoptosis in the TRAIL-sensitive cell lines A-375 and Mel-HO as well as enhanced the proapoptotic effects of TRAIL in these cells. In A375-TS, Alisertib was able to overcome selected TRAIL resistance. Addressing the pathways, activation of the extrinsic caspase cascade via caspase-8 and caspase-3 was shown. In contrast to the response of melanoma cells to inhibitors of other kinases, no loss of the mitochondrial membrane potential and no production of reactive oxygen species (ROS) were seen as direct effects of AURKA inhibitor, thus no indication of an activation of mitochondrial apoptosis pathways. Further investigation of the pathways is in progress.
Conclusions: Aurora kinase A inhibitors appear as alternative effective strategies for targeting melanoma cells. In particular, the strongest effects were seen in combination with TRAIL, thus suggesting the consideration of AURKA and TRAIL pathways as additional targets in melanoma therapy.

P235 (O05/01)

Resistance to T cell therapy through reciprocal interactions between melanoma, endothelial and immune cells in the perivascular niche

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Tumor regression, remission and relapse after successful immunotherapy with adoptively transferred T-cells (ACT) targeting melanocytic antigens can be recapitulated in the Hgf-Cdk4 mouse melanoma model. Previously, we could show that primary Hgf-Cdk4 and transplantable HCmel3 melanomas can resist T-cell therapy (ACT) through inflammation-induced reversible dedifferentiation. Surprisingly, histopathological analyses of ACT resistant melanomas frequently revealed melanoma cells clearly cuffing vessels at the invasion front or at least 1 mm away from the main tumor. This phenomenon was originally described as angiotropism in human melanomas by histopathologist and is associated with poor prognosis. We hypothesize that angiotropic growth of melanoma cells contribute to inflammation-induced ACT resistance. Therefore, we analyzed in detail ACT resistant and untreated melanomas histopathologically for signs of angiotropic growth. ACT resistant primary melanomas revealed in 8/15 melanoma cells clearly cuff blood vessels at some distance (>1 mm) away from the main tumour mass. In contrast, only 2/15 of untreated primary melanomas showed this phenomenon. Similar results were obtained with the transplantable HCmel3 melanoma cell line. In 7/15 ACT resistant, but only in 1/15 untreated HCmel3 melanomas we could detect angiotropism. Whole genome and RT-PCR mRNA expression analyses of control and ACT resistant HCmel3 melanomas identified a set of cell adhesion, migration and angiogenesis genes that are up-regulated in relapsed HCmel3 melanomas *in vivo*. These gene expression changes can be largely recapitulated *in vitro* in HCmel3 cells upon treatment with the proinflammatory mediator TNF. In transwell assays, we observed that preincubation of HCmel3 cells with neutrophil-conditioned medium enhanced their migration towards endothelial cells. Time-lapse video microscopy revealed that HCmel3 selectively migrated with increased velocities and for longer distances on endothelial cell surfaces when compared to surfaces of keratinocytes or purified extracellular matrix components alone. This effect was enhanced by TNF treatment.
Taken together, these experimental results indicate that reciprocal interactions between melanoma, endothelial and immune cells contribute to melanoma progression and therapy resistance in the perivascular niche.

P236

HMGB1 release from UVB-irradiated epidermal keratinocytes drives spontaneous melanoma lung metastasis in a TLR4-dependent manner

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Background: We found that repetitive UVB-irradiation induces a Tlr4-dependent skin inflammatory response which drives the development of spontaneous lung metastasis in mice bearing serial HGF-CDK4(R24C) melanoma skin transplants. We hypothesized that UV damage of epidermal keratinocytes leads to cytosolic translocation of the nuclear protein high-mobility group box 1 (HMGB1) which acts as an endogenous TLR4 ligand capable of triggering inflammation following its release into the extracellular space.
Methods: We investigated how genetic or pharmacologic blockade of the HMGB1/ TLR4 signalling axis impacts skin inflammatory responses and melanoma metastasis induced by two consecutive sunburning doses of 4.5 kJ/m2 UVB on the back skin of mice.
Results: Immunofluorescence and immunoblot analyses indeed provided evidence for HMGB1 cytosolic translocation and release in epidermal keratinocytes in mice upon UVB irradiation. HMGB1 cytosolic translocation and release in response to UVB irradiation was confirmed in primary keratinocyte cultures *in vitro*. Furthermore, treatment of mice with recombinant BoxA or glycyrrhizin, two functional inhibitors of extracellular HMGB1, or with CLI-095, a specific inhibitor of TLR4 signalling, largely abrogated UVB-dependent inflammation and reactive epidermal keratinocyte hyperproliferation. Importantly, pharmacologic blockade of the HMGB1/TLR4 signalling axis also largely abrogated the metastasis-promoting effects of UVB irradiation in mice bearing serial HGF-CDK4(R24C) melanoma skin transplants.
Conclusions: Taken together, our results demonstrate HMGB1-dependent mechanistic link between UVB-induced DNA damage of epidermal keratinocytes and TLR4-dependent neutrophilic skin inflammation which drives metastatic progression of melanoma.

P237

Immune cell subsets as markers of response to ipilimumab in metastatic melanoma

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Intravenous anti-CTLA-4 antibody (ipilimumab) is a recently approved immunotherapy that is implicated with improved overall survival (OS) of patients with metastatic melanoma. An establishment of markers of early response to ipilimumab would improve the clinical management of patients. However, these markers are still elusive.
In this prospective study, 41 patients were included that were diagnosed with unresectable stage III or IV melanoma. Four courses of ipilimumab were administered at a dose of 3 mg/kg every 3 weeks. Response was measured following RECIST 1.1 and immune-related response criteria. Median OS was 8.9 months after first infusion of ipilimumab with an overall response rate of 20.4%.
An increased eosinophil count after the first and before the second infusion was associated with a favorable response. Moreover, an increased neutrophil count after the first and before the second infusion was associated with an unfavorable response and worsened OS as well as worsened progression-free survival (PFS).
We describe immune cell subsets and relevant soluble inflammatory factors at the time after the first and before the second ipilimumab infusion as early response markers, thereby shedding light on the mechanism of action of ipilimumab therapy.

P238

CD73 correlates with an inflammatory mesenchymal cell state in melanoma and is regulated via MAPK signaling

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Background: CD73 is a cell surface 5′ ectonucleotidase expressed by melanoma and immune cells that converts extracellular AMP to immunosuppressive adenosine and hence represents a promising new immunotherapeutic target. However, its regulation in melanoma is unknown and we reasoned that it is a critical determinant for clinical strategies.
Methods: We used an integrative approach of global gene expression analysis, pharmacological and genetic perturbations as well as FACS-based cell state characterization.
Results: Gene set enrichment analysis of melanoma cell line panels revealed that CD73 levels correlate with a dedifferentiated mesenchymal phenotype driven by inflammatory and mitogenic signaling. We found that the melanocytic growth factor HGF and the proinflammatory cytokine TNF-alpha synergistically induced CD73 in a MEKERK signaling dependent manner. Consistently, many melanoma cell lines with activating mutations in BRAF or NRAS exhibited high basal CD73 expression that was robustly suppressed by the treatment with BRAF or MEK inhibitors. In line, CD73 levels were restored in BRAF inhibitor resistant cells generated by CRISPR/ Cas9-mediated deletion of the negative RAS regulator and tumor suppresser NF1 (neurofibromatosis 1). Using a genetically engineered mouse model, we previously showed that murine melanomas resist T-cell based immunotherapy by inflammationinduced dedifferentiation. Now we demonstrate that these relapse tumors express high levels of CD73 in contrast to untreated controls and cell cultures established thereof had high inflammatory and mitogenic signaling activity.
Conclusions: Our findings link immunosuppressive CD73 expression by melanoma cells to oncogenic MEK-ERK signaling and further support the rationale to combine BRAF inhibitors with immune checkpoint blockade.

P239

Melanoma-macrophage fusion can result in stealth tumor cells

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Local recurrence can develop following the surgical removal of a primary melanoma with histologically tumor-free resection margins. A potential explanation is that melanoma cells fuse with macrophages and adopt their phenotype but still have tumorous features. We aimed to investigate whether such cells are present in human melanoma tissue samples and if they can result from cell fusion.

To study spontaneous cell fusion *in vitro*, human melanoma cell lines stained with the fluorescent vital dye CellTracker Orange were co-cultured for 24 h with freshly isolated human monocytes stained with CellTracker Green. Hybrid cells were detected in all melanoma cell lines based on double positivity by fluorescent microscopy. Not only the morphology but also the phenotype of hybrid cells could resemble macrophages: certain hybrids were negative for MelanA, and they could be positive for CD68. Such melanoma-macrophage hybrids could also be identified in ex vivo BRAFV600E mutant human melanoma tissue samples, as certain peritumoral MelanA-CD68+ macrophages, isolated with laser capture microdissection, contained BRAFV600E mutation on a genetic level. Besides this, we detected MelanA+ circulating tumor cells among circulating CD14+ monocytes in a stage IV melanoma patient.

In conclusion, we showed that melanoma cells can fuse spontaneously with macrophages, and thereby tumor cells can acquire macrophage morphology and lose the melanoma-specific marker MelanA, while still carrying the oncogenic BRAFV600E mutation. Therefore, melanoma-macrophage fusion might play a role in missing tumor cells by routine histological assessment and might explain an alternative mechanism of tumor dormancy and spreading.

Miscellaneous

P240

StarPEG-heparin-based hydrogels as modulator of human inflammatory M1 macrophage responses

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Impaired wound healing is a problem of immense clinical and economic relevance. Persistent inflammation in impaired wound healing is primarily driven by unresisted inflammatory M1 macrophage (infM1) activation. Therefore, the principal objective of this project is the development of immunomodulating wound dressings capable to bring unopposed inflammation under control by repressing infM1 activity and thus enabling inflammatory resolution. Here, we use biohybrid hydrogels formed by crosslinking of amine functionalized star-shaped poly(ethylene-glycol) (starPEG) and carbodiimide/N-hydroxysulfosuccinimide (EDC/s-NHS)-actived heparin which have been shown to encourage angiogenesis. Since both reduction of uncontrolled inflammation and induction of vascularization are suggested to improve an impaired healing response, we tested different 3D starPEG-heparin-based hydrogels with respect to their immunomodulating capacity on infM1.

In the present study infM1 were derived from human CD14+ monocytes by differentiation with GM-CSF for 6 days and subsequently seeded on different starPEG-heparin-based hydrogels. The hydrogels were composed of either standard heparin (SH) or desulfated derivatives of heparin (DSH) and functionalized with RGD adhesion sites. After 3 days culture on the hydrogels survival (XTT cell viability assay) and adhesion of infM1 were assessed and inflammatory functions (cytokine response) upon stimulation with LPS were determined (ELISA, qPCR). Additionally, interaction of the hydrogels with infM1-derived mediators was analyzed in a cytokine adsorption assay.

Our results show that starPEG-heparin-based hydrogels functionalized with RGD adhesion sites promote survival and adhesion of infM1. StarPEG-heparinbased hydrogels modulate the cytokine response of infM1 via two manners: 1) downregulation of cytokine expression and release in infM1 and 2) binding of cytokines released from infM1. Interestingly, modulation of the cytokine response is determined by the sulfation level of heparin. Hydrogels based on desulfated heparin predominantly reduce production of pro-inflammatory cytokines in infM1 as seen by reduced expression and release of TNF, IL-12p40, IL-6 and MCP-1, one of the key chemokines regulating migration of monocytes/macrophages. In contrast hydrogels based on standard heparin predominantly bind and sequester inflammatory mediators including IL-12p40, IL-8 and MCP-1 released from infM1.

From these results we conclude that starPEG-heparin-based hydrogels provide immunomodulating capacities on infM1 which may be fine-tuned by usage of heparin derivatives with adjusted levels of sulfation. We suggest that the hydrogels are capable to modulate unopposed infM1 activity. We are therefore currently investigating immunomodulating effects of the different starPEG-heparin-based hydrogels in *in vivo* mice models of normal and impaired wound healing.

P241

In vitro evaluation of the capacity of a monofilament debrider* to remove biofilm and the efficacy of different wound dressings to prevent biofilm regrowth

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Introduction: Development of biofilms on wounds is a major impediment to wound healing. Therefore, current research targets antibiofilm strategies to restore an optimal wound-healing environment. Combined treatment involving debridement and addition of antibacterial agents may provide the highest success rates. A monofilament debrider* consisting of polyester fibers presents a fast and almost painless option for debridement and removal of biofilm. We have then analyzed the re-growth properties of biofilm underneath different wound dressings.

Methods: A *S. aureus* biofilm was cultivated on glass plates. The monofilament debrider* was used to wipe the glass plates under standardized conditions (P = 0.067N/cm2, v = 1.6 cm/s). Afterwards, glass plates were covered with various antimicrobially active wound dressings† and incubated for 24 h at 37°C. Then, dressings were removed and glass plates further incubated for 48 h. Biofilm on the glass plates was evaluated directly after dressing removal and following 48 h regrowth period using the fluorescent alamar blue assay.

Results: It was shown that the monofilament debrider* effectively removed biofilm *in vitro*. Furthermore, it was observed that subsequent treatment with wound dressings reduced formation of new biomass. Significantly fewer bacteria were found after incubation with dressings containing antimicrobials like silver or polihexanide. Polihexanide-containing dressings further exhibited a persistent decrease of biofilm re-growth, while biofilm quickly reformed in untreated controls and after removal of antimicrobial-free and silver-containing dressings.

Conclusions: It can be concluded that the combination of biofilm removal on the infected or critically colonized wound using a monofilament debrider* and subsequent treatment with antimicrobial dressings presents a successful antibiofilm strategy.

*Debrisoft® (Lohmann & Rauscher).

†Vliwasorb® (Lohmann & Rauscher), Vliwaktiv® (Lohmann & Rauscher), Vliwaktiv® Ag (Lohmann & Rauscher), Suprasorb® A (Lohmann & Rauscher), Suprasorb® A+Ag (Lohmann & Rauscher), Suprasorb® X (Lohmann & Rauscher), Suprasorb® X+PHMB (Lohmann & Rauscher), Suprasorb® P (Lohmann & Rauscher).

P242

Efficacy of antimicrobial wound dressings against *S. aureus*, *E. coli* and *K. pneumoniae* as well as their resistant kinsmen MRSA and NMD-1 strains *in vitro*

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Introduction: Bacteria that are resistant to common antibiotics such as the methicillinresistant *Staphylococcus aureus* (MRSA) or *E. coli* and *K. pneumonia* strains that carry the metallo-beta-lactamase-1 gene (NMD-1) are increasingly isolated from chronic wounds. Therefore, special care has to be taken to accomplish both, treatment of the wound infection at hand but also to prevent the spread of this pathogen. Hence, dressings containing antimicrobial substances or with inherent antimicrobial properties are increasingly utilized. Also these dressings should provide a general broad antimicrobial activity against both, sensitive and resistant bacteria species, concerns have been raised whether they are indeed effective against MRSA and NMD-1-carrying strains. Here, we have rated the antibacterial activity of various dressings* against *S. aureus*, MRSA, *E. coli*, NMD-1-carrying *E. coli*, *K. pneumoniae*, and NMD-1 carrying *K. pneumoniae* using the JIS L 1902 standard test which allows a direct evaluation of the dressing's effects on the micro-organisms.

Methods: The determination of antimicrobial activity was performed according to the Japanese Industrial Standard (JIS L 1902: 2002, 'Testing method for antibacterial activity of textiles'). Culture medium was inoculated with the test microbes and cultivated for 24 h at 37°C under aerobic conditions. For experiments, 400 mg samples of the wound dressings* were incubated with each test microbe (200 μL) for 24 h at 37°C under aerobic conditions. Polyester was used as reference material.

Results: All dressings containing an antimicrobial substance such as PHMB (SXP) or silver (SAA and VWA) exerted a distinct antimicrobial effect against all test strains used that could be rated a strong antimicrobial activity according to JIS L 1902 (log reduction >3). Furthermore, the alginate dressing (SA) was able to efficiently bind and inhibit bacteria progeny. Similarly, strong antibacterial activity was observed for the SAP-containing dressing (FV) against the gram-negative bacteria (log reduction >3) while it demonstrated a significant activity against *S. aureus* and MRSA (log reduction = 1.4 and 2.0, respectively). However, no effect on bacterial growth was found for the dressing containing just activated carbon in a viscose matrix w/o silver (VW).

Conclusions: It could be shown that dressings with an inherent antibacterial activity such as alginate or SAP-containing dressings or dressings with an antimicrobial substance such as PHMB or silver are equally effective against sensitive strains of *S. aureus*, *E. coli* and *K. pneumonise* as well as their resistant kinsmen MRSA and NMD-1 strains. Hence, it seems to be safe to use these dressings in treatment of infected chronic wounds.

*(FV) Flivasorb® (Lohmann & Rauscher), (VW) Vliwaktiv® (Lohmann & Rauscher), (VWA) Vliwaktiv® Ag (Lohmann & Rauscher), (SA) Suprasorb® A (Lohmann & Rauscher), (SAA) Suprasorb® A+Ag (Lohmann & Rauscher), (SXP) Suprasorb® X+PHMB (Lohmann & Rauscher).

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Sap from leaves of *Isatis tinctoria* and Tryptanthrin exhibit strong antimycotic properties against yeast and dermatophytes

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Introduction: Infectious diseases, especially of the skin, have been treated with phytomedicine throughout human history and long before anti-infectious agents had been developed. One example of such plants is woad, *Isatis tinctoria* L. (Brassicaceae) which is known for its blue indigo dye and for its medical properties. The application of woad for treatment of wounds and skin rashes as well as ulcers and haemorrhoids was described. In addition, a strong preservative effect against fungal decay of wood has been observed. Active compounds, such as tryptanthrin, indole-3-acetonitrile and p-coumaric acid ester, have been shown to exhibit antimicrobial activity against bacteria, yeast and dermatophytes. Hence, it is of great interest to investigate the antimycotic effect of woad especially for its usage in formulations for adjuvant treatment of wounds and skin diseases. In the present study, we determined the antifungal activity of two saps of woad leaves and tryptanthrin, against the yeasts *Candida albicans* and *Malassezia pachydermatis* as well as the dermatophyte *Trichophyton rubrum*.

Method: The antimycotic activity of sap of fresh woad leaves (FW) and of dried fermented woad leaves (DFW) as well as a DMSO extract of tryptanthrin against *C. albicans* DSM 1386, *M. pachydermatis* DSM 6172 and *T. rubrum* DSM 16111 was analysed *in vitro* by microplate laser nephelometry (NEPHELOstar Galaxy; BMG LABTECH, Germany). Moreover, the antifungal capacity was determined by chemiluminescent measurement of the fungal ATP content (BacTiter-Glo(TM); Promega, Germany) using a luminometer (LUMIstar Galaxy; BMG LABTECH, Germany). For control, DMSO without tryptanthrin was included.

Results: Tests showed that both saps of woad leaves exhibit a significant fungicide capacity against yeast and dermatophytes. Though, growth inhibition by FW was substantially higher than by DFW. Moreover, the fungicide potential towards *T. rubrum* (IC50 (DFW) 1.70.9%) (FW) 0.90.0%) and *M. pachydermatis* (IC50 (DFW) 16.82.7%, (FW) 9.90.6%) was higher than towards *C. albicans* (IC50 (DFW) 53.614.3%, (FW) 13.23.6%). Similar results were obtained using the DMSO extract of tryptanthrin against the fungi tested. Thus, a significantly higher antimycotic effect of tryptanthrin was observed against *T. rubrum* (IC50 1.84 μg/ml) and *M. pachydermatis* (IC50 0.40 μg/ml) in comparison to *C. albicans* (IC50 11.5 μg/ml). Although the DMSO control exhibited a slight antifungal activity itself at about 5% (v/ v), the observed antimicrobial activity could be fully attributed to tryptanthrin as acting DMSO concentrations in the preparations tested were considerably lower than 5%.

Conclusions: These *in vitro* experiments demonstrate a strong fungicide capacity of sap of woad leaves against yeast and dermatophytes. Because of its likewise strong antimycotic effects it can be assumed that tryptanthrin is involved in the antifungal activity of woad. However, tryptanthrin is formed after harvest during processing and therefore should only be present in sap of dried fermented woad. However, sap of fresh woad showed the higher potential and therefore it is to be hypothesized that other active compounds or interaction of the plant matrix is responsible for the fungicidal effects of woad. Hence, further investigations are required. Nonetheless, these results are crucial evidence that woad could be a natural source for antimycotic agents.

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Drug repurposing of approved therapeutics for the treatment of chronic and autoimmune skin diseases

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The development of new therapeutics is usually a long-lasting and cost-intensive process. Only a small amount of therapeutics that is effective in animal models can find their way to phase I studies in humans. Especially for the treatment of rare diseases like autoimmune skin blistering diseases hardly any new therapeutics are approved because of the cost-benefit considerations for pharmaceutical companies. A fast and cost-effective alternative is the use of drug repurposing for the therapy of these diseases. Here, we use a commercial available chemical library with 1200 approved drugs that are excluded from patent protection (Prestwick Chemical Library, PCL) for the analysis of drugs that can be utilized by off-label use for the treatment of chronic and autoimmune skin diseases. We screened human polymorphonuclear cells (PMNs), T cells and B cells by semi-high throughput screening for the inhibitory activity of the PCL substances on the activation of the respective cell types. 28 B cell-, 32 PMN- and 41 T cell-inhibitory substances were identified and further validated *in vitro*. Effects of the 32 screened substances on PMNs were analysed by ROS activity measurement, CD62L and CD66b expression, toxicity and proliferation assays in a dose-dependent manner and revealed 6 potential candidate substances for further validation *in vivo*. The first 3 substances were tested in an antibody-transfer model for autoimmune blistering skin diseases as a typical neutrophil-dependent disease. 2 out of these 3 substances revealed so far unknown therapeutic effects on disease manifestation. The 41 T cell inhibitory substances were also further validated *in vitro* and *in vivo*. Here, T cells were isolated and the effects of the PCL substances were analysed in a dose dependent manner on IL-2 expression, proliferation and toxicity. 15 substances were *in vitro* validated. 4 out of them were tested in a mouse model of the T cell-dependent ALDARAinduced psoriasiform dermatitis and one of them showed significant migitation of the disease progression. The B cell inhibitory substances were also validated using proliferation and toxicity assays in a dose dependent manner and 5 out of them are now available for testing in *in vivo* models. Taken together, as shown here the screening of approved substances in cell-based assays is a promising tool for the therapy of rare diseases as well as other diseases that are currently difficult to treat.

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Inter-alpha-trypsin inhibitor heavy chain 5 (ITIH5) affects epidermal morphology in constitutive knockout mice and could be a novel key player in delayed type hypersensitivity responses of the skin

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Inter-alpha-trypsin inhibitors (ITIs) are protease inhibitors that are thought to be important regulators in various acute-phase processes. They are composed of one light chain (bikunin) and different heavy chains (ITIHs). The only function known so far of ITIHs is the covalent linkage to hyaluronic acid (HA). Using GeneChip® Human Exon 1.0 ST expression profiling we identified ITIH5 as the major ITIH family member expressed in human skin. To investigate the role of ITIH5 in skin we established a new ITIH5−/− mouse model. We detected that the skin of ITIH5−/− mice as well as corresponding *in vitro* 3D-skin-equivalents exhibited structural abnormalities. In both models a significantly reduced epidermal thickness and absence of a stratified structure as well as a complete lack of the stratum corneum was observed. Interestingly, using a Van-Gieson staining we detected different extracellular matrix (ECM) structures in skin-equivalents of ITIH5−/− and wild type mice. First results indicate a mechanistic link between the ability of ITIH5 to stabilize the ECMcomponent HA and the impaired ECM structure if ITIH5 is lacking. Moreover, ITIH5 expression is significantly up-regulated in various inflammatory skin diseases including allergic contact dermatitis (ACD). To understand more precisely the role of ITIH5 in ACD we used the contact hypersensitivity (CHS) mouse model, where a role for HA degradation in modulating the inflammatory response has been recently described. Preliminary studies revealed that ITIH5−/− mice showed significantly reduced CHS responses. In consideration of these observations we assume that ITIH5 could be a novel key player in delayed type hypersensitivity (DTH) responses of the skin.

Taken together, our experiments revealed to our knowledge for the first time the specific and strong expression of ITIH5 in human skin. Preliminary evidence indicates that ITIH5 forms complexes with HA, thereby on the one hand facilitating the formation of a normal ECM structure and on the other hand modulating CHS responses.

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Conflicting results of kappa opioid receptor (KOR) activation in human skin organ culture versus mini-pig skin *in vivo* point to a key role for intact sensory innervation in cutaneous opioid and mast cell biology

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Many cell types of human skin express kappa opioid receptor (KOR), which is known to modulate multiple skin functions, including keratinocyte proliferation/differentiation (Tominaga et al., 2007; Cheng et al., 2008), and neurogenic inflammation. Reportedly, KOR activation down-regulates cytokine and chemokine release, as well as neuropeptide release from sensory nerve fibers under inflammatory conditions, and thus influences the maturation and activation of different immune cells. However, the effects of KOR-mediated signaling on human skin mast cells (MCs; which are key players in neurogenic inflammation) *in situ* remain unclear. Moreover, although a few studies on mice suggested that KOR agonists may influence vascular biology (Yamamizu et al., 2013), the effect of KOR activation on angiogenesis was not yet investigated in human skin. Therefore, in order to reveal effects of KOR activation on keratinocyte proliferation, MC functions and angiogenesis, we treated 4 mm punches of full-thickness denervated human skin in serum-free organ culture with a new, highly selective KOR agonist (KORA).

We found that, under these *in vitro* conditions, administration of KORA (200 nM or 1 M for 48 h) significantly increased epidermal thickness by inducing epidermal hyperplasia (higher number of DAPI+ nuclei and increased % of Ki-67+ epidermal cells). Although this was partially counterbalanced by a slight increase in the ratio of apoptotic (i.e. TUNEL+) cells, the overall effect of KOR stimulation was one of proliferation-driven epidermal hyperplasia.

Interestingly, c-Kit immunofluorescence (marking both immature and mature MCs) revealed that KORA treatment significantly decreased the number of detectable cutaneous MCs. Although the number of mature MCs (as measured by toluidine blue histochemistry and tryptase immunohistochemistry) was also reduced upon KOR activation and MC degranulation was dose-dependently increased by KORA. Finally, the number of CD31+ cells and of CD31+ blood vessel cross-sections was reduced by KORA, suggesting inhibition of angiogenesis.

This partially conflicted with previous *in vivo* reports that had suggested primarily anti-inflammatory skin responses to KOR activation (e.g. Earl et al., 1996). Repeated topical KORA administration was tested on mini-pig skin over 28 days *in vivo*. Interestingly, our preliminary results did not show a marked difference between MC degranulation, epidermal hyperplasia, and Ki67+ cells in the skin of vehicle versus KORA treated mini-pigs *in vivo*. First clinical results in humans support these findings.

These apparently conflicting MCs results between the two models raise the question whether the biological effects of KOR agonists greatly depend on the presence of intact sensory innervation, e.g. during MC-dependent neurogenic skin inflammation, and how the absence/presence of functional sensory nerve fiber-MC interactions impacts on the constitutive release/activation characteristics, and the pro- versus anti-inflammatory phenotype of MCs in human and porcine skin.

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Analysis of key epidermal lipid enzymes in rosacea patients

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Rosacea is a common, chronic, inflammatory skin disease of unknown aetiology. Recently, impairments of the skin barrier function resulting in higher transepidermal water loss were reported in rosacea patients. Thus, we asked here, whether rosacea also shows a dysregulation of skin barrier related epidermal lipid enzymes. Using immunohistochemistry and microarray analysis, we found that the expression of selected enzymes in different epidermal lipid pathways was only slightly changed on the protein- and mRNA-level. Microarray analysis revealed an increase of the expression of fatty acid synthase and minor changes in epidermal lipid transport proteins. Moreover, enzymes involved in cholesterol-, steroid-, ceramide- and sphingosine-metabolism were moderately changed in all rosacea subtypes. These mRNA-findings were confirmed by immunohistochemical stainings for key enzymes of the fatty acid-, steroid-, ceramide- and sphingosine-pathways. Interestingly, we could not demonstrate significant differences in the regulation of these enzymes in rosacea subtypes, but instead we show a rather uniform pattern of up- and downregulation of the various enzymes. In sum, our findings provide a deeper insight into the pathophysiology of rosacea and argue for a rather limited impact of dysregulated epidermal lipid enzymes in this disease.

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PGC1 alpha – a common regulator of mitochondria and ribosomes?

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Muscle atrophy, weight loss and cachexia are well known symptoms and negative prognostic factors in motorneuron diseases (MND) traced back to an impaired metabolism. PGC1 alpha, a transcriptional co-factor plays an important role in regulation of mitochondrial energy supply and metabolic adaptability to varying energy requirements. In MND patients and in the respective mouse models, mitochondrial biosynthesis is impaired and expression of PGC 1 alpha reduced. Unfortunately, clinical trials to increase mitochondrial energy production by supplementation of creatine, ubiquitin among others have so far failed to delay or even suppress disease progression. Therefore, we addressed the question whether apart from mitochondria other or additionally affected cell-organelles are responsible for metabolic homeostasis.

Ribosomes and ribosomal biogenesis essentially required for protein biosynthesis are closely regulated dependent on different demands to the cell. A common denominator for the regulation of mitochondrial and ribosomal biogenesis is currently elusive. Therefore, we investigated a possible interaction of PGC1 alpha with the rDNA that is transcribed into the 47S rRNA by RNA polymerase I, a key component for ribosomal biogenesis.

Double immunostaining of PGC1 alpha and nucleolin in HEK-cells showed nucleolar localization of the co-transcriptional factor as a first hint for a possible role in ribosomal biogenesis. A physical interaction and binding of PGC1 alpha mainly to the rDNA promotor and to a minor extent to gene-internal rDNA sequences was detected in chromatin immunoprecipitations. Investigation of PGC1 alpha in skin, brown adipose tissue, liver, heart, muscle and brain revealed decreased amounts of 47S rRNA in samples of PGC 1 alpha knock-out mice in comparison to wild type in known PGC1 alpha relevant tissues, providing evidence that binding to the rDNA has also functional relevance.

Thus, we here found a previously unreported role for PGC1 alpha in ribosomal biogenesis and this may represent – apart from the known impact of PGC1 alpha in mitochondrial energy supply – an alternative or additional link to metabolic dysregulation in motorneuron diseases.

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