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P001

Age and sex dependence of tryptase according to a retrospective survey on 1092 tryptase values from non-mastocytosis patients

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Introduction: The tryptase extent as predictor of severe anaphylactic reactions inpatients with hymenoptera venom allergy supports decision about duration of immune therapy. Patients with high levels have to be treated lifelong. Unfortunately, an elevated tryptase is up to now not defined. A value of 11.4 μg/L is proposed, butmastocytosis is also known in patients with much lower values. We performed a retrospective survey about 1092 non-mastocytosis patients (2001–2007) concerning age distribution of the tryptase extent. False positivity was considered by determination of heterophilic interference.

Methods: Tryptase determination was based on the Phadia test kit (UniCAP 100® and ImmunoCAP® Tryptase, Phadia, Uppsala, Sweden). Re-testing in subjects with elevated tryptase was performed with the same test kit after pre-incubation with heterophilic Blocking Tubes® (Scantibodies Laboratory, Santee, USA).

Results: Tryptase average in total test population amounted 5.13 μg/L, the 95th percentile 10.8 μg/L, and standard deviation 3.05 μg/L. Averages increased with age (subjects aged 15–34 years: 4.53 μg/L, 35–64 years: 5.20 μg/L, >64 years: 6.26 μg/L). These differences were statistically significant (Mann–Whitney test, Holm α adjustment).Frequency of subjects with tryptase >8.75 μg/L and 11.4 μg/L in different age classes was also determined. 5.9% of the subjects aged 15 to 34 showed values >8.75 μg/L. In contrast, 15.8% of the subjects older than 64 years showed a tryptase >8.75 μg/L. This increase was statistically significant (chi quadrate test). When11.4 μg/L was chosen as upper limit, an increase was also detectable, but not statistically significant. Altogether, only 11 patients showed false elevated tryptase values due to heterophilic interference.

Conclusions: The extent of tryptase is age depending and increases with age. Older patients show more often elevated tryptase values. False positive values due to heterophilic interference were found rarely and only in subjects with initial values between 8.86 μg/L and 12.45 μg/L. Thus, decrease of tryptase values below the upperlimit after re-testing is possibly due to common variability of biological lab data.

P002

Bcl-3 is overexpressed in atopic dermatitis and acts as a transcriptional modulator of innate immune responses in human keratinocytes

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Atopic dermatitis (AD) is a chronic inflammatory skin disease that is often associated with skin infections. Recent studies have demonstrated that the Th2 type cytokines interleukin (IL)-4, IL-10 and IL-13 can downregulate antimicrobial peptide expression in AD and that this phenomenon may account for the observed propensity towards skin infections in these patients. We report here the identification of B cell leukemia (Bcl)-3 as a transcriptional modulator of antimicrobial peptide expression in human keratinocytes. Bcl-3 is structurally related to the IκB inhibitors but is distinct in several critical ways. Bcl-3 contains domains that interact predominantly with p50 and p52 homodimers, and has been reported to directly or indirectly transactivate or repressgene expression via κB elements. In this study, we demonstrate that Bcl-3 is inducible by the Th2 cytokines interleukin (IL)-4 and IL-13 in keratinocytes and is overexpressed in lesional skin of AD patients. Bcl-3 was shown to be important to cutaneous innate immune responses as small interfering RNA (siRNA) silencing ofBcl-3 reversed the downregulatory effect of IL-4 and the upregulatory effect of TNFα on human beta defensin (HBD)3 expression. Furthermore, Bcl-3 silencing enhanced 1,25-D3-induced gene expression of cathelicidin antimicrobial peptide in keratinocytes, suggesting a negative regulatory function on cathelicidin transcription. In summary, we identified Bcl-3 as an important modulator of cutaneous innate immune responses and its possible therapeutic role in atopic dermatitis.

P003

Differences in allergen uptake by human epithelial cells of the respiratory tract and the skin

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Airborne antigens like pollen derived allergens are mainly exposed to two types of epithelial surfaces: the skin and the respiratory tract. In the skin, keratinocytes act as a physical, chemical and immunological barrier. In contrast, respiratory tract epithelia consists of many different specialised epithelial cell types. Here we analysed the uptake of allergens by keratinocytes and airway epithelial cell lines of different origin (A549, Calu-3, NCI-H727). Fluorescently labelled timothy grass pollen allergens were used as a model: Phl p 1 as a protein with glycosylations and disulfide bridges and Phl p 6 lacking posttranslational modifications. Non-allergenic proteins (HSA and HRP) were used as control. The uptake was analysed by flow cytometry and fluorescence microscopy. Uptake of allergens and non-allergenic proteins by keratinocytes increased constantly over time. Inflammatory conditions simulated byIFN-γ stimulation lead to enhanced uptake of proteins. Furthermore, by keratinocytes internalised allergens are localised in lysosomes making processing and MHC-dependent presentation by keratinocytes probable. Uptake of proteins was also observed in airway epithelial cell lines. The respiratory epithelial cell line A549 showed a constant level of protein uptake over time, no lysosomal localisation. Internalised proteins were exocytosed rapidly indicating a transcytosis mechanism for proteins to pass the respiratory epithelial barrier. In NCI-H727 cells uptake of proteins was similar to A549 cells but Calu-3 showed differences like an increasing uptake over time. In contrast to keratinocytes, IFN-γ stimulation had no impact on protein uptake by airway epithelial cell lines. These differences between respiratory and dermal epithelial cells in the uptake of pollen allergens indicate distinct mechanisms of allergen uptake and processing in the epithelia examined. The higher uptake of allergens by keratinocytes in inflammatory status suggests a higher susceptibility of inflamed skin for uptake of allergens and possibly a higher risk for sensitisation under natural exposure conditions such as chronic atopic eczema.

P004

Hapten-specific CD4+CD25+ regulatory T cells are critical for low zone tolerance to contact allergens and induction of CD8+ suppressor T cells

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Specific immune suppression and induction of tolerance are essential processes in the regulation and circumvention of allergies. Low zone tolerance (LZT) to contact allergens, induced by topical application of subimmunogenic doses of haptens, results in the generation of CD8+ suppressor T cells which prevent the development of contact hypersensitivity (CHS). However, the precise mechanisms of LZT are not yet understood. In this study, we analyzed the role and function of naturally occurring CD4+CD25+FOXP3+ regulatory T cells (nTregs) in LZT. We observed significantly increased numbers of nTregs during the induction of LZT. Furthermore, depletion ofCD4+CD25+ Tregs during tolerization (by anti-CD25-Ab or cyclophosphamide) induced an elevated CHS response as revealed by a significant ear swelling, hapten-specific T cell proliferation and Tc1 cytokine pattern. Notably, transfer experiments revealed that CD8+ T cells purified from these Treg-depleted and tolerized mice totally lost their capacity to act as suppressor T cells in LZT, demonstrating an abrogated development of CD8+ suppressor T cells in the absence of CD4+CD25+ T cells. Adoptive transfer experiments of CD4+CD25+ Tregs obtained from tolerized animals into naïve mice that are subsequently sensitized clearly showed that CD4+CD25+ T cells exhibited regulatory functions for the transfer of LZT to allergens. In addition, sensitization of recipients of CD4+CD25+ Tregs with a second, unrelated hapten, completely abolished the tolerance reaction, indicating an allergen-specificity of CD4+CD25+ Tregs in LZT. Our data demonstrate that hapten-specific CD4+CD25+ Tregs play a privotal role in tolerance to contact allergens as important modulators in the network of CD8+ T cell-related immune responses.

P005

Concentration of the major birch pollen allergen bet v 1 indifferent fractions of ambient air deviates from pollen counts in Munich, Germany

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Exposure to allergens is one of several factors determining sensitization and symptoms in individuals. Exposure to aeroallergens from pollen is assessed by counting allergenic pollen in ambient air. However, proof is lacking that pollen count is representative for allergen exposure, also because allergens were found in non-pollen bearing fractions. We therefore monitored simultaneously birch pollen count and the major birch pollen allergen Bet v 1 in different size fractions of ambient air in Munich from 2004 till 2007.

Methods: Ambient air was sampled in Munich on the university campus at 510 m above sea level, 1.8 m above ground, with a Chemvol® high-volume cascade impactor equipped with stages PM>10 μm, 10 μm > PM > 2.5 μm, 2.5 μm > PM > 0.12 μm. The polyurethane impacting substrate was extracted with 0.1 M NH4HCO3 pH8.1. The major birch pollen allergen Bet v 1.0101 was determined with a Bet v 1specific ELISA. Pollen count was assessed with a Burkard pollen trap.

Results: The four studied years were all strong birch pollen flight years for Munich, Germany. In those years 93 ± 3% of Bet v 1 was found in the PM > 10 μm fraction, the fraction containing birch pollen. On none of the days did we find any Bet v 1 in 2.5 μm > PM > 0.12 μm (all <0.5%). We found that Bet v 1 could have absorbed to diesel particles that also land in this fraction. On average pollen released 215% more Bet v 1 in 2007 than the same amount of pollen in 2004. Also within one year, the release from the same amount of pollen varied several fold between different days. This variation could be explained by the phenomenon that Bet v 1 from pollen within catkins increased from zero to 9200 ng/10 mg pollen in the last week before pollination when each day anthers could have pollinated, depending on the weather.

Conclusion: Bet v 1 was only found in the pollen containing fraction. Pollen from different years, different trees and even different days released up to 10-fold different amounts of Bet v 1. Thus exposure is poorly monitored by only monitoring birch pollen count. Monitoring the allergen itself in ambient air might be an improvement in allergen exposure assessment.

P006

Birch pollen allergics with pollen associated oral allergy syndome to hazelnutand carrot show cross reactive T cell responses

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In European countries food allergy affects as much as about 4–6% of children and1–3% of adults. Apart from primary food allergy which is initiated by the respective food allergen, secondary food allergy develops after primary sensitization by airborne pollen allergens (e.g. birch pollen-fruit syndrome. Increasing evidence has shown that this is caused by IgE and T cell cross reactivity to proteins, homologous to pollen allergens, which are found in food causing the pollen associated oral allergy syndrome (OAS). Birch pollen allergy often results in cross allergy to Bet v 1homologous proteins of the pathogen related protein family 10 (PR-10) like Cor a1. in hazelnuts, Dau c1 in carrots, Mal d1 in apple, Api g1 in celery and others. Former studies showed cross reactive IgE in patients suffering from OAS, and allergen specific responses of *in vitro* generated T cell clones and T cell lines, but still little is known of primary T cell reactivity in OAS. Therefore, we analyzed the T cell response to recombinant Bet v 1, Cor a1 and Dau c1 in a panel of patients with proven birch pollen allergy and food allergy to hazelnuts and carrots (patient history, prick test, EAST) after stimulation with allergen loaded mature monocyte-derived dendritic cells(DC) *in vitro*. The optimal concentration of Bet v 1, Cor a1 and Dau c1 for loading of DC and subsequent stimulation of isolated CD4+ T cells, determined as 5-20 μg/ml was non toxic. In primary stimulation of CD4+ T cells with Bet v 1 or Cor a1 loaded DC significant amounts of IL-5, IL-13 and little IFN-γ or IL-10 were induced as compared to unloaded DC, showing a strong bias towards TH2 cytokines. With Dauc1 significant allergen-specific Th2 cytokine secretion was observed though little proliferative response could be determined. In restimulation experiments, we analyzed T cell proliferation after restimulation with DC loaded with identical antigen as in primary stimulation or with the respective cross reactive allergen and could thus identify a group of birch pollen allergic patients with T cell cross reactivity to Cor a1and Dau c1. This shows the role of T cell responses in the pathogenesis of pollen associated OAS and emphazises the importance to target T cells with novel therapeutic approaches.

P007

The wheal-size of skin prick tests does not correlate with specific IgE levels inpatients with type-I allergy

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The wheal-size of skin prick tests or allergen-specific IgE serum levels are often regarded as surrogate markers for the symptom severity of type-1 allergic diseases. We investigated whether the wheal-sizes of SPTs with allergen were correlated with sIgE levels and whether they were influenced by symptom severity. We re-analysed a data set of 126 patients. Briefly, SPTs and measurement of sIgE with the ImmunoCAP (TM) system (Phadia AB, Uppsala, Sweden) and the ISAC system (vbc-genomics, Vienna, Austria) had been performed in 126 subjects with allergic/non-allergic rhinoconjunctivitis (83.3%) and/or bronchial asthma (34.9%)and/or atopic dermatitis (5.6%). There were 34 controls and 84 with a history matching a sensitization to at least one of five. The five study-allergens cat, dust mite, birch, grass or mugwort pollen labelled 'allergic'. Another eight patients were labelled 'irrelevant positive' with a positive SPT but without an allergen-specific history and added to the controls. SPTs had been measured in millimetre square with a semi-automated system. Disease activity in allergic patients had been classified according to the ARIA recommendations. We used SPSS 15.0 for Windows for the graphical re-analysis of the data set. We used the Wilcoxon test for assessing differences of means. Box plot analyses revealed that the symptom severity (mild versus moderate/severe) did neither influence the outcome of the wheal-sizes nor the sIgE levels ($P > 0.025$)except for the subgroup of mugwort-allergic patients whose sIgE were significantly higher in the moderate/severely affected patients than in the mildly affected ones ($P < 0.025$). The wheal size did not differ in both groups. None of the allergen-specific wheal-sizes were correlated significantly with the sIgE level in the allergic patients except for house dust mite allergic patients ($P < 0.01$). The sIgE levels are only scantily correlated with the wheal-sizes of SPTs and both are hardly influenced by symptom-severity in individuals suffering from ARC. Hence, defining specific *in vivo* or *in vitro* cut-offs for clinical tests may be without clinical relevance and should not be used as a surrogate marker for symptom severity inpatients.

P008

Analysis of differential gene expression in dendritic cells activated by potent skin sensitizers

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Allergic contact dermatitis (ACD) is caused by skin contact with protein-reactive chemicals in the environment. ACD is suggested as the most frequent manifestation of immunotoxicity in humans and the initial step in specific sensitization is the activation of dendritic cells in the skin. To study the molecular events in dendritic cell activation, we established an *in vitro* cell culture model. We first generated immature DCs from human peripheral blood monocytes by depletion of CD2, CD7, CD19, CD56, CD16, CD235a positive leukocytes and consecutive incubation with GM-CSF and IL-4. Secondly, at least 6×106 cells were stimulated with the test compounds for 30 h. Our test substances consisted of two model sensitizers (2,4,6-trinitrobenzene sulfonic acid-TNBS (200 mg/ml) and cinnamic aldehyde (0.1 mM)) and two model irritants (sodium dodecylsulfate–SDS (5 mg/ml) and cinnamic aldehyde (0.1 mM)). Sensitizing or irritative effects of the substances were confirmed by demonstration of IL-8 expression (sensitizers) and lack thereof (irritants) with quantitative RT-PCR analysis. In order to elucidate the molecular events specific for sensitization we compared gene expression in cells treated with a sensitizer to the gene expression after treatment with the irritant compounds using Affymetrix exon arrays. Three biological replicates were performed for each pair of substances. Data analysis was performed with respect to differentially expressed genes combining the data of all biological replicates. Among the strongest differentially expressed genes are prominent members of the oxidative stress response related genes (i.e. AKR1C2) and genes involved in immunologic responses, particularly DC maturation. Effects seen in the microarray analysis were verified by quantitative RT-PCR analysis. In conclusion, we identified a set of genes differentially expressed in two sensitizer/irritants pairs identifying molecular processes specific for allergic contact sensitization, independent of the substance used in the assay.

P009

Mast cells can modulate the phenotype of Tregs by lowering their numbers andCD25 expression

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Natural regulatory T cells (Tregs) express high levels of CD25 and Foxp3, the Treg-specific transcription factor crucial for their development and function. Interestingly, numbers of cutaneous Tregs are significantly reduced in atopic dermatitis and psoriasis, and Treg function seems to be impaired in allergic asthma. As these conditions are (co)mediated by mast cells (MCs), we asked whether MC scan modulate Treg numbers and/or function. We depleted non-CD4 T cells fromC57BL/6 splenocytes by MACS separation technology and then selected positively for CD4+CD25+-T cells (purity: >90%). Peritoneum-derived cultured MCs (PCMCs) were then coincubated at a 1:1 ratio with CD3/CD28-preactivated CD25 negative ('Teff') or CD25 positive ('Treg') cells for 3 days followed by FACS analyses (CD25and Foxp3) and supernatant cytokine measurements. As result, MCs induced a dramatic and significant decrease of CD25 expression in the 'Treg' population, but not in 'Teff' cells when compared to the respective MC-free T cell controls (MFICD25: 'Teff' 7466 versus 'Teff' + MCs 6345; $P = 0.04$ and 'Treg' 7369 vs 'Treg' + MCs 3880; $P = 0.0009$). Furthermore, this was paralleled by a pronounced drop of T cell numbers in the 'Treg'-MC coculture, but not in the 'Teff'-MC coculture (CD4+CD25+ labelled cells: 'Teff' 14968 vs 'Teff' + MCs 11808; $P = 0.02$ and 'Treg' 16962 vs 'Treg' + MCs 5552; $P = 0.004$). Similar, albeit less prominent MC-driven effects on 'Tregs' were seen at 10:1 'Treg'-MC ratios (MFI CD25: 'Treg' 7369 vs 10:1 'Treg' + MCs 5258 and CD4+CD25+ cells: 'Treg' 16962 versus 10:1 'Treg' + MCs 9623). Strikingly, Foxp3 expression levels and IL-2 production were unaltered and stable in all 'Treg'-related culture samples. In summary, these data implicate that MCs can downregulate Treg numbers and CD25 expression, which may critically influence the course of Treg-modulated inflammatory reactions.

P010

Interleukin-1-beta derived from human primary keratinocytes is an important factor for induction of Interferon-gamma production by autologous T-cells

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In a number of inflammatory skin diseases such as eczema or psoriasis infiltrating lymphocytes are found in close vicinity to keratinocytes, enabling interaction of these two cell types. It has been proposed that keratinocytes rather support a Th2 response by interacting lymphocytes. However, all of these studies used cell lines or cells from different individuals. We now examined this hypothesis with autologous cultures of keratinocytes derived from the outer root sheet of the hair follicle co-cultured with CD3+ T cells from the same donor. During the co-culture either Staphylococcus enterotoxin B or tetanus toxoid were added. In different experimental approaches the addition of T-cells to keratinocytes resulted in higher production of IFN-gamma by T cells. Furthermore we established an experimental set up with addition of autologous, antigen-pulsed monocytes as well. Here the induction of IFN-gamma by the presence of keratinocytes resulted in a marked and significant increase of IFN-gamma production by T cells. We could show that this effect is mediated by soluble factors as well as direct cell–cell contact. Furthermore we saw an outstanding role of Interleukin-1-beta in this process. We conclude from our study that keratinocytes rather support a Th1 than a Th2 local response pattern in part due to secretion of IL-1-beta. This property of keratinocytes may account for the observed cytokine switch in allergic eczematous skin from a Th2 like micromilieu in acute towards a Th1 dominated milieu in chronic lesions. The suppression of IL-1-betaproduction may act as a new therapeutic target.

P011

Nickel (Ni) exposed PBMC of Ni allergic individuals but not of healthy controls lead to cytokine production in human fibroblasts

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Introduction: In the general population the prevelance of metal sensitivity is approximately 10% to 15%; with nickel (Ni) sensitivity at the first place followed by cobalt (Co) and chromium (Cr). It is still an open question if metal allergy can lead to a hypersensitivity reaction against metal implants followed by inflammation and osteolysis.

Method: PBMC were obtained from five individuals allergic to Ni (patch test and lymphocyte transformation tests (LTT) positive) with total joint arthroplasty, five Ni allergic patients (patch test and LTT positive) without implant, two patients with total joint arthroplasty and no patch test reactivity but positive LTT, five Ni non-allergic patients with implant, and five Ni non-allergic patients without implant. Five healthy non-allergic volunteers served as control blood donors. LTT was done by stimulation of PBMC with NiSO4, CoCl2 and CrCl3 in 96-well cell culture plate for 5 days and radioactive labelling with 3H-Thymidin for 18 h. The cell culture supernatant of a nonradioactive parallel culture was used for stimulation of fibroblast cultures obtained from five individuals. RNA was isolated and analysed with Realtime PCR for the expression of IL6, IL8, MMP2, MMP9, IL13 and IL15.

Results: In contrast to controls, PBMC of Ni allergic (positive patch test) patients show strong proliferation to Ni (mean SI = 10.35) as well as Ni allergics with metal implants (but to a lesser extent; mean SI = 4.8). Fibroblasts stimulated with the supernatants of Ni exposed PBMC of Ni allergics both with and without implants – but not of non-allergics–show a strong expression of IL13 and IL15. Expression of MMP2, MMP9 and IL6 was not elevated in these groups.

Conclusion: Ni exposed PBMC of metal (Ni) allergic patients with metal implants may eventually lead to a periimplant inflammatory response also by possibly influencing the cytokine production of fibroblasts.

P012 (V03)

Induction of regulatory T cells during wasp venom immunotherapy

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The induction of regulatory T cells (Treg) are thought to play an important role in thecourse of hymenoptera venom immunotherapy (VIT) leading to long-lasting immune tolerance to venom allergens. However, the underlying mechanisms are not yet entirely clarified. In this study, we longitudinally characterized the impact of VIT on the pool of peripheral Treg of 21 wasp venom-allergic patients with severe reactions (grades II-IV). Before and one month after starting rush wasp VIT freshly isolated peripheral blood mononuclear cells (PBMC) were analysed for CD4, CD25, CD45RO- and Foxp3 expression, the latter the most reliable marker for regulatory T cells *in* and *ex vivo*. Furthermore, T cell subsets were characterized for the usage of the T cell receptor VB chain family via flow cytometer analysis. We demonstrate that VIT expanded the pool of memory CD4+/CD25+/Foxp3+ Treg and in parallel induced the frequency of the VB2+ and VB5.1+ Treg in the course of VIT suggesting a wasp venom specific oligoclonal expansion of effector Treg. Strikingly, these changes were accompanied by a decline in the frequency and absolute numbers of CD4+/Foxp3+ as well as CD4+/CD25+/Foxp3+ Treg after 1 month of VIT. However, the capacity of effector Treg to suppress proliferative responses of PBMC to wasp venom *in vitro* was enhanced, in line with a marked increase in IL-10 production, further supporting an enhanced effector phenotype of Treg during VIT. Taken together, wasp VIT is associated with an enhanced effector phenotype of oligoclonally expanded CD4+/CD25+/Foxp3+ Treg, most likely through an enhanced recirculation of these cells to secondary lymphoid organs.

P013

Impact of specific immunotherapy and environmental birch pollen exposure on Bet v 1-specific humoral and cellular profiles in birch pollen allergics and healthy individuals

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While the clinical efficacy of specific immunotherapy (SIT) has been well established, the immunological mechanisms leading to allergen tolerance are still focus of intensive research. In particular, the modulation of immune responses by the balance between adaptive allergen-specific regulatory T (Treg) cells and T helper (Th) cells is of special interest. Healthy and allergic individuals exhibit three distinct allergen-specific T cell subsets (Th1, Th2 and type 1 Treg (Tr1) cells) in different frequencies suggesting that the ratio of these T cell populations may be decisive in the development of allergy and recovery. In the present study, we investigated the occurrence and frequency of birch pollen allergen (Bet v 1)-specific Th1, Th2 and Tr1-like cells in birch pollen allergic individuals either treated only symptomatically ($n = 8$) or by birch pollen SIT ($n = 15$) and healthy controls ($n = 8$) outside and in the following birch pollen season. The different T cell populations were distinguished according to their secreted cytokine profiles by *ex vivo* ELISPOT analysis of peripheral blood mononuclear cells.
Both birch pollen allergic groups showed an augmented Th2/Th1- and Th2/Tr1-ratio compared to healthy individuals. However, in contrast to allergic patients treated only symptomatically, patients receiving SIT showed a decreased Th2/Tr1-ratio during the birch pollen season with a relative increase of the IL-10-secreting T cells compared to the Th2 cells. On the humoral level, baseline titres of birch pollen-specific IgE did not change in both SIT-treated patients and healthy controls in contrast to birch pollen allergic subjects not treated by SIT. Of note, an increase of birch pollen specific 'blocking' IgG4-antibodies was solely detectable in patients undergoing SIT.
These data confirm that induction of allergen-specific Treg accompanied by an increase of allergen-specific IgG4 represent decisive changes induced by SIT inpatients suffering from pollen allergy. Furthermore, the ratio of T effector to Treg cells seems to be a pivotal indicator determining an allergic or healthy constitution.

P014 (V01)

Epicutaneous priming induces IL-17-producing CD4+ Th cells

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T helper (Th)17 cells play a crucial role in immune mediated inflammatory diseases as shown in experimental models of multiple sclerosis, colitis, arthritis and psoriasis. Recent findings suggest that Th17 cells are also involved in the pathogenesis of classic Th2 diseases like asthma and atopic dermatitis. Destructive tissue inflammation during the chronic phase of these diseases is caused by a combine dinfiltrate of Th17 and Th1 cells, indicating an important function of their lineage-specific cytokines IL-17 and IFN-gamma. Even though the role of Th17 andTh1 cells has been intensively studied in inflammatory bowel disease or psoriasis, little is known on the generation of IL-17 and IFN-gamma after epicutaneous antigen priming. In our study, we analyzed antigen-specific migration of skin or gut primed ovalbumin (OVA)-specific T cells from OVA T cell-receptor transgenic mice after transfer into naïve BALBc mice following epicutaneous OVA exposure. We observed specific enrichment of OVA-reactive T cells after epicutaneous OVA exposure predominantly in the skin draining lymph nodes whereas PBS exposure resulted in random distribution of OVA-specific T cells. Both investigated T cell populations, from original skin or from original gut tissue, migrate with similar frequency but only skin primed T cells were able to induce protein contact dermatitis in BALBc mice after adoptive transfer and epicutaneous antigen exposure. Eczema occurred by peptide challenge without additional stimuli like Toll-like receptor activation. Importantly, neither transfer of gut primed OVA T cells followed by epicutaneous OVA peptide challenge, nor challenge with non-specific peptides or PBS did induce eczema. The antigen and organ-specific migration of OVA-specific T cells after epicutaneous antigen exposure was associated with high expression of IFN-gamma in the skin-draining lymph nodes, independently from the transfer of skin or gut primed Thc ells. In contrast, epicutaneous OVA exposure induced high levels of IL-17 and theTh17-cytokine IL-22 in the skin-draining lymph nodes when skin-primed OVA-specific Th cells were transferred but not after transfer of gut primed OVA-specific Th cells. These results suggest, that co-expression of IFN-gamma, IL-17 and IL-22 are responsible in regulating local tissue inflammation and protein contact-dermatitis.

P015

Patients with primary lymphedema show polymorphisms in the podoplanin gene, but no pathogenic mutations

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The lymphatic vascular system maintains tissue fluid homeostasis and mediates the afferent immune response. The transmembrane glycoprotein podoplanin is specifically expressed by lymphatic, but not blood vascular endothelial cells. Our previous studies demonstrated that podoplanin null mice develop congenital lymphedema and that podoplanin heterozygous knockout mice show enhanced lymphvascular diameters of the intestine due to a 50% reduction of the protein expression level of podoplanin. In human primary lymphedema patients, increased lymphvascular diameters and malfunction of intestinal lymphatic vessels followed by an impaired absorption of fatty acids have been described. Therefore, the podoplanin gene was screened for sequence variations in 70 primary lymphedema patients of 66 non-related individuals. PCR amplification of all six exons and the adjacent intronic sequences of the podoplanin gene, followed by direct DNA sequencing revealed six different polymorphisms of the podoplanin gene. Three of these are novel, while the rest were already described in the literature. The sequence variants were present at a frequency of 1–2% in both the patients and the control group. We did not find evidence for pathogenic mutations in the human podoplanin gene in this group of patients. Taken together, these data suggest that primary genetic defects of podoplanin do not play a disease-causing role in patients with primary lymphedema.

P016

Influence of age-associated hormone decline on human dermal fibroblasts

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Within the scope of the Explorative Project 'Genetic aetiology of human longevity' supported by the German National Genome Research Network 2 (NGFN-2) an *in vitro* model of human hormonal ageing has been developed. Human epithelial cells, SZ95 sebocytes, were maintained under a hormone-substituted environment consisting of growth factors (GH, IGF-I) and sexual steroids (17β-estradiol, progesterone, DHEA and testosterone) in concentrations corresponding to those circulating in 20- (f20) and in 60 (f60)-year-old women. In order to decipher the effects of hormones and their decline occurring with age on skin dermis, the biological activity and the gene expression pattern of human dermal fibroblasts were analyzed using the *in vitro* model mentioned above. The corresponding hormone receptor expression was confirmed by RT-PCR, Western blotting and immunocytochemistry. Fibroblast proliferation, measured by 4-methylumbelliferyl-heptanoate assay, was significantly stimulated under the respective hormone treatments ($P < 0.001$). In addition, f60-fibroblasts showed lower content of neutral lipids ($P < 0.01$) in contrast to f20-fibroblasts by means of nile red microassay. Expression profiling employing a cDNA microarray composed of 15, 529cDNAs identified ca. 300 genes with altered expression levels at f20 versus f60.Confirmation of gene regulation was performed by real-time RT-PCR. The functional annotation of these genes identified pathways related to cell metabolism and organization, cell cycle and growth, and oxidative stress. 34 common genes were found to be differentially expressed in hormonally aged SZ95 sebocytes and indermal fibroblasts. Our data demonstrate that biological activity and transcriptome of dermal fibroblasts may be affected by the age-associated hormone decline, they illustrate hormone-dependent genes and signalling pathways in human dermal cells and display the importance of hormones in human endogenous skin ageing.

P017

SEBACEOUS GLAND RECEPTORS

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Due to its strong biological and metabolic activity and the involvement in multiplemolecular pathways the sebaceous gland has been suggested to be 'the brain of the skin'. This organ is target for several–some of them unexpected–hormones and expresses relevant receptors (Rc), whereas its function plays a major role in skin ageing. Rc ligands are peptides (such as neurotransmitters), hormones, pharmaceutical drugs and/or a toxins, whereas 'binding' ordinarily initiates a cellular response. Three of four groups of peptide/neurotransmitter Rc, the so-called serpentine Rc group are present (corticotrophin-releasing hormone Rc 1 and 2, melanocortin-1and 5 Rc, μ-opiate Rc, VPAC Rc, cannabinoid Rc 1 and 2, vascular endothelial growth factor Rc and histamine 1 Rc). The single-transmembrane domain Rc are represented by the insulin-like growth factor-I Rc and the third group, which does not possess intrinsic tyrosine kinase activity by the growth factor Rc. Nuclear Rc expressed in sebocytes are grouped into two major subtypes. From the steroid Rc family, the androgen Rc and the progesterone Rc are expressed. The thyroid Rc family includes the estrogen Rc (α and β isotypes), the retinoic acid Rc (isotypes α and γ) and retinoid X Rc (isotypes α, β, γ), the vitamin D Rc, peroxisome proliferator-activated Rc (isotypes α, δ and γ) and the liver X Rc (α and β isotypes). At last the vanilloid Rc belongs to the transient ion channels and is expressed in differentiating human sebocytes. Rc-ligand interactions control sebocyte proliferation, differentiation and lipid synthesis. However, not every ligand that binds to a sebocyte Rc also activates the Rc, such ligands are antagonists and inverse agonists.

P018

Human skin stem cells and the ageing process

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In healthy individuals, skin integrity is maintained by epidermal stem cells which self-renew and generate daughter cells that undergo terminal differentiation. Despite accumulation of senescence markers in aged skin, epidermal stem cells are maintained at normal levels throughout life. Therefore, skin ageing is induced by impaired stem cell mobilisation or reduced number of stem cells able to respond to proliferative signals. In the skin, existence of several distinct stem cell populations has been reported. Genetic labelling studies detected multipotent stem cells of the hair follicle bulge to support regeneration of hair follicles but not been responsible for maintaining interfollicular epidermis, which exhibits a distinct stem cell population. Hair follicle epithelial stem cells have at least a dual function: hair follicle remodelling in daily life and epidermal regeneration whenever skin integrity is severely compromised, e.g. after burns. Bulge cells, the first adult stem cells of the hair follicle been identified, are capable of forming hair follicles, interfollicular epidermis and sebaceous glands. In addition - at least in murine hair follicles -, they can also give rise to non-epithelial cells, indicating a lineage-independent pluripotent character. Multipotent cells (skin-derived precursor cells) are present in human dermis, dermal stem cells represent 0.3% among human dermal foreskin fibroblasts. A resident pool of progenitor cells exists within the sebaceous gland, which is able to differentiate into both sebocytes and interfollicular epidermis. The self-renewal and multi-lineage differentiation of skin stem cells make these cells attractive for ageing process studies but also for regenerative medicine, tissue repair, gene therapy and cell-based therapy with autologous adult stem cells not only in dermatology. In addition, they provide *in vitro* models to study epidermal lineage selection and its role in the ageing process.

P019

Prolactin has regulatory effects on keratins k6 and k19 expression in the human hair follicle

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Keratins serve crucial roles in normal skin and hair physiology, providing not only structural mechanical support, but also have important functions in regulating cell growth and hair follicle (HF) cycling. So far, (neuro-) endocrine controls of keratin expression have been sparsely investigated. The pituitary hormone prolactin (PRL) is a source for a wide range of bio regulatory effects in humans, including strong effects on hair growth. Recently, PRL and PRL receptors were found to be expressed inhuman skin and normal human scalp HFs. Here, we aimed to clarify whether intrafollicular-generated PRL signals also impact keratin expression. Using gene expression analysis of HFs we have identified a large subset of keratins and keratin-associated protein genes to be differentially regulated by PRL. To further explore PRL effects on human HF keratins by quantitative immunofluorescence we evaluated the effects of PRL and PRL receptor antagonists on expression of keratin6 (K6), as well as keratin 19 (K19). Treatment of organ-cultured human scalp HFs with high dose PRL (400 ng/ml) resulted in a significant down-regulation of K6 expression and up-regulation of K19 expression in the outer root sheath. This effect was blocked with the addition of PRL receptor antagonist to the culture medium. Addition of the PRL receptor antagonist alone to the culture medium led to a significant up-regulation of K6 expression in HFs cultured for 9 days. These changes in keratin expression were accompanied by hair shaft elongation in PRL-treated HFs, along with increased proliferation of hair bulb keratinocytes (Ki-67 staining). In this study we provide first evidence for the potential regulation of PRL on keratin expression in the HF. In addition, these results lend further support to the key role that PRL plays in human HF growth and function.

P020

Tropisetron modulates TGF-β-induced collagen synthesis in human dermal fibroblasts possibly via a 5-HT3 receptor-independent mechanism

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The serotoninergic system has been implicated in the pathogenesis of scleroderma and scleroderma-like skin changes for years. Tropisetron (TRO) is a serotonin (5-HT) receptor antagonist and known as an antiemetic. Interestingly, casuistical observations described an improvement of symptoms in patients with progressive systemic sclerosis after intake of TRO. Further, TRO treatment appears to have beneficial effects in rheumatic diseases such as rheumatoid arthritis and fibromyalgia. Of note, the serotoninergic system is expressed in human skin. The effects of 5-HT are mediated by at least 15 different 5-HT receptor subtypes whereas TRO elicits its antagonistic effects via binding to 5-HT3-receptors. Here we investigated the effect of TRO on transforming growth factor-β1 (TGF-β1)-induced collagen synthesis in human dermal fibroblasts (HDF) *in vitro*. We also investigated the impact of TRO on SMAD-signaling, a pathway involved in TGF-β1-mediatedcollagen synthesis. In addition, we analysed the expression of 5-HT receptor subtypes in HDF. TRO reduced TGF-β1-induced collagen mRNA expression and intracellular collagen protein amounts as shown by real-time RT-PCR and Western immunoblotting. In addition, TRO suppressed secretion of procollagen-I-C-peptide in a dose-dependent fashion as determined by ELISA. Regarding the SMAD pathway we further showed that TRO in contrast to TGF-β1 neither affects SMAD3 phosphorylation, nuclear translocation of SMAD2/3 nor SMAD3/4-dependent promoter activity. Surprisingly, expression of 5-HT3-receptors was undetectable although 5-HT1B, 5-HT2A/B and 5-HT7 receptors were found in HDF suggesting that TRO presumably acts in a 5-HT3 receptor-independent manner. In summary, our results highlight a potent *in vitro* suppressive activity of TRO on TGF-β1-inducedcollagen synthesis and shed light into the possible mechanism of TRO-mediated effects in HDF *in vitro*. These data are also encouraging for a detailed investigation of the 5-HT system in fibrotic skin diseases and furthermore may point to novel therapies in the treatment of patients with such disorders.

P021 (V15)

KdPT, a tripeptide derivate of alpha MSH, suppresses IL-1-induced cytokine expression, signaling and oxidative stress via a non-melanocortin-1 receptor-mediated mechanism

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Interleukin (IL)-1 and P. acnes are crucial players in the pathogenesis of acne by inducing proinflammatory cytokines which subsequently recruit inflammatory cells and lead to abscess formation of the pilosebaceous unit. Although sebocytes express melanocortin receptors (MC-Rs) and alpha-melanocyte-stimulating hormone(alpha-MSH) has potent immunomodulatory effects its usefulness as an anti-inflammatory therapeutic agent in acne is hampered due to its sebotrophic and melanotropic actions via binding to MC-Rs. Using the immortalized human sebocyte cell line SZ95 we examined the effects of KdPT, a stereo isomeric peptide derivative of the three last amino acids of the C-terminal domain of alpha-MSH with replacement of Thr for Val, on IL-1-mediated cytokine expression and signal transduction. KdPT potently suppressed IL-1beta-induced IL-6 and IL-8 mRNA expression and protein secretion. The peptide likewise inhibited cytokine induction by P. acnes but did not attenuate IL-6 and IL-8 expression induced by TNF-alpha or LPS. Mechanistically, KdPT reduced IL-1beta-mediated IkB alpha degradation, attenuated DNA binding of NF-kB and reduced intracellular reactive oxygen species which are known to activate the redox-sensitive transcription factor NF-kB. Importantly, KdPT did not bind toMC-1R as shown by blocking experiments of sebocytes with the MC-1R antagonist agouti signal protein as well as by competitive KdPT/NDP-alpha-MSH radioligand binding assays of sebocytes and MC-1R expressing B16.F1 melanoma cells. In accordance with the latter findings, KdPT lacked any pigment-inducing effect in both normal human melanocytes and B16.F1 melanoma cells. Taken together, these findings show potent anti-inflammatory *in vitro* effects of a new tripeptide, KdPT, and point towards novel future directions in the treatment of inflammatory skin diseases such as acne with this agent.

P022

Modulation of anti-oxidative enzymes and oxidative stress in human dermal fibroblasts–a novel cytoprotective facet of the neuropeptide alpha-MSH

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Alpha-melanocyte-stimulating hormone (α-MSH) is crucially involved in the cutaneous tanning response after ultraviolet (UV) light exposure. This is demonstrated by its direct melanotropic effect but also in individuals with distinct melanocortin-1 receptor (MC-1R) polymorphic alleles. Recently, we could demonstrate a direct cytoprotective effect of α-MSH due to inhibition of UVB-induced apoptosis and DNA damage. Here, we hypothesized that α-MSH and MC-1R may also protect from UVA-induced oxidative stress since photoageing is typically more prominent in the red hair fair skin phenotype, i.e. in individuals with non-functional MC-1R. As a first approach to test this hypothesis we analysed the *in vitro* effects of α-MSH in human dermal fibroblasts (HDF) on the expression and activity of key enzymes implicated in removal of UVA-induced reactive oxygen species (ROS).α-MSH was capable of potently upregulating mRNA and protein expression of superoxide dismutase 2 (SOD2) as well as of total SOD enzyme activity in HDF. Moreover, α-MSH increased total catalase activity which however was not associated with increased mRNA or protein expression and which occurred at a later time point compared with SOD upregulation by α-MSH. In silico promoter analysis confirmed the presence of both CRE and TRE consensus sites within the promoter region of SOD2 but not within the catalase gene suggesting a cAMP-PKA-CREB- andAP1-dependent mechanism of transcriptional activation of SOD2 by α-MSH. The significance of the upregulating effect of α-MSH on both SOD and catalase in HDF was subsequently investigated in HDF exposed to UVA. Pretreatment of HDF with α-MSH reduced UVA-induced intracellular accumulation of ROS and this effect was paralleled by reduced expression of matrix metalloproteinase-1, a central mediator of photoageing. Moreover, the effect of α-MSH in HDF appeared to be mediated byMC-1R as 3T3 fibroblasts stably expressing human wild-type MC-1R accumulated significantly less intracellular ROS than vector-alone transfected cells. These findings highlight a novel facet of α-MSH in cutaneous biology and may point towards novel future strategies in the prevention of photoageing.

P023 (V12)

Angiotensin AT2 receptor stimulation or AT1 receptor blockade act anti-inflammatory and anti-fibrotic in a mouse model of scleroderma

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Skin harbours a complete renin-angiotensin-system (RAS). There is evidence for an upregulation of the RAS in scleroderma lesions. Our project aimed at investigating whether pharmacological interference with the RAS (angiotensin AT1 receptor blockade or AT2 receptor simulation) may be effective in reducing inflammation and fibrosis in a scleroderma model in mice. Female C3/H mice were treated with bleomycin injections (100 μl of a 100 μg/ml solution s.c.) every second day over a period of 4 weeks. Animals were divided into the following treatment groups ($n = 6$ each; daily treatment): (i) untreated animals, (ii) bleomycin + vehicle, (iii) bleomycin + AT2R-agonist Compound 21 (C21) (0.3 mg/kg bw s.c.), (iv) bleomycin + AT1R-blocker Candesartan (0.1 mg/kg bw s.c.).Subsequently, tissue samples were collected and analysed for markers of inflammation and fibrosis by real-time RT-PCR, Western Blotting and conventional histological staining (HE). After 4 weeks of bleomycin injections, histological analysis showed an increase in extracellular matrix primarily within the subdermal layers. This fibrotic reaction was ameliorated by C21 and Candesartan treatment. Histological reduction of fibrosis as a result of C21 or Candesartan treatment coincided with a reduced expression of pre-collagen I α and TGFβ as estimated by western blot. Furthermore, bleomycin elicited an increase in IL-6 and MCP-1 mRNA expression, which could be significantly reduced by C21 and Candesartan. Our data indicate that pharmacological interference with the cutaneous RAS byAT1R-blockade or AT2R-stimulation are potential therapeutic approaches to reduce inflammation and fibrosis in scleroderma and potentially in other pathological settings with similar pathomechanisms.

P024

Glycyl–histidyl–lysine suppresses neutral lipid synthesis in sz95 sebocytes

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Intracellular cytoplasmic accumulation of neutral lipids is characteristic for sebocyte differentiation. Sebaceous lipid synthesis is downregulated by hydrocortisone (HC), retinoids and estrogens; however it is upregulated by fatty acids and androgens. The combination of Glycyl–histidyl–lysine (GHK), a tripeptide with a high affinity to Cu2+,with Cu2+ (GHK-Cu) is a potent chemoattractant for macrophages, mast cells and monocytes and is possibly related to wound healing processes in human skin. Inhuman plasma and wound areas, GHK is likely to exist as a mixture of GHK and GHK-Cu. GHK-Cu stimulates collagen synthesis and modulates wound healing properties directly or indirectly. SZ95 human sebocytes not only synthesize neutral lipids but also produce themselves inflammatory cytokines, such as IL-6 and IL-8. In this work the properties of GHK in influencing neutral lipid and cytokine synthesis inSZ95 sebocytes were investigated. 40,000 SZ95 sebocytes/well were seeded and cultured in 96-well plates. After 1 h pre-incubation with 10-4 M linoleic acid (LA), cells were treated with 10-4 M HC or 10-3 M GHK for 24 h at 37°C, 5% CO_2. Then cells were examined for intracellular polar and neutral cell lipids and cell vitality. Treatment with 10-3 M GHK significantly inhibited neutral lipids by 15% ($P < 0.01$), but not polar lipids (3%). GHK (10-4 M) did not exert measurable effects on either type of lipids. No lipid inhibition was measured without pre-stimulating with LA (SZ95 sebocyte lipids are 10–20% decreased by HC and 40% increased by LA). Cell viability and proliferation remained unchanged for all data sets. After 24 h treatment no cytotoxicity was detected. Additionally, an inhibition of cytokine IL-6 and IL-8 release in human SZ95 sebocytes was found. In conclusion, GHK selectively reduced the lipid content of SZ95 sebocytes stimulated to produce lipids, i.e. antagonized the fatty acid effect. In addition, it reduced the sebocyte inflammatory potential. The mechanism of these GHK effects is not identified, but its biological actions may be interesting for oily and inflammatory skin treatment.

P025

Parts of the bothrops moojeni snake venom activated lipid synthesis and ppar α, β, γ in transiently transfected sz95 sebocytes

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With progressing ageing human sebocytes reduce lipid production. However, the influence of certain aging mechanisms on sebaceous lipid synthesis as well as the ways to influence the latter are not fully identified. Certain lipids like fatty acids regulate neutral lipid stimulation and act as ligands of nuclear receptors such as PPAR. Arachidonic acid (AA) is identified as a natural PPAR ligand. Based on the knowledge gained from transient transfected HeLa cells a new transient transfection method for SZ95 sebocytes has been developed. SZ95 sebocytes were transiently transfected with pSG5 expression vectors containing PPARα, *β*, *γ*2. After transfection cells were treated with Botmo GF fractions 11-101, 11-117 and *Naja mossambica* sPLA2 as positive control. The enzymatically active Bothrops moojeniGF11-101 and 11–117 PLA2 (100 μg/ml) increased significantly neutral lipids synthesis up to 200% compared to untreated SZ95 sebocytes without inducing toxic or apoptotic effects. Botmo GF 11–101 is a non enzymatically active PLA2, which was not able to activate any PPAR isotype. Botmo GF 11-117 and Naja mossambicamossambica sPLA2 led to comparable results. However, both molecules induced asignificant PPARα activation in SZ95 sebocytes. In conclusion, our results suggested that enzymatically active PLA2 Botmo GF 11–117 is able to activate lipid synthesis inSZ95 sebocytes by utilizing or activating the AA metabolism. Enzymatically active sPLA2 IIA like Botmo GF 11–117 and Naja mossambicamossambica sPLA2 showed a significant PPARα activation in SZ95 sebocytes. These enzymatically active PLA2may directly activate the AA pathway and consequently PPARα. Lipid synthesis and PPAR activation of PLA2 in SZ95 sebocytes seem to be sPLA2 class-dependent.PLA2 biological actions may be interesting for investigations on lipogenesis, which may be valuable for the development of pharmaceutical agents against skin dryness and ageing.

P026

Human organ-cultured hair follicles from men with androgenetic alopecia express a CRH-mediated stress response that is modified by caffeine

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Human hair follicles show a local stress response that is equivalent to the hypothalamic-pituitary–adrenal (HPA) axis, and CRH, its key stress mediator, had been shown to inhibit human hair growth *in vitro* and therefore might be involved in stress-included deterioration of androgenetic alopecia *in vivo*. The aim of the study was to test, whether hair follicles from men with androgenetic alopecia (AGA) would be responsive to CRH-mediated stress and if this reaction would be counteracted by caffeine. Scalp skin biopsies were electively taken from the balding vertex area of men with AGA (Hamilton stage III–VI) after informed consent, and hair follicles were extracted and organ-cultured over 120 h in medium with or without CRH. Caffeine was added to one group of CRH-treated hair follicles to test for stress-protective effects. CRH (10-7 M) suppressed hair shaft elongation, induced catagen, inhibited matrix keratinocyte proliferation and induced apoptosis. Caffeine (0.001%) inhibited these effects and even stimulated growth over untreated (non-CRH-treated) control level. The catagen inductor TGF-2 was up-regulated by CRH and down-regulated by caffeine, while the effects on growth factor IGF-1 were vice versa. Further stress parameters within the HPA-axis such as cortico-releasing-hormone-receptor-1(CRH-R1), inositol-1,4,5-phosphate (IP-3), adreno-corticotropic hormone (ACTH) and its cognate receptor, melanocortin-receptor-2 (MC-R2) were shown to be responsive to CRH-mediated stress and were modified by addition of caffeine. Also, HPA-axis-independent stress-related receptors such as Trk A and p75NTR were involved in CRH-induced stress and modulated by caffeine. Thus, the human hair follicle specifically reacts to CRH-induced stress which is modified by caffeine in a highly differentiated manner. These observations may contribute to understand the clinical process of androgen-dependent miniaturization of genetically predisposed hair follicles in men with AGA, and using an energy promoting substances such as caffeine may represent an effective adjuvant therapeutic principle in AGA.

P027

Ligand-induced modulation of peroxisome proliferator-activated receptor (PPAR)- and vitamin D receptor (VDR)-signaling pathways in melanoma cell lines *in vitro*

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Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor superfamily of transcriptional regulators that regulate lipid, glucose, and amino acid metabolism. In recent studies it also has been shown that these receptors are implicated in tumor progression, cellular differentiation, and apoptosis and modulation of their function is therefore considered as a potential target for cancer prevention and treatment. Using real time PCR (light cycler), we characterized expression of VDR, PPARa, d and g in primary cultured normal melanocytes and in melanoma cell lines. We show that VDR is strongly expressed in melanoma cell lines and have characterized VDR polymorphisms. We show that PPARd is the strongest expressed PPAR in these cells. PPAR ligands and other agents influencing PPAR signalling pathways have been shown to reveal chemopreventive potential by mediating tumour suppressive activities in a variety of human cancers and use of these compounds may represent a potential novel strategy to prevent melanoma pathogenesis and to inhibit melanoma progression. In addition, transcription of PPARs has been shown to be directly regulated by 1,25(OH)2D3. We demonstrate antiproliferative effects of various PPAR-ligands and/or 1,25(OH)2D3 on melanoma cells. In conclusion, we here show interaction of VDR- and PPAR- signaling pathways and our data support the concept that VDR and PPARs may be of importance for growth regulation of melanoma cells, opening new perspectives for melanoma therapy.

P028

Modulation of the NOTCH–signaling pathway by 1,25-dihydroxyvitamin D3 in cultured human keratinocytes and sebocytes *in vitro*

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The NOTCH–signaling pathway has been shown to be of critical importance for the embryonic development and the growth of human skin cells, including keratinocytes and sebocytes. NOTCH signaling depends on the presence or absence of several specific receptor proteins and corresponding ligands. We have characterized the expression of key components of the NOTCH–signaling pathway (NOTCH receptors1; corresponding ligands delta, jagged 1,2) in cultured human keratinocytes (HaCaT,SCL-1) and sebocytes (SZ95) using real time PCR and western analysis. We found strong expression of NOTCH 1–3 and jagged 1, 2 on the RNA and the protein levels in all cell lines analyzed. *In vitro* treatment of SZ95 sebocytes with1,25-dihydroxyvitamin D3, the biologically active form of vitamin D, resulted at low concentration (10-10 M) in elevated RNA expression of jagged 1 and NOTCH 1.Interestingly, treatment with 1,25-dihydroxyvitamin D3 modulated expression of key components of the NOTCH signaling pathway differentially in spontaneously immortalized and non-malignant HaCaT keratinocytes as compared to the cutaneous squamous cell carcinoma cell line SCL-1. Treatment of HaCaT cells with1,25-dihydroxyvitamin D3 in high concentration (10-6 M) resulted *in vitro* both in inhibition of cell proliferation and in reduced RNA and protein expression of jagged 1.RNA expression of Notch 1 was inhibited as well, while protein content was only marginally affected. In SCL-1 cells, RNA expression of NOTCH 1 was slightly reduced after treatment with 1,25-dihydroxyvitamin D3 in high concentration (10-6 M), while protein content was only marginally altered. In conclusion, our results point at a cross talk between vitamin D–and NOTCH–signaling pathways while regulating the growth of keratinocytes and sebocytes. Our findings point at a differential response of non malignant and malignant keratinocytes to the biologic effects of vitamin D analogs that involve the NOTCH–signaling pathway. Moreover, we conclude that both vitamin D–analogs and pharmacologic modulation of NOTCH–signaling may open new therapeutic perspectives for the treatment of hyperproliferative skin diseases and sebaceous gland disorders

P029

Analysis of the NOTCH–signaling pathway in malignant melanoma

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The NOTCH–signaling pathway has been shown to be of critical importance for the embryonic development and the growth of human melanocytes. NOTCH signaling depends on the presence or absence of several specific receptor proteins and corresponding ligands. We have analyzed the immunohistochemical staining pattern of NOTCH receptors 1 and 2 in malignant melanoma, skin and lymphnode metastases of malignant melanoma and in benign acquired melanocytic nevi. Additionally, we investigated expression of NOTCH receptor 1, 2, 3 and 4 and their corresponding ligand jagged 1 and 2 in a vitamin D–sensitive human melanoma cell line (MeWo) using real time PCR and western analysis. We found a differential immunohistochemical staining pattern of NOTCH receptors 1 and 2 in tissues analyzed and strong expression of Notch 1–3 and jagged 1,2 on the RNA and protein levels in MeWo melanoma cells. Interestingly, treatment of melanoma cells with1,25-dihydroxyvitamin D3 (10-10 M–10-6 M), the biologically active form of vitamin D, resulted *in vitro* both in a dose-dependent inhibition of cell proliferation and in reduced RNA and protein expression of NOTCH receptor 1 (10-6 M). Treatment of MeWo cells with 1,25-dihydroxyvitamin D3 at low concentration (10-10 M) resulted *in vitro* in an increased expression of jagged 1. In conclusion, our results point at across talk between vitamin D–and NOTCH–signaling pathways while regulating the growth of melanoma cells. Moreover, we conclude that both vitamin D–analogs and pharmacologic modulation of NOTCH–signaling may open new therapeutic perspectives for the treatment of malignant melanoma.

P030

Thyrotropin releasing hormone (trh) and thyroid stimulating hormone (tsh) regulate the expression of keratins k5 or k6 in human hair follicle and epidermis

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The skin and hair are prominently affected by a range of thyroid disorders, and thyroid hormone (TH) is known to have profound effects on keratinocytes. Nevertheless, the role of other members of the hypothalamic-pituitary-thyroid axis: i.e. thyrotropin releasing hormone (TRH) and thyroid stimulating hormone (TSH), inhuman skin and hair biology, is still largely unknown. We have recently shown that human hair follicles (HFs) express functional receptors for both TRH and TSH. Keratins were previously reported to be affected by TH (e.g. TH upregulates K6). By microarray analysis we detected that a defined subset of keratins and keratin-associated protein genes is differentially regulated by TSH and/or TRH. Consequently, we have further characterized the effects of TRH and TSH on keratin expression in situ in human epidermis and HF. We demonstrated by quantitative immunohistomorphometry that treatment of human scalp skin or HFs organ culture with TRH (1–100 ng/ml for 6 days) significantly downregulated K6 immunoreactivity in suprabasal layer keratinocytes of both the epidermis and outer root sheath (ORS).TSH treatment (10 or 100 mU/ml for 5 days), instead, upregulated K5 expression by basal layer epidermal keratinocytes and hair matrix keratinocytes in situ, but did not change K5 expression in the ORS. This study was complemented by qPCR of isolated ORS keratinocytes. Hence, we provide here the first evidence that TSH and TRH regulate keratin expression in human skin, on the gene and protein level.

P031 (V06)

A new role for Substance P: induction of regulatory T-cells during stress-adaptation

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Stress is accepted as a potent immunomodulator and aggravator of allergic inflammation mainly through neuropeptides-dependent neurogenic inflammation. However, more and more data accumulates that indicates a protective, anti-inflammatory role of certain stress paradigms, the mechanisms of which have not yet been fully elucidated. In a combined murine model of allergic dermatitis (AD) and perceived stress (noise) we repeatedly exposed AD mice to stress prior to sensitisation to ovalbumine. We found, that under these conditions stress markedly increased dendritic cell (DC) migration and maturation in a substance P (SP) but not calcitonine gene related peptide (CGRP) dependent manner. Moreover, the effects of stress were selectively abolished when animals were treated with SP-receptor NK1antagonist. Interestingly, in stressed AD mice, the number of regulatory CD4+CD25+T cells (Tregs) was increased, while other T cell subsets (CD8+ cytotoxic T-cells, CD4+CD25- T cells) were decreased. When dendritic cells from stressed AD mice were co-cultured with T lymphocytes we measured higher medium concentrations of TH1 (IFNγ and TNFz) versus TH2 (IL-4 and IL-5) cytokines, as well as higher levels ofIL-2 indicating promotion of 'anti-allergic' TH1 and T regulatory phenotypes. Concordantly with the stress and SP dependent alterations in DC-T cell interaction disease severity (eosinophilic infiltrates, epidermal thickening) was significantly reduced and the number of FoxP3+ T-regulatory in skin increased. This was abrogated in mice treated with NK1 antagonist. Taken together, our data show a new role for the sensory neuropeptides SP during stress-induced habituation to allergen and provide new leads for the development of successful therapeutic strategies in the management of allergic disease.

P032

Do neurotrophins cooperate with cytokines in epithelial and melanocyte growth control?

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In analogy to their role in the nervous system, neurotrophins in skin are potent regulators of epithelial growth and pigmentation. Lately, evidence accumulates, that shows close interaction between neurotrophins and cytokines in neuronal regeneration. Here we investigate if a similar co-operation exists in epithelial and melanocyte growth control. To this end we cultivated fully pigmented human scalp skin anagen hair follicle in medium substituted with insulin, glutamate, hydrocortisone and antibiotics and added NGF and TNF-α either alone or in combination at concentrations of 0.25 ng/ml each. Hair follicle growth, pigmentation and cycle stage were evaluated. After seven days of culture, follicles treated with either TNF-α or NGF/TNF-α  showed significant growth retardation as compared to controls, while pigmentary status or hair cylce stage did not differ between test and control. However, immunohistochemistry revealed an increase in total bulbar melanocyte number in the group treated with NGF/TNF-α. In addition we observed sporadic apoptosis (TUNEL-labelling) and proliferation (Ki67) of melanocytes in these hair follicles. These preliminary observations suggest a new role for neurotrophins in concert with cytokines. While growth of epithelial cells of the hair follicle is inhibited the hair follicle melanocyte population may be protected. Further experiments are required to confirm this hypothesis.

P033

Is substance P involved in stress-induced premature graying?

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The accumulation of oxidative stress induces apoptosis of hair follicle melanocytes in the pigmentary-unit during senile hair graying in a process analogue to the mitochondrial theory of aging. Thus, disturbance of well-balanced melanocyte stress-regulatory mechanisms by oxidative stress caused e.g. by cutaneous inflammation or emotional stress may lead to untimely death of melanocytes and premature graying. We exposed C57BL/6 mice to 24 h of noise-stress or a bolus injection of the neuropeptide stress-mediator Substance P (SP) during the early growth phase (anagen) of the depilation-induced hair cycle. Back skin was harvested72 h later and melanocyte markers such as tyrosinase related peptide (TRP) 1, TRP 2 and c-kit as well as TUNEL-labelling were assessed. Exposure to stress or SP resulted in an increase of c-kit positive melanocytes in the stem cell harbouring bulge region while SP treatment also led to melanocyte-apoptosis in the developing pigmentary-unit of early anagen hair follicles. These findings suggest a differentiated susceptibility of distinct melanocyte populations in the hair follicle to stressors. On the one hand pigment-producing melanocytes of the pigmentary-unit show increased vulnerability towards stress-mediators, with subsequent premature death during early anagen. On the other hand stress-mediators have a stimulatory effect on the melanocytes in the stem cell containing hair follicle bulge region. Together this suggests an increased cell turn-over under stress. Preliminary data from murine telogen full skin microarray analysis also head for similar two-sided results. Stressed skin shows up-regulated genes for cell differentiation, proliferation and immune responses while it shows down-regulated genes for intracellular signalling cascades and pigmentation. Our results suggest a precocious exhaustion of the melanocyte stem cell pool due to an enhanced turnover of pigment cell precursors as a pathway involved in premature canities.

P034

Stress sensitive differentiation in expression of nerve fibers and antigen presenting cell and Substance P sensitive alteration of dendritic cell subpopulations in spleen

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Substance P (SP)–a sensory neuropeptide–was revealed as an important stress mediator with its own stress axis in the skin. In this context, we were able to show, that stress-dependent SP affects the migration of dendritic cell subpopulations to skin draining lymphnodes with subsequently altered T-cell activation and disease activity in a mouse model for allergic dermatitis (AD). Here we postulate that stress-dependent communication between nerve fibres and immune-competent cells with effect on the course of inflammatory skin diseases can also occur in spleen. To address this question, AD was induced in C57BL/6 mice by double sensitization (i.p) and an intradermal challenge using chicken egg ovalbumin. Animals were additionally exposed to sound stress for 24 h prior to challenge. In this model, stress lead to a relative hyperinnervation of the immune-competent areas of the spleen. At the same time, an increased number of antigen-presenting cells (APC) could be observed in these areas and contacts between nerve fibres and APC were found. Substance P *in vitro* had the capacity to raise the number of antigen presenting cells in spleen and increased the number of CD4+ T-cell-stimulating CD4-CD8- dendritic cells and further the number of CD25+ T-regulatory cells. *In vivo* we found stress depend end shift of cytokine mRNA levels towards a TH-1 cytokine profile. Under same conditions we were able to show increasing NK1-receptor mRNA amounts and basely PPT1 mRNA levels. Further analysis of quality and function of neuro-immune interactions in the spleen will reveal the role of the observed stress-induced alterations in the spleen in atopic disease.

P035

Alpha-MSH: a protective endogenous hormone in chemotherapy-induced hair follicle damage?

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Effective, safe, and well-tolerated therapeutic and/or preventive regimen against chemotherapy-induced hair loss still remain to be developed. Recently, we have established the first human *in vitro* model for chemotherapy-induced hair follicle (HF) dystrophy, which employs a key cyclophosphamide metabolite(4-hydroperoxycyclophosphamide, 4-HC). Using this model we asked whether exogenous alpha-melanocyte-stimulating hormone (alpha-MSH), which we had also shown to be endogenously produced within human scalp HFs and to exert cytoprotective effects against ultraviolet-induced apoptosis and DNA damage, may protect against 4-HC induced HF damage. HFs were microdissected, pretreated with alpha-MSH (10-7 M) and then exposed to 4-HC in the continued presence of alpha-MSH. We compared hair follicle elongation, proliferation and apoptosis of matrix keratinocytes and melanin clumping (as a sensitive indicator of HF dystrophy) between alpha-MSH-treated and vehicle-treated HFs from three different female patients. We observed that alpha-MSH significantly reduced melanin clumping in 50% of the patients. Also, 4-HC-induced premature HF regression (catagen) and excessive hair matrix apoptosis were decreased. In order to shed light into the possible mechanisms of alpha-MSH-mediated protection, expression of the transcription factors Nrf1/2 and its dependent anti-oxidative enzymes (GCS, GSTpi, HO-1) was studied. Among these factors, heme oxygenase-1 (HO-1) was most robustly induced by 4-HC, but also by alpha-MSH alone, while joint application of4-HC and alpha-MSH had an additive stimulatory effect on the expression of this well-known 'guardian of tissue damage'. In conclusion, our currently available datapoint towards a novel cyto- and tissue-protective dimension of alpha-MSH in skin biology: protection against chemotherapy-induced HF damage. Given that alpha-MSH is considered a relatively toxicologically safe and well-tolerated, this has encourages one to explore this intrafollicularly produced neuropeptide hormone as a protectant in chemotherapy-induced alopecia.

P036

Functional regulation of primary keratinocytes by somatostatin

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Somatostatin (SST) is a peptide hormone that inhibits secretion of several hormones and has antiproliferative effects. It acts as a regulator in the central nervous system as well as peripheral tissues and has been shown to be present in the skin. SST mediates its physiological functions through five receptor subtypes (SSTR1-5), which belong to the family of seven transmembrane domain G protein-coupled receptors (GPCR). All five SSTR subtypes are expressed in the stratum granulosum of the human epidermis. Epidermal hormone receptors are known to control the physiological function of keratinocytes, therefore we investigated the regulation of keratinocytes by somatostatin. Stimulation of primary keratinocytes with SST leads to a decrease of cell proliferation and cell migration, whereas transepithelial resistance of differentiated keratinocytes increases significantly. The activation of endogenous SST receptors was confirmed by measurements of the second messenger cyclic AMP, as SSTRs downregulate cAMP signalling through inhibitory G proteins. Migrating keratinocytes show altered cytoskeleton dynamics with delayed lamellipodia formation after SST stimulation. Our data show that somatostatin receptors can regulate diverse aspects of keratinocyte function.

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P037

The cAMP pathway in fibroblasts is a potent but differential and promoter-specific regulator of various TGF-β mediated effects involved in ECM homeostasis

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cAMP is a key messenger of a variety of hormones and neuropeptides some of which are able to modulate the composition of extracellular matrix (ECM). Using various approaches and the well established impact of transforming growth factor-β (TGF-β)we investigated the effects of cAMP on key functions of fibroblasts including collagen synthesis, proteoglycan expression, collagen contraction and closure of mechanically induced cell layer wounds. Treatment of human dermal fibroblasts with for skolin, an artificial cAMP inducer, induced CREB phosphorylation at serine 133 and induced CREB-dependent promoter activity. db-cAMP and for skolin strongly antagonized the inductive effects of TGF- β on the expression of collagen, connective tissue growth factor, tissue inhibitor of matrix metalloproteinase-1 and plasminogen activator inhibitor type I, four prototypical TGF- β responsive genes. Increased intracellular cAMP prevented TGF- β-induced SMAD-specific gene transactivation, while-TGF- β-mediated SMAD phosphorylation and nuclear translocation remained unaffected. The relevance of these TGF-β antagonistic effects of cAMP was extended by two functional *in vitro* assays. Increased intracellular cAMP levels suppressed the inductive activity of TGF-β to contract mechanically unloaded collagen lattices. In addition, treatment of cells with db-cAMP resulted in an attenuation of fibroblast migration of mechanically induced cell layer wounds. However, cAMP antagonized by no means all TGF-β-mediated effects as shown by synergistic effects of increased cAMP levels on hyaluronan synthase 2 expression and hyaluronan secretion. The latter effect is presumably mediated by putative CREB binding sites adjacent to SMAD binding sites within the hyaluronan synthase 2promoter. Our findings identify the cAMP pathway as a potent but differential and promoter-specific regulator of extracellular matrix components and other key fibroblast effector functions.

P038

Atypical manifestations of tinea corporis–importance of histology

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Objectives: We report four cases of atypical tinea corporis, where the initial clinical diagnosis was different from dermatophytosis. The differential diagnoses and the diagnostic difficulties related to atypical manifestations of fungal infections are discussed. Our cases emphasize the importance of conventional histological examination, which enables a fast correct diagnosis.

Methods: The following diagnostic tools were applied: Native preparation, macroscopic and microscopic examination of the culture and histological examination of the skin.

Results: The diagnosed fungi were *Microsporum canis*, *Trichophyton rubrum* and *Trichophyton interdigitale*. In one case, the fungal culture was negative in spite of positive native preparation and detection of PAS positive hyphens in histology.

Conclusions: Even though all four shown cases of tinea corporis presented clinically quite different, histological examination revealed stereotypical findings:

The epidermis was characterized by acanthosis, spongiosis. Neutrophils were found within a parakeratotic stratum corneum. The dermis revealed infiltrates with lymphocytes and partially neutrophils or eosinophils. Hyphae and spores could be identified in the stratum corneum already in H&E stain, although a PAS staining facilitated the detection of fungi. Nevertheless, there were some histological differences between the cases. Patient 1and 2 displayed more extensive spongiosis and/or subepidermal edema as well as amore impressive dermal infiltrate. Such presentation is a good hint for an infection with zoophilic dermatophytes. Antropophilic fungi in most cases cause a much lessintense inflammatory reaction, therewith accounting for a histologically so called 'invisible dermatosis'. A special feature of patient 4 is the dense subcornealaccumulation of neutrophils mimicking histologically a pustular psoriasis or IgA pemphigus.

P039 (V31)

Interleukin 17 in atopic eczema: linking allergen-specific adaptive and microbial-triggered innate immune response

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The pathogenesis of atopic eczema (AE) is based on complex interactions between genetical predispositions and environmental factors. Th17 cells are involved in antimicrobial first line defence that is imbalanced in AE patients. To investigate a possible role of IL-17 in AE, T cells were isolated from atopy patch test (APT) reactions to Dermatophagoides pteronyssinus and characterized for cytokine profile and response to microenvironmental stimuli. Interactions with keratinocytes and antigen presenting cells were investigated. We found that nearly ten percent of lymphocytes infiltrating APT reactions releasedIL-17 upon PMA/ionomycine activation. Among these were the known subpopulations Th17 and IFN-γ coproducing Th1/IL-17 cells, and the newly characterized subtype IL-4/IL-17 producing T cells (Th2/IL-17). Interestingly, T cell clones triggered by cognate antigen secreted Th2 and Th1 cytokines, but not IL-17.Additional exposure to *Staphylococcus aureus*-derived SEB, but not to IL-1β, IL-6 and IL-23,markedly enhanced IL-17, but not IL-4 release. In turn, SEB-induced IL-17 promoted β-defensin-2 expression in AE keratinocytes. This effect was partially inhibited by IL-4and IL-13. *In vivo*, sequential application of SEB at APT sites increased IL-17 and β-defensin-2, but not IFN-γ and IL-4 expression. In summary, our data demonstrate that full expression of IL-17 by Der p 1-specific-lymphocytes is tightly regulated and requires additional tissue-derived stimuli, such as microbial danger signals. The IL-17/HBD-2 axis links adaptive and innate immunity. This mechanism is partially impaired by Th2 cytokines, leading to persistence of microbial pro-inflammatory products in AE.

P040

Role of Protease-activated Receptors in bleomycin-induced skin fibrosis

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Aims: The aim of this study was to evaluate the role of protease-activated receptors (PARs) in murine skin fibrosis induced by bleomycin.

Background: PARs are G protein-coupled receptors involved in a variety of processes such as body homeostasis and thrombosis as well as in inflammatory and proliferative responses triggered by tissue injury.

Methods: In order to induce skin fibrosis, bleomycin was injected subcutaneously every other day into the shaved back skin of wild type, PAR1- and PAR2-deficient mice for five weeks. Bleomycin is a frequently used anti-tumour antibiotic for various kinds of cancers which was originally isolated from the fungus *Streptomyces verticillus*. Lung fibrosis is a well-known side effect of bleomycin. To investigate the effect of PAR1- and PAR2-deficiency in the development of skin fibrosis, we determined the levels of fibrotic features such as extracelullar matrix accumulation and degradation, bundle thickness of collagen fibres, amount of myofibroblasts as well as the number of inflammatory infiltrates in wild type and PAR-deficient mice.

Results: In wild-type mice of both genders a significant increase of dermal thickness, high amounts of collagen accumulation and thickening of vessel walls was observed as compared to controls. Furthermore, the sclerotic changes in wild-type mice were characterized by a significant loss of hair follicles. In contrast, PAR1-and PAR2-deficient mice responded in a less severe manner in this scleroderma model. Of note, PAR1-deficient mice differed from PAR2-deficient ones in several aspects: the number of inflammatory cells such as macrophages was higher inPAR2-deficient mice whereas PAR1-deficient mice showed higher amounts of alpha smooth muscle actin, a classical marker protein of myofibroblasts. Furthermore, MMP9 expression was elevated in wild-type and PAR2-deficient mice, whereas-PAR1-knockout mice lacked MMP9 expression.

Conclusion: Our data clearly indicate that PAR-deficient mice are protected against skin fibrosis. These results suggest that PAR1 and PAR2 and their ligands exert pro-fibrotic effects in murine skin. Thus, targeting PAR1 and PAR2 signaling in skin fibrosis may be a novel therapeutic approach in scleroderma.

P041

Kindlins: novel proteins involved in epidermal adhesion and skin ageing

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Kindlins (also known as fermitin family homologues) are a novel family of cellular adaptor proteins in integrin-containing focal adhesions. Kindlin-1 and -2 are expressed in the skin and seem important for epidermal adhesion and dermal-epidermal communication, but their expression patterns, regulation or functions remain poorly understood. Most information has been derived through studies on Kindler syndrome (KS), a rare autosomal recessive disorder caused by loss of function of kindlin-1. KS manifests with an evolving phenotype: initial skin blistering is followed by photosensitivity, early progressive poikiloderma, atrophy and skin cancer, features also seen in aged skin. Here we have combined genetic, recombinant expression, iRNA and antibody studies to illustrate differential expression and functions of Kindlin-1 and -2 in the skin. Kindlin-1 is an epidermal-specific protein facing the basal surface in basal keratinocytes. In contrast, kindlin-2 is expressed at high levels in all major skin cell types keratinocytes, fibroblasts and melanocytes. In the epidermis, it appears at the entire cell periphery of basal keratinocytes, partially co-localizing with e-cadherin and alfa6 integrin, but not with laminin 332. In keratinocytes, both kindlins are phosphorylated by anEGF-mediated process indicating functions signal pathways controlling integrin-mediated cell-extracellular matrix adhesion and spreading. The biological phenotype in KS suggests a pivotal role for the kindlins in epithelial-mesenchymal communication. This may involve secondary mediators, as suggested by the fact that *in vitro*, PDGF and FGFb, but not TGF-beta, suppress kindlin-2 expression in fibroblasts significantly within 24 h. Functionally the kindlins do not seem to compensate for each other, since in KS skin kindlin-2 is not upregulated or redistributed. Taken together, here we have generated the molecular tools to demonstrate differential expression and functions of kindlin-1 and -2 in the skin and lay a basis for further studies on the physiological and pathogenetic role of kindlins, not only in KS but also in common conditions, such as skin ageing.

P042

T cells in psoriasis lesions lack ICOS expression

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ICOS is the third member of the CD28 superfamily and the ICOS-B7RP-1 pathway is important for T cell co stimulation. The ICOS receptor is expressed on activated T cells, e.g. on T cells in allergic airway disease. Its ligand, B7RP-1, is found on antigen presenting cells. Although previously implicated in Th2 stimulation, it has now been shown that ICOS stimulates both Th1 and Th2 cytokine production. Therefore, we asked the question whether ICOS is involved in T cell activation in psoriatic skin. We investigated five skin samples each from normal donors (skin obtained by breast reduction surgery), psoriasis lesions as well as non-lesional skin from psoriasis patients by both conventional light microscopy and dual-color confocal fluorescence microscopy. Using conventional immunostaining for the ICOS receptor we found no expression of the receptor in normal skin, in contrast to psoriasis lesions. To further characterize the cells that express ICOS in psoriasis skin, we performed double immunofluorescence staining against ICOS receptor and CD2+ as marker for T cells. We found that T cells were almost always negative for double staining. The same holds for mast cells, identified by tryptase staining. However, ICOS positive cells were also positive for KiM1P staining, indicating that these cells may be macrophages. These data show that T cells in psoriasis are negative for ICOS receptor staining. The results are in good agreement with a newly described role of ICOS as an important activator of regulatory T cells expressing IL-10, with an important functional involvement in self tolerance.

P043

Activated innate immune response pathways in cutaneous lupus erythematosus

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Background: Chronic discoid lupus erythematosus (CDLE) is an autoimmune skin disorder that is characterized by a cytotoxic immune response invading the basal epidermal layer accompanied by hydropic degeneration and apoptosis of keratinocytes ('interface dermatitis'). Recent studies provided evidence for an important pathogenic role of the type I interferon system in this skin disease, but the detailed components of the proinflammatory network remained undiscovered.

Methods: Laser microdissection of cryofixed skin biopsies taken from six CDLE patients with active disease was performed. Epidermal, junctional and dermal cells were extracted. mRNA was isolated, amplified and used for microarray gene expression analyses. Immunohistochemistry was performed to confirm the results on the protein level. In situ hybridization for IFNα1 and IFNβ was established to identify lesional IFN producing cells in the skin. *In vitro* stimulation of keratinocytes was done to demonstrate their capacity to produce type I IFNs.

Results: Our analyses demonstrated a strong activation of innate immune response pathways (TLR, JAK/STAT, MAP-kinase, NFkB) accompanied by a lesional cytokine-storm (e.g. CCL5, CCL20, CCL22, CXCL9, CXCL10, CXCL11, IL3, IL7,IL12, IL18), recruitment of cytotoxic immune cells (granzyme B, perforin, Tia1,NKG2D) and induction of keratinocytic apoptosis. In the skin of CDLE patients we identified an 'interferon-signature' (e.g. IFIT1, IFITM2/3, AIM2, IRF7, STAT1) that closely resembles the expression pattern described in the blood of patients with active systemic disease (SLE). Additionally we were able to show that keratinocytes are producers of type I IFN in cutaneous LE skin lesions.

Conclusion: Our results demonstrate that inappropriately activated innate immune pathways are involved in the pathogenesis of CDLE skin lesions. We assume that these mechanisms drive a cytotoxic cellular immune response, which is responsible for tissue destruction and the scarring character of this disease.

P044

Novel genes associated with malignant melanoma

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Purpose: Malignant melanoma is an aggressive cancer with increasing incidence. The process of malignant transformation, progression and metastasis mechanisms are still poorly understood. To gain new insights into the involved genes we conducted gene expression profiling of frozen tissues of 18 melanocytic nevi (MN), 20 primary melanomas (PMM) and 20 metastatic melanomas (MMM).

Methods: Gene expression patterns were analyzed using Affymetrix Human GenomeU133A 2.0 genechip arrays. Statistical analysis was performed with Genomatix Chip inspector, bioconductor package MCR estimate, ingenuity software and Genomatix Bibliosphere. Expression levels of selected gene products were verified on RNA level by RT-PCR and Taq Man PCR, as well as immunohistochemically on tissue microarrays with more than 300 unrelated MN/MM/MMM cases with known clinical outcome.

Results: A total of 285 differentially expressed genes was detected in PMM compared to MN, 191 genes in PMM compared to MMM and 586 genes in MN compared to MMM, respectively. By means of repeated cross validation we were able to classify all samples correctly according to their genetic profiles. Novel potentially important genes were further validated. For some of those a crucial function in MM progress can be envisioned.

Conclusion: We were able to verify decreased expression levels of FRZB, an antagonist of Wnt induced cytosolic accumulation of beta-catenin and of TLE1, which was shown earlier to function as a transcriptional repressor in Wnt signalling. Furthermore we could show for the first time high expression levels of two genes, which belong to the serine proteinase inhibitor family, Serpin B3 and B4, in PMM compared to MN. Also for the first time we could show high expression levels of GDF 15, a downstream target of p53, in a large scale of primary melanoma tissue sections.

P045

Restricted ige recognition of bullous pemphigoid (bp) 180 and bp230 in patients with bp and elderly individuals with pruritic dermatoses

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Background: BP is the most common autoimmune blistering disease in the elderly characterized by IgG autoantibodies against hemidesmosomal proteins, namely BP 180 and BP230. Classical BP is characterized by tense bullae associated with itching which maybe preceded by urticarial or eczematous skin lesions. Recently, elderly individuals with pruritic, polymorphic dermatoses were found to carry IgG against BP230 and, less frequently, BP180. The pathogenetic role of IgE against the BP autoantigens is a matter of debate in light of the frequently observed blood eosinophilia and eosinophilic spongiosis of lesional skin. The injection of IgE from BP sera into mice has been shown to reproduce clinical and histological features of BP.

Objective: The purpose of this study was to characterize the epitope specificity of IgE againstBP180 and BP230 in classical BP and in elderly patients with pruritic disorders. Utilizing the two major subdomains of BP230 and six major epitopes of the BP180ectodomain, we analyzed IgE autoantibodies in 30 BP sera and sera from 15 patients with pruritic dermatoses and 25 patients with immediate type allergic reactions.

Results: We identified IgE against the COOH-terminus of BP230 in 10/17 BP sera and theNH2-terminus of BP180 in 9/22 BP sera. In contrast, only 2/17 BP sera showed IgE reactivity against the COOH-terminus of BP180 and 2/17 against the NH2-terminusof BP230. Noteworthy, two of four sera from patients with pruritic disorders also showed IgE reactivity against BP180-NC16a.

Conclusion: The findings of the present study suggest that - in contrast to IgG reactivity–IgE selectively targets epitopes in the COOH-terminus of BP230 and the NH2-terminus ofBP180. IgE recognition of the BP autoantigens is presumably an early pathogeneticevent since some elderly patients with pruritic dermatoses ('pre-pemphigoid') have already IgE against the NH2-terminus of BP180. Thus, IgE may be a relevant effector molecule in the pathogenesis of BP.

P046

Therapeutic B cell depletion induces strong elevation of BAFF and mediates opposed effects on autoreactive and pathogen-specific serum IgG in pemphigus vulgaris

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Pemphigus vulgaris represents a severe autoimmune bullous skin disorder which is primarily associated with autoantibodies against desmoglein3 (dsg3). Although PV is considered a B cell mediated autoimmune disease, over expression of crucial factors for growth and survival of B cells, i.e. BAFF (B cell activating factor) or APRIL (aproliferation inducing ligand) has not been described, yet. In the present study, we investigated the effect of immunosuppressive treatment alone (five patients) or in combination with immunoadsorption (IA) (six patients) and rituximab (11 patients), respectively, on serum BAFF and APRIL levels. Circulating titers of dsg3-specific autoantibodies, Varizella–Zoster virus (VZV)- and Epstein–Barr virus (EBV)-IgG, respectively, were determined by ELISA. Immunosuppressive drugs alone and adjuvant IA, respectively, did not show any effects on serum BAFF and APRIL levels in PV patients compared to healthy controls. In contrast, rituximab led to strong and significant elevation of BAFF, but not of APRIL, in serum of PV patients. BAFF elevation showed a strong inverse correlation to peripheral B cell counts, since on recovery of peripheral CD19+ B cells serum concentrations of BAFF normalized to pre-treatment levels.

Moreover, rituximab induced a significant decrease of dsg3-specific circulating autoantibodies, which was accompanied by clinical remission. In contrast, titers of anti-VZV- and anti-EBV-IgG were significantly increased for up to 6 months after rituximab therapy, whereas immunosuppression and adjuvant IA, respectively, did not alter VZV- and EBV-reactive IgG serum levels. Thus, rituximab probably exerts a differential effect on autoreactive and pathogen-specific plasma cells, respectively, possibly a result of distinct CD20expression on these two cell populations. Finally, our results suggest that combining rituximab treatment with a BAFF antagonist could prolong the period of peripheral B cell depletion, due to reduced de-novo generation of B lymphocytes. This novel therapeutic strategy may therefore improve the clinical response in otherwise refractory PV patients.

P047

Genetic modifiers other than filaggrin mutations in X-linked ichthyosis

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Ichthyosis vulgaris (IV) and X-linked ichthyosis (XLI) are the most prevalent skin disorders of cornification and share a similar phenotype of generalized fine scaling. IV is due to loss-of-function mutations in the filaggrin (FLG) gene on 1q21 and XLI is caused by deletions/mutations in the steroid sulfatase (STS) gene on Xp22.32.Concurrent mutations in both gene loci have been associated with a more severe scaling phenotype. Here, we report a pedigree with family members harbouring the same concurrent mutations in the FLG and STS genes who exhibit a variable scaling phenotype despite comparable environmental surroundings. Thus, phenotypic variation in these individuals is not sufficiently explained by interaction between XLI and FLG mutations. Instead, we propose that additional genetic modifiers, possibly other genes within the epidermal differentiation complex, may play a role in dictating phenotypic severity in XLI. Sequencing of the potential candidate gene loricrin did not reveal any mutations. Future analyses of other candidate genes are needed to solve this mystery.

P048 (V24)

Conditional collagen VII inactivation reveals high anchoring fibril stability *in vivo*: implications for molecular therapies

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Recessive dystrophic epidermolysis bullosa (RDEB) is in the prime focus of therapy development for genetic skin diseases. Caused by mutations in the COL7A1 gene, absence of collagen VII as the main component of anchoring fibrils leads to a stigmatizing and cancer prone condition with extensive skin blistering, mucosal involvement, soft tissue scarring, alopecia, nail dystrophy and development of mittende formities of hands and feet. To analyse disease mechanisms and develop biologically valid therapies we generated several transgenic models for RDEB. A collagen VII hypomorph with only 10% residual protein led to discovery of continuous inflammation and contractile fibrosis as a cause for development of mitten deformities and, possibly, prerequisite for epithelial carcinogenesis. The skin fragility was strongly reduced after intradermal injection of fibroblasts, demonstrating the feasibility of cell therapy for RDEB. Major questions in therapy development concern the quantity of collagen VII required for a therapeutic effect and the stability of the protein *in vivo*. To address these questions we designed a Col7a1 allele for inactivation by a tamoxifen-inducible Cre-recombinase. Ubiquitous gene inactivation in 14 days old Col7a1 fl/fl mice lead to strong reduction of collagen VII levels and to microblistering in skin and lingual mucosa within 5 weeks. In contrast, keratinocyte-restricted Col7a1 inactivation using a K14 promoter-driven Cre-recombinase caused a clearly slower loss of anchoring fibril function: microblisters occurred after 10-12 weeks. Interestingly, the amount of collagen VII required for epidermal-dermal adhesion varies between localizations. In the tongue, reduction of collagen VII to about half of normal levels produced microblisters, but the back skin was more stable and a reduction of 65–75% was required for blister formation, suggesting a stabilizing effect of the greater attachment surface of the hair follicles. These observations demonstrate that in the skin both keratinocytes and fibroblasts are significant sources of the anchoring fibrils. Moreover, collagen VII has a low turnover rate *in vivo*, thus providing an important basis for design of molecular and cellular therapy protocols for RDEB.

P049

Treating immune diseases with gene reprogramming

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Psoriasis is a cutaneous disorder characterized by inflammation and abnormal epidermal proliferation with a prevalence of 2–3% in the caucasian population. Like other immune diseases, psoriasis is caused by genetic and environmental factors. A genetic predisposition is also known. Typical attributes are keratinocyte hyperproliferation, epidermal influx of polymorphonuclear leukocytes and an infiltration of the papillary dermis and the epidermis with mononuclear leucocytes. T-cells and cytokines play an essential role in the pathogenesis of this chronic skin disease. A relative deficiency of IL-10 in psoriasis skin can be observed by contrast of an overexpression of numerous proinflammatory cytokines as IL-2, IL-6, IL-8 and IFN-γ. Our strategy for the therapy of psoriasis bases on gene reprogramming by 3'trans-splicing. By reprogramming ICAM-1 mRNA, upregulated in psoriasis, via trans-splicing to IL-10 mRNA we intend to downregulate ICAM-1 and increase the expression level of IL-10. A rapid, FACS (fluorescent activated cell sorting) based high-throughput screen was used to identify and select the most efficient and specific reprogramming molecules. Out of a binding domain library we identified molecules with over 65% trans-splicing efficiency in our test system. These potential constructs were modified to deliver theIL-10 coding sequence in ICAM-1 (over)expressing endothelial cells. We tested these RTMs in human dermal microvascular endothelial cells (HDMEC) and performed real-time PCR analysis. The generated IL-10 was quantified and tested for functionality by ELISA. This approach opens a novel way to treat psoriasis.

P050

Gene therapy for autosomal dominant diseases

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Mutations in the KRT14 gene underly different types of the blistering skin disease epidermolysis bullosa simplex (EBS), which is mostly inherited in an autosomal dominant way. In genetherapy, treatment of dominantly inherited disorders is still a challenge to be overcome. We chose spliceosome mediated RNA trans-splicing (SmaRT) to repair the KRT14 missense mutation R125P. Trans-splicing uses the cell's spliceosome to recombe two distinct mRNA molecules, of which one is an engineered pre-trans-splicing molecule (PTM). Crucial for the functionality and efficiency of a specific trans-splicing process is a binding domain (BD) included in the PTM, which has the task of bringing PTM and endogenous target into near proximity to allow trans-splicing to take place. We developed a screening method based on fluorescence molecules to identify highly functional PTMs, differing in their binding domain specific for intron 7 of the KRT14gene. Trans-splicing was detected by fluorescence microscopy and FACS analysis. Subsequently isolated PTMs showed a high trans-splicing efficiency in HaCat cells, revealing specific trans-splicing into exon 8 of the endogenous KRT14 gene. Successful trans-splicing in this model constitutes a novel approach to treat autosomal dominant diseases.

P051

3' Trans-splicing as a tool for gene therapy in dystrophic epidermolysis bullosa

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Introduction: Mutations on the COL7A1 gene are responsible for functional defects in type VII collagen, the major component of anchoring fibrils. Abnormalities or lack of those structures lead to the inherited blistering skin disorder dystrophic Epidermolysis bullosa (DEB). Gene therapy efforts for DEB are presently focused on the transfer of a wild type cDNA into affected cells, which is still accompanied by a number of technical challenges. In this study we have shown, that Spliceosome Mediated RNA Trans-splicing (SMaRT) is an alternative tool to restore type VII collagen expression in an *in vitro* DEB model.

Methods: SMaRT provides intron-specific gene-correction at the pre-mRNA level. A pre-trans-splicing molecule (PTM) is designed to exchange parts of the coding sequence of the endogenous transcript by the wild type sequence. In our gene therapy model we used primary and immortalized keratinocytes from a recessive DEB (RDEB) patient, carrying two heterozygous nonsense mutations in COL7A1 exons 14 and 104 that provoke collagen VII deficiency. These cells were retrovirally transduced with a 3' PTM encoding COL7A1 wild type exons 65–118.

Results: Retroviral transduction of the cells resulted in correction of the 3' portion of theCOL7A1 transcript via trans-splicing. Consequent restoration of type VII collagen expression was detected by immuno-labelling of transduced RDEB Null keratinocytes. The reverted cells also regained their ability to secrete and deposit type VII collagen at the dermal-epidermal junction (DEJ), which was analyzed in artificial skin equivalents. Anchoring fibrils-like structures were visualized at the DEJ by electron microscopy.

Conclusion: In this work we demonstrated that 3' trans-splicing within the endogenous COL7A1gene is functional. Thus SMaRT may be a new gene therapy approach for treatment of DEB.

P052

Using RNA Trans-splicing to correct mutations in the plectin gene

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Spliceosome Mediated RNA Trans-splicing (SMaRT) is a new gene-therapeutical approach for the correction of large genes. In this approach the endogenous splicing machinery is utilized to recombine a target cellular pre-mRNA and apre-trans-splicing molecule or PTM by trans-splicing, replacing the disease causing parts of a gene by their wildtype copy. In a previous reported *in vitro* model we have demonstrated, that PTMs can be designed to trans-splice to the 5' region of a transcript, replacing the 1026 nt long sequence encoding exons 2–9 of the geneplectin. Thereby all possible mutations in the 5' portion of the gene, leading to the rare variant of the genodermatosis epidermolysis bullosa Simplex with late onset muscular dystrophy (EBS-MD), can be corrected with one single trans-splicing construct. Starting from this model we have developed a high throughput screen for finding of the most specific and efficient PTMs to optimize the gene correction. By using a fluorescent reporter system we are able to rapidly evaluate the effect of various PTM binding domains on trans-splicing functionality. After double transfection of single PTMs and a target molecule containing the intron of interest, the best PTMs can be identified by FACS. Up to date we have identified several PTMs with improved efficiency to trans-splice into the plectin gene in cell culture experiments. The identification of the most efficient PTM will increase the endogenous correction of the plectin gene at them RNA level and should enable us to establish a gene therapy approach for patients suffering from EBS-MD.

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P053

Novel and recurrent COL17A1 mutations and genotype-phenotype correlations in a large European cohort of junctional epidermolysis bullosa

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Junctional epidermolysis bullosa is a heterogeneous group of blistering skin diseases with dermal-epidermal separation. Most cases are caused by mutations in the genes encoding laminin 332 or collagen XVII, both components of the hemidesmosome-anchoring filament complex of the skin. Here we report COL17A1mutations in a large cohort of 34 patients with moderate and mild variants of JEB, JEB-other. PCR amplification of all 56 COL17A1 exons and subsequent automated DNA sequencing revealed a total number of 27 different mutations. Two recurrent mutations were disclosed: the non-population-specific nonsense mutation p.R1226Xand another nonsense mutation, p.G803X. Furthermore, we identified seven novel mutations: two nonsense mutations (p.W464X, p.R154X), four frameshift mutations (p.N854fsX109, p.D1289KfsX3, p.G999fsX21, p.R1183fsX68) and one splice-site mutation (c.1849-2A>C). Six of them lead to reduced levels or absence of collagen XVII in the skin, as shown with immunofluorescence staining. The spectrum of genotype-phenotype correlations was expanded by careful clinical examination. An unusual phenotypic constellation was identified in a large family with three mildly affected siblings, in which the diagnosis of the mentally retarded index patient was only established at the advanced age of 79 years. She was compound heterozygous for the COL17A1 nonsense mutation (p.R1169X) and a splice-site mutation (c.1849-2A>C). Immunofluorescence staining showed reduced levels of collagen XVII and junctional blister formation in the skin. Clinically, localized non-scarring blistering, but no alopecia or mucosal involvement, were present. The two other affected siblings showed a similar skin phenotype. These findings expand the COL17A1 mutation data base and broaden the spectrum of minimal or mild EB phenotypes which sometimes are mistaken for acquired diseases and goun diagnosed for extended periods of time.

P054

Identification of an oncostatin m receptor mutation linked with familial primary cutaneous amyloidosis

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Familial primary cutaneous amyloidosis (FPCA) is a rare skin disease in Caucasians. Recently, two different missense mutations in the oncostatin M receptor gene (OSMR) were postulated to be causative for FPCA in a Brazilian pedigree.

In this report a mutational analysis was performed of the OSMR gene as well as the transthyretin, apolipoprotein (Apo) A1-, lysozyme-, and fibrinogen alpha-genes in three affected and two unaffected individuals of a Caucasian family with FPCA by direct sequencing. Moreover, immunohistochemistry was performed to classify the deposited amyloid. Molecular analysis identified a previously unknown mutation in the OSMR gene (p.Tyr710Cys) within the first extracellular fibronectin type III-like (FNIII) domain. The amyloid deposits only stained with an antibody directed against amyloid P component, while no staining was observed with antibodies directed against a large variety of known extracerebral amyloid diseases. In addition, no staining was found with antibodies directed against keratin (amyloid K). Our study identifies a novel germline mutation in the OSMR gene linked with FPCA in a Caucasian family. An amyloid profile untypical for cutaneous amyloidosis was detected.

P055

Fibroblast therapy enhances dermal-epidermal stability in dystrophic epidermolysis bullosa: long term effects and molecular mechanisms

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Recessive dystrophic epidermolysis bullosa (RDEB) is an incurable skin fragility disorder caused by mutations in the collagen VII gene, COL7A1. It has a severe impact on the life of the patients, therefore, causal therapies are urgently needed. Our pilot experiments with a viable RDEB mouse model, the collagen VII hypomorph, suggested that allogeneic fibroblast injections present a promising therapeutic approach. Here we investigated long-term efficacy, mechanisms and adverse effects of this cell therapy regimen. 5 week-old hypomorphic mice were injected intradermally, into a 2×3 cm dorsal skin area, with 20 million EGFP+ fibroblasts. The animals were sacrificed 1–100 days post-injection, and skin specimens from injected, adjacent, and untreated areas were subjected to (immuno) histopathological and to RNA expression analysis. 24 h post-injection, the EGFP+ fibroblasts were found spread throughout the dermis within the injected area, but they had not migrated into the neighbouring areas of the skin. Cell numbers gradually decreased until 28 days after treatment, when no EGFP+ expressing cells were observed any more. The fibroblasts actively synthesized collagen VII. The mRNA expression levels were strongly increased at 7 days after injection and returned to basic levels within 28 days. Collagen VII protein at the dermal-epidermal junction, as measured with semiquantitative confocal laser scanning microscopy, was significantly increased for more than 70 days after treatment, but started slowly decreasing at 100 days post injection. Functionally, the mechanical stability of fibroblast-treated skin areas was increased for more than 70 days, as compared to untreated areas. No significant adverse effects were observed. Injections of allogeneic fibroblasts only lead to a transitory mild inflammatory infiltrate, but not to fibrotic processes or to myofibroblast differentiation. Moreover, no specific immune response to collagen VII was observed. These observations clearly demonstrate that intradermal injection of allogeneic fibroblasts is efficacious in increasing dermal-epidermal stability for about 3 months and that no significant adverse effects are to be expected. Therefore, this study paves way to development of a clinically feasible and effective causal treatment regimen for RDEB.

P056

A frequent functional SNP in the MMP1 promoter as a disease modifier in alarge European cohort of dystrophic epidermolysis bullosa

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Dystrophic epidermolysis bullosa (DEB) is a heritable skin disorder, characterized by dermal blistering following minor trauma and healing with scarring. All forms are caused by mutations in the collagen VII gene, COL7A1, with clinical severity depending on the nature and localization of the mutations. Still, inter-individual differences in patients harbouring identical COL7A1 mutations suggest that additional genetic and environmental disease modifiers exist. A polymorphism influencing the transcription of matrix metalloproteinase 1 (MMP1) has recently been identified as a possible genetic disease modifier of recessive DEB in a French patient cohort. The SNP 1G/2G in the MMP1 promoter leads to enhanced transcription of the proteinase, which can cleave collagen VII. Here we used a rapid genotyping method to evaluate the status of this SNP in a large cohort of 77 European DEB patients. This cohort, comprising 31 dominant DEB, 17 recessive DEB-other and 29 recessive DEB-severe generalized patients, underwent careful clinical characterization. Collagen VII expression was assessed by indirect immunofluorescence or electron microscopy, and COL7A1 mutations were determined by direct sequencing to allow for genotype-phenotype correlations. For the dominant DEB group, the status of the SNP did not significantly differ from a matched control group. Nevertheless, within this group, the more active SNP status (1G/2G or 2G/2G) was associated with a higher disease severity. The status of the SNP in recessive DEB-other differed significantly from the controls. All patients in this group had the more active variant (1G/2G or 2G/2G). In recessive DEB-severe generalized, the SNP distribution was also significantly different from controls. Only two patients in this group harboured mutations predicted to lead to remnant collagen VII in the skin (missense mutations), with a possible disease modifying role of more MMP1. In one of them, the more active 2G/2G MMP1 promoter SNP is likely to be associated with the severe phenotype. In the other, the presence of the less active 1G/1G SNP suggests that yet other disease modifiers, genetic or environmental, play a role in DEB. Taken together, the frequent SNP in the MMP1 promoter represents one, but certainly not the only disease modifier in DEB.

P057

Distal and proximal interleukin-10 promoter polymorphisms associated with risk of cutaneous melanoma development: a case-control study.

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Inherited promoter polymorphisms of the IL-10 gene resulting in altered IL-10 production may contribute to a genetic susceptibility for melanoma. We investigated the role of distal as well as proximal polymorphic alleles [-7400InDel, -6752AT (rs6676671), -3538AT (rs1800890), -1087AG (rs1800896), -597AC (rs1800872)] of the IL-10 promoter in a hospital-based case-control study of 165 Caucasian patients from Germany with cutaneous melanoma and 162 healthy cancer-free Caucasian control subjects from the same area matched by age, gender, and colour of hair and eyes. Using multivariate logistic regression analyses to control for number of nevi and skin type the IL-10 'higher producing' promoter genotypes -6752TT, -3538AA, and -597CC were significantly associated with a reduced risk of melanoma (OR 0.56,95% CI: 0.34–0.92, $P = 0.022$; OR 0.52, 95% CI: 0.32–0.86, $P = 0.011$; OR 0.34, 95%CI: 0.13–0.88, $P = 0.026$ respectively). Although our findings need to be confirmed by independent and larger studies we have described for the first time the association of distal gene variants of the IL-10 gene as an independent risk factor for melanoma.

P058

Novel filaggrin mutations in German ichthyosis vulgaris patients and high presence of CD1a+ cells in the epidermis of the atopic subgroup

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Ichthyosis vulgaris (IV) is a common genetic skin disease with a prevalence of1:250–1,000. Filaggrin (FLG) mutations cause the disorder and at the same time predispose to atopic diseases. We evaluated a cohort of 25 IV patients from Germany, the clinical presentation, epidermal ultrastructure, histology and filaggrin antigen. Restriction enzyme analyses was performed to pre-screen the prevalent mutations 2282del4, R501X and R2447X. In a second step the complete FLG sequencing analysis revealed the presence of five novel mutations. The null alleles 424del17 and 621del4 are located in the profilaggrin S100 domain, 2974delGA in filaggrin repeat 2, R3766X in repeat 101 and E4265X in repeat 102. The combined prevalence of these mutations in the German control population was below 1%. Two patients showed the previously described mutation S3247X. Moreover, we wondered whether the suggested epidermal barrier defect in IV is associated with different numbers of dendritic cells in the epidermis. Interestingly, CD1a+ cell counts between non-atopic and atopic IV patients showed a significant difference for atopic patients with eczema as well as atopic patients without eczema supporting the hypothesis that there is a primary barrier defect that predisposes to atopic manifestations, possibly independent of atopic eczema.

P059

Recessive epidermolytic hyperkeratosis caused by a novel termination codon mutation in the keratin 10 gene

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Epidermolytic hyperkeratosis (EHK; OMIM 113800), also termed bullous congenital ichthyosiform erythroderma (BCIE), is a keratinization disorder that typically presents with erythema and widespread blister formation at birth. The cause of EHK are mutations in the genes encoding keratins K1 and K10 which leads to clumping of keratin intermediate filaments (KIF) in suprabasal keratinocytes. The majority of mutations are located at the highly conserved helix boundary motifs of both keratins and a genetic 'hotspot' has been identified in K10 affecting an evolutionarily highly conserved arginine residue (p.Arg 156). We have previously identified a recessive form of EHK due to a nonsense mutation in the KRT10 gene leading to loss of K10expression in the affected homozygous individuals (p.Gln434X). We now report another family with recessive EHK and have identified a novel premature termination codon mutation (PTC) (p.Lys439fsX6) which is located five amino acids downstream of the previously reported mutation. In this family, ultrastructural analysis also showed sparse keratin filaments and keratin clumps that show a nearly homogenous, amorphous structure. We therefore suggest that this characteristic ultrastructural picture should prompt detailed analysis of the pedigree to search for parental consanguinity and a recessive inheritance. Interestingly, p.Gln434X andp.Lys439fsX6 together with a recently reported mutation (p.Cys427X) are all located in close proximity in the 2B domain of K10 suggesting a genetic hot spot in recessive EHK. Expanding the catalogue of known mutations in this disorder is important with respect to molecular diagnosis and genetic counselling.

P060

5# Trans-splicing in the type vii collagen gene–development of a mRNA based gene therapy approach for dystrophic epidermolysis bullosa

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Mutations in COL7A1, the gene coding for type VII collagen, are the cause of dystrophic epidermolysis bullosa, a heritable mechanobullous skin disease. Because of its size COL7A1 exceeds the integration capability of most viral vectors commonly used for delivery in gene therapy. Furthermore endogenous regulation of expression is crucial in tissue with a complex differentiation program. Therefore we chose an mRNA based gene therapy to repair defects in COL7A1. In this approach a wild type copy mRNA of the mutated gene is trans-spliced to the target gene. In this project we corrected mutations in the 5# part of COL7A1. We tested rationally constructed pre-trans-splicing molecules (PTMs), which showed a trans-splicing efficiency of about 40% in our test system, regarding their endogenous trans-splicing potential in HaCaTs. After amplification of cDNA with specific primers we were able to detect endogenous trans-splicing in HaCaTs by sequencing. With a fluorescence based screening procedure we are maximizing efficiency and specificity to much higher levels than 40%. The long term goal of our approach is an ex vivo gene therapy, in which skin grafts taken from patients are transfected with specific PTMs and are then retransplanted to the patients.

P061

Suicide gene therapy for RDEB SCC

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Recessive dystrophic epidermolysis bullosa (RDEB) is an inherited blistering disorder often associated with cutanous squamous cell carcinoma (SCC), which displays a life threatening factor in this patient group. EB SCC is characterized by its rapid tumour growth as well as a high metastatic rate and does not respond to conventional chemo- or radio therapeutical therapy. Therefore we developed a cancer suicide gene therapy approach using SMaRT technology (splicesosome mediated RNA trans-splicing). Previous data identified MMP-9 as possible target gene in a RDEB SCC cell line for further *in vitro* studies of pre mRNA trans-splicing molecules (PTMs). Vectors carrying intronic regions of MMP-9, essential trans-splicing motifs and cDNAs of cell-death inducing peptides were constructed. After transfection of cancer cells the pre mRNA of MMP-9 is trans-spliced with the mRNA of a peptide, e.g. herpes simplex virus thymidine kinase (HSV-tk), leading to its specific expression. As a result tumour cells should be killed by producing their own death signals. In preliminary experiments we observed correct trans-splicing of the target gene MMP-9 and cell death inducing peptide on the mRNA level. Moreover cell culture tests indicate the functionality of resulting MMP-9/toxin fusionprotein in tumour cells.

P062

Analysis of four filaggrin loss-of-function mutations (R501X, 2282del4, R2447Xand S3247X) in Austrian and German atopic dermatitis patients

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Background: Recently, mutations in the filaggrin gene (FLG) have been shown to be a major predisposing factor for atopic dermatitis (AD). In this study we sought to evaluate the influence of four prevalent mutations (R501X, 2282del4, R2447X andS3247X) on AD in Austria and Germany.

Methods: We genotyped a large cohort of 462 Austrian and German AD patients and402 control individuals. Mutation analysis was performed using size analysis of fluorescently labelled PCR products for 2282del4 as well as a Taqman based allelic discrimination assay for R501X, R2447X and S3247X.

Results: All four mutations occurred more frequently in patients with AD than in controls. We observed a highly significant association of the combined genotype with AD. Subgroup analysis revealed a significant overrepresentation of mutation carriers among adult patients with early age of onset of the disease. When analyzing atopicco-morbidities, we found a higher frequency of null alleles in AD patients with concomitant asthma than in those without this co-morbidity, although these results did not reach statistical significance. Additionally, a significant association of high total serum IgE levels with the FLG mutations was observed. Furthermore, we have identified a novel null mutation at amino acid position 488 (1464delC) in one patient.

Conclusions: Our data point towards a key role of the mutations in the pathogenesis of early onset AD and support the hypothesis of a facilitated allergic sensitization via an impaired epidermal barrier. With the 1464delC variant we have found a novel FLG mutation, which deserves further evaluation in other study populations.

P063

Characterization of Psoriasis Susceptibility Locus 6 (PSORS6) in Patients with Early Onset Psoriasis and Evidence for Interaction with PSORS1

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Psoriasis is a genetically complex, chronic inflammatory skin disease. We have previously identified a susceptibility locus on chromosome 19p13 (PSORS6). In a follow-up linkage disequilibrium (LD) study in an independent family-based cohort, we found evidence for association to a newly discovered microsatellite at this locus (D19SPS21, P <5.3*10-5). An LD-based association scan in 300 trios revealed association to several single SNPs in one LD block. When we stratified this cohort for carrying the PSORS1 risk allele at the HLA-C locus, evidence for association became much stronger at single SNP and haplotype levels (P-values between 1.0*10-4 and 8.0*10-4). In a replication study of 1,114 patients and 937 control individuals, evidence for association was also observed after stratification to the PSORS1 risk allele. In both study groups, logistic regression showed evidence for interaction between the risk alleles at PSORS1 and PSORS6. The associated LD block did not comprise any known genes. Interestingly, an adjacent gene, MUC16, coding for a large glycosylated protein expressed in epithelia and of unknown function, could be shown to be also expressed in tissues relevant for pathogenesis of psoriasis such asskin and thymus. Immunohistochemical analyses of skin revealed focal staining forMUC16 in suprabasal epidermal cells. Further functional studies are required to clarify its potential role in psoriasis and identify the causal variant(s) at this locus. Our data establish PSORS6 as a confirmed psoriasis susceptibility locus showing interaction with PSORS1.

P064

Comparative genomics and gene expression analysis reveal that the evolutionary origin of hair keratins preceded the appearance of mammalian hair

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The presence of hair is one of the specific characters of mammals and, hence, the origin of hair has been a central event in mammalian evolution. The main components of hair are cysteine-rich alpha-keratins, also known as hard keratins or 'hair keratins'. The same proteins are also present in mammalian claws. To gain insights into the evolutionary history of these important structural proteins, we compared the genomic loci of the human hair keratin genes with the homologous loci of the green anole lizard *Anolis carolinenis*. Indeed, the genome of the lizard contained two type I and four type II hair keratin-like genes. Gene locus synteny, conserved exon–intron organization and a high level of sequence similarity demonstrated that these genes were orthologous to mammalian hair keratin genes. mRNA transcripts of the lizard hair keratin-like genes were detected in abdominal skin samples and, at higher levels, in the digits of the lizard. Immunohistochemistry with antibodies against two lizard hair keratin-like proteins revealed specific expression of these proteins in keratinocytes that form the claws. We conclude that hair keratins are not restricted to mammals and that at least some hair keratins of mammals and reptiles are preferentially expressed in the claws. Taken together, our results indicate that hair keratins have been derived from cysteine-rich alpha keratins present in the claws of the last common ancestor of mammals and reptiles.

P065

Xeroderma pigmentosum group C and G gene polymorphisms, alternative splicing and functional DNA repair in multiple melanoma patients

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In the rare disease xeroderma pigmentosum (XP) defects in nucleotide excision repair (NER) genes lead to an >1000-fold increased skin cancer risk including melanoma. To investigate if genetic variances in XPC and XPG genes might contribute to the development of melanoma we investigated polymorphisms, alternative splicing and functional NER in a high-risk group of 30 patients with sporadic multiple melanomas and 30 matched healthy individuals. We analyzed 3 XPC gene polymorphisms (intron 11 C-6A, exon 15 A2920C and intron 9 poly AT) which are in linkage disequilibrium. The intron 11 -6A allele within the XPC intron 11 splice acceptor site leads to a spontaneously increased exon 12 skipping in XPC mRNA. In fibroblast cell lines this resulted in diminished DNA repair but primary lymphocytes from probands with or without melanoma have not yet been tested. In all but one of our 60 individuals the three XPC polymorphisms were in linkage disequilibrium. Using quantitative real-time PCR we found that the intron 11 -6A alleleled to a significantly increased expression of the exon 12 deleted XPC mRNAisoform. The isoform expression was about two-fold higher in individuals carrying theA/A genotype compared to C/C carriers ($p<0.0001$). Using host cell reactivation inprobands# lymphocytes we measured a relative NER of 26.1% in C/C carriers and16.1% in A/A carriers. Regarding an association with melanoma risk we could not detect significant differences in these factors between patients and controls with this number of probands. We also assessed an XPG gene polymorphism (exon 15 C3507G). This was not distributed differently between patients and controls and did not influence functional NER in the probands# lymphocytes. In addition, we assessed a spontaneously alternatively spliced XPG mRNA isoform containing a 109 bp cryptic exon in intron 1 and leading to a frameshift after the insert which results in a stop codon two amino acids downstream. Its functional relevance is still unclear. Using quantitative real-time PCR we found that this isoform was >2-foldhigher expressed in multiple melanoma patients than in controls ($p=0.0401$). This alternatively spliced XPG mRNA isoform might serve as a molecular marker for an increased melanoma risk.

P066

Frequent somatic mutations of GNAQ in uveal melanoma do not impact patient survival

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In contrast to cutaneous melanoma, BRAF and NRAS mutations are absent in uveal melanoma. The heterotrimeric G protein alpha subunit q (GNAQ) was recently discovered as an oncogene frequently mutated in uveal melanoma and blue nevi. The mutations occur exclusively in codon 209, the ras-like domain, and result inconstitutive activation of GNAQ with consecutive activation of the MAP-kinase-pathway. We studied a cohort of 75 uveal melanomas and correlated GNAQ mutation status and chromosomal aberrations with patient survival. We found activating GNAQ mutations in 53% of the tumours. After stratification for copy number of chromosome3, GNAQ mutation status was not correlated with patient survival. Similarly to BRAF and NRAS mutations in cutaneous melanoma, the missing prognostic relevance may indicate that GNAQ mutations are an early event. This is also consistent GNAQ mutations also being present in most blue nevi.

P067

Association of the FAS/CD95-promoter single nucleotide polymorphism -670A/G and lupus erythematosus in a German cohort

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Aim of this investigation was to clarify by examination of a functional single nucleotide polymorphism (SNP) at position -670 in the promoter of the apoptosis-mediating gene FAS/ CD95 /APO-1, whether immunogenetic variation can influence the characteristics of an autoimmune reaction reflected by different clinical disease patterns of lupus erythematosus. In 107 German lupus erythematosus (LE) patients, who were previously classified due to clinical feature, systemic involvement, profile of auto antibodies and phototesting, and 96 controls, the genotype frequencies of the FAS promoter alleles -670 A/G were determined by allele-specific polymerase chain reaction (PCR). A connection between lupus erythematosus and an A-homozygote genotype of the FAS promoter SNP was observed. A significant difference was found for patients with systemic lupus erythematosus and positive anti-Ro auto antibodytitres. Comparison of patients# phototesting and allelic variants showed no statistical significance that could imply causality between photosensitivity or development of skin lesions and the FAS promoter SNP. A-homozygocity of the -670 FAS gene promoter single nucleotide polymorphism is a risk factor for developing LE, especially SLE and a positive titre for anti-Ro antibodies. These results confirm that apoptosis in general and the FAS receptor in special may contribute to the development of autoimmune reactions in lupus erythematosus.

P068 (V36)

IL-17A induces cathelicidin antimicrobial peptide in keratinocytes through a vitamin D dependent mechanism

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Cathelicidin is strongly expressed in lesional skin in psoriasis and may play an important role as both an antimicrobial peptide and as an autoinflammatory mediator in this chronic skin disease. The mechanism of increased cathelicidin in psoriatic keratinocytes is not known but recent observations have found psoriasis has abundant Th17 cells which produce interleukin (IL-) 17A and IL-22. We found that human keratinocytes stimulated with supernatants from T cells isolated from lesionalpsoriatic skin increased expression of cathelicidin when stimulated in the presence of 1,25-dihydroxyvitamin D3 (1,25D3). This induction was signalled through the IL-17receptor A (IL-17RA). *In vitro*, IL-17A, but not IL-22, increased cathelicidin mRNA and peptide expression in keratinocytes dependent on the presence of 1,25D3. At the same time, co-incubation with 1,25D3 blocked induction of human β-defensin 2(HBD2), IL-6 and IL-8, which are other target genes of IL-17A. Act1, which is associated with IL-17RA and essential for IL-17A signaling, mediated cathelicidin induction, as its suppression by siRNA inhibited HBD2 and cathelicidin. Both 1,25D3and IL-17A signalled cathelicidin induction through MEK-ERK. These results suggest that increased IL-17A in psoriatic skin increases cathelicidin through a vitamin D3, Act1 and MEK-ERK dependent mechanism. Therapies targeting this cathelicidin regulating system might be beneficial in patients suffering from psoriasis.

P069

The *in vivo* expression and secretion of different antimicrobial peptides is induced in patients with atopic dermatitis independently of *S. aureus* colonization

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Human skin has the ability to defend itself against potentially invading microorganisms by production of highly effective antimicrobial peptides (AMP) as part of the 'chemical barrier'. Recently, reduced expression of AMP in patients with atopic dermatitis (AD) compared to *psoriasis vulgaris* has been described. This observation was discussed as a possible explanation for the high microbial infection rate of AD patients. Aim of our study was to analyze comparatively the *in vivo* expression and secretion of different AMP in AD patients and healthy controls. Standardized skin derived washing fluids from lesional and non-lesional skin as well as nasal swabs were collected from untreated AD-patients ($n = 38$) and matched controls ($n = 25$). Qualitative and (semi-)quantitative analysis of microbial colonization with *Staphylococcus aureus* (SA) and coagulase negative Staphylococci was performed. The release of human beta defensin (hBD)-2, hBD-3, psoriasin (S100A7), and RNase seven was determined by ELISA using specific antibodies. In addition, biopsies were taken from AD patients and controls to investigate AMP expression by immunohistochemistry. Nasal and/or skin colonization with SA was observed in 87% of AD patients and 32%of healthy controls. Immunohistochemistry revealed an induced expression level for all AMP under investigation in lesional and partly in non-lesional skin. All AMP were detectable in skin-derived washing fluids as well as in nasal secretion. The median AMP-release in lesional skin of AD patients was significantly higher for hBD-2, psoriasin and RNase seven when compared to non-lesional skin and healthy controls. No correlations between SA colonization and individual AMP levels were observed, whereas SA colonisation was positively correlated with the severity of AD as determined by the 'scoring of atopic dermatitis' (SCORAD). Nasal fluids of AD patients and controls showed no significant differences in hBD-2 and -3, psoriasin and RNase seven levels. In contrast to previous observations this study indicates that the antimicrobial response in AD is not generally impaired, but greatly differs according to the type of AMP produced by skin.
B B

P070 (V33)

SlanDCs (6-sulfoLacNAc+ dendritic cells), a novel proinflammatory cell type in systemic lupus erythematodes

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Systemic lupus erythematoses (SLE) is characterized by high levels of serum TNF-zand IFN-α, as well as activated dendritic cells (DCs), B cells and T cells. In this study we asked, whether 6-sulfoLacNAc expressing DCs (slanDCs), which have an outstanding capacity to produce proinflammatory cytokines and to programme Th1 cells, may play a role in human SLE. Immunohistochemical studies of affected skin samples (SLE $n = 10$, CDLE $n = 10$, SCLE $n = 10$) revealed increased numbers of slanDCs in the dermal inflammatory infiltrate. SlanDCs specifically clustered within pseudo follicular structures with CD3+ T cells and CD20+ B cells. A common proinflammatory signalling pathway in SLE is induced by autoimmune complexes containing single stranded (ss) RNA which bind to toll-like receptors (TLR) 7 and 8.Quantitative and qualitative PCR analysis of FACS-sorted slanDCs revealed the expression of TLR7 as well as of TLR8. This combined expression is in contrast toCD1c+DCs and to pDCs that either express TLR7 or TLR8. In line with this, only slanDCs displayed a strong production of TNF-α and IL-12 after stimulation with selective ligands for TLR7 as well as for TLR8. SlanDCs also produced by far superior levels of the proinflammatory cytokines TNF-z, IL-6, IL-12/IL-23p40, IL-12p70 but not IFN-α after stimulation with the dual TLR7/8 ligand. Hence, slanDCs showed a massive response to ssRNA, the natural ligand of TLR7 and TLR8.Hydroxychloroquine is a classic therapeutic option in SLE and is known to inhibit signalling via TLR7 and TLR8. By studying TLR7/8-stimulated blood samples obtained before and during oral treatment with hydroxychloroquine we obtained first evidence of a reduced production of TNF-a by slanDCs during treatment with hydroxychloroquine. Taken together these data provide strong evidence that slanDCs may play an important role as proinflammatory effector cells in human SLE and may be an important target for therapeutic approaches.

P071

Suppression of melanoma tumour growth with murine DEC205 single chain fragment variable fusion protein.

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The ability of immunotherapy to directly and specifically target antigen expressing tumour cells is of premiere importance in order to minimize the number of side effects one induces while mounting an immune response towards cancerous tissue. We have utilized two different transplantable melanoma models to investigate the efficacy of a single chain fragment variable (scFv) antigen fusion protein in a therapeutical setting. We created a scFv for the antigen uptake receptor DEC-205, which is expressed exclusively on dendritic cells (DC) and fused the melanoma antigen gp100 to it. Initially we tested for specific binding of the scFv to 6 day cultured bone marrow derived dendritic cells. The cells were incubated with PBS, scFv-gp100 or non-binding control scFv-β-Gal then analyzed via FACS. Results indicated the binding affinity of the scFv to be 46% compared with a non-binding scFv-β-Gal 0.8%, while the monoclonal antibody stained 86% compared to isotype controls, 0%. InC57/Bl6 mice, initial immunohistochemical staining of cytospins from CD11c+isolated cells displayed a positive staining of scFv comparable to the monoclonal antibody. In further experiments, C57/Bl6 mice were injected with the scFv-gp100 fusion protein or control saline subcutaneously. Using immunohistochemistry the mice injected with scFv-gp100 had positively stained DCs in the proper axillary lymphnodes, as shown by an anti c-myc antibody specific for the scFv, in comparison to untreated or scFv-β-Gal injected littermates. Furthermore the induction of gp100 specific CD8+ T cells examined via ELISPOT IFN-γ assays indicated the superior antigen targeting and presentation of the scFv-gp100 fusion protein via DC. We then examined the tumour suppressive effects of the scFv-gp100 in transplantable melanoma tumour models. When the vaccination regime was implemented after a visible tumour has formed at the site of s.c tumour cell injection (5X105 cells), we observed a concentration dependent suppression of tumour growth from thescFv-gp100 in comparison to gp100 peptide injected, scFv-β-Gal injected or WT mice. Together our data indicates that the scFv is a viable method of targeting various tumour antigens to DCs in order to elicit a specific immune response to cells which over express endogenous antigens.

P072 (V30)

A humanized mouse model to study human regulatory T cells *in vivo*

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CD4+CD25+Foxp3+ regulatory T cells (Tregs) suppress efficient immune responses against melanoma. In the opposite, their activity is mandatory to prevent autoimmunity and allergy. However, functional studies on human Tregs are restricted so far to *in vitro* investigations. To overcome this limitation we established a simple and robust humanized mouse model to study the complex functional properties of human Tregs *in vivo*. Transfer of human peripheral blood cells (PBMC) into newborn NOD-Scid mice resulted in a lethal graft-versus-host disease (GvHD) characterized by decelerated growth, reduced mobility, and mortality of treated animals within 2 months. The development of GvHD was accompanied by massive cellular infiltration of human immune cells into multiple organs resulting in chronic hepatitis, colitis and inflamed skin. However, a single transfer of additional human Tregs suppressed the GvHD in a dose-dependent manner. Mice that received increased numbers of human Tregs showed strongly reduced signs of infiltration and inflammation and lacked disease symptoms and mortality up to 100 days after engraftment. Prevention of GvHD by Tregs was associated with decreased early expansion of human immune cells *in vivo*, particularly CD4+ T cells in lymphoid tissues, indicating preferential effects on CD4+ T-helper cell function. These data show that the functional properties of human Tregs can be efficiently analyzed in this humanized mouse models *in vivo* and open new opportunities for pre-clinical testing of novel biologics for immunotherapy of allergic diseases, autoimmunity and skin cancer.

P073 (V29)

Sildenafil treatment prolongs survival and reduces immune suppression in melanoma bearing mice

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Using the ret transgenic melanoma mouse model we studied the influence of Myeloid Derived Suppressor Cells (MDSC) on tumour immunity *in vivo*.
Tumour growth in these mice strongly reflects the clinical situation with regard to genetic background, risk factors, tumour antigen expression and tumour localization. Overexpression of the human protooncogene ret in melanocytes leads to melanoma development in 30% of transgenic mice in the first 60 days of life, whereas other transgenic littermates remain free of clinically visible tumours. Myeloid derived suppressor cells are known to be enriched in lymphatic organs and tumour infiltrate of tumour bearing hosts. They are negatively influencing zeta chain expression in the T-cells receptor, causing anergy and immune suppression. In our model, secondary lymphoid organs as well as tumours of ret transgenic tumour bearing mice accumulated significantly higher numbers of myeloid derived suppressor cells as compared to BL/6 wild type mice. In addition, quickly progressing tumours accumulate higher numbers of MDSC then slower growing tumours. The number of tumour infiltrating MDSC can therefore serve as a marker of tumour progression rate. Moreover, we observed significantly reduced zeta chain expression in T cells from lymphoid organs. Analyzing tumour infiltrating lymphocytes (TIL), we found that TILs from larger tumours show significantly stronger zeta chain down-regulation then TILs from smaller tumours. *In vitro* co-incubation experiments revealed that MDSC are responsible for zeta chain down-regulation in T cells. MDSC are known to influence T cells via NO secretion. To overcome the suppressive environment, we treated mice with PDE-5 inhibitor Sildenafil (Viagra®). This drug maintains high intracellular cGMP-level and negatively regulates NO-production. We could show that this treatment is able to significantly prolong survival of tumour bearing mice. Lymph node metastasis of Sildenafil treated mice are stronger infiltrated by T cells and zeta chain levels in T-cell receptors are restored. Furthermore, lower quantities of MDSC can be found. The identified mechanism to overcome the suppressive effect generated by MDSC provides new possibilities to improve human melanoma immunotherapy.

P074

UV-induced regulatory T cells switch antigen-presenting cells from a stimulatory into a regulatory phenotype

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UV-induced regulatory T cells (UV-Treg) inhibit sensitization in an antigen(Ag)-specific fashion. The migratory behaviour of UV-Treg can be reprogrammed by tissue-specific antigen-presenting cells (APC) in an Ag-specific manner. We recently observed that UV-Treg in turn can influence APC, since upon co-incubation with activated UV-Treg hapten-coupled bone marrow-derived dendritic cells (DC) lost their capacity to induce hapten-sensitization when injected subcutaneously (s.c.) intonaïve mice. The inhibitory effect of UV-Treg on DC was dependent on interleukin (IL)-10 since it could be prevented by a neutralizing anti-IL-10-antibody. UV-Treg but not DC appear to be the relevant source of IL-10 as the inhibition was still observed when DC from IL-10 knock-out mice were used. Despite the crucial role of IL-10cellular contact is essential because the inhibitory effect was not observed in transwell experiments. To determine how UV-Treg influence APC and their Ag-presenting capacity, activated UV-Treg were cocultured with DC. After depletionof T cells, FACS-analysis of DC was performed, revealing down-regulation of B7-2and MHC class II but induction of the inhibitory molecules B7-H3 and B7-H4. To determine whether APC pretreated with UV-Treg in turn are able to induce Treg, DC were coincubated with activated UV-Treg. DC were isolated, hapten-coupled and injected s.c. into naïve mice. 5 days later ear challenge with DNFB was performed. Splenocytes and lymph node cells were obtained from these recipients and injected into naïve mice which were hapten-sensitized 24 h later. Upon adoptive transfer, contact hypersensitivity response was significantly suppressed in the secondary recipients, indicating the presence of Treg in the pool of transferred cells. Together, this demonstrates that UV-Treg can switch APC from a stimulatory into a regulatory phenotype, which further induces Treg.

P075

Stabilin-1 – multitasking receptor on macrophages linking uptake and secretion

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The multifunctional scavenger receptor stabilin-1 is expressed on macrophages in placenta and adult tissues as well as on sinusoidal endothelial cells and macrophages in lymph nodes – primary sites for melanoma metastasis. We showed that stabilin-1 mediates endocytosis of extracellular ligands acLDL, regulator ofECM-remodelling and cell adhesion SPARC, and placental lactogen (PL) which belongs to the growth hormone (GH) family. Both SPARC and a cLDL are targeted viastabilin-1 for the degradation in lysosomes. In contrast, a portion of PL escapes degradation and is delivered into novel storage vesicles. These vesicles do not belong to classical endosomal/lysosomal system. However, these vesicles communicate with trans-Golgi network (TGN). Stored PL can be secreted back to the extracellular space. Next, we found that stabilin-1 shuttles between TGN and endosomes. This trafficking pathway is mediated by GGAs, clathrin adaptors that interact with the DDSLL motif in the cytoplasmic tail of stabilin-1. Here stabilin-1 is involved in delivery of the novel chitinase-like protein SI-CLP from TGN to the endosomal/lysosomal compartment. SI-CLP was identified by us as a binding partner for stabilin-1 in yeast two-hybrid screening. SI-CLP contains a conservative enzymatically silent Glyco_18 domain, similarly to YKL-40 and Ym1/Ym2. Endogenous SI-CLP is over expressed in human macrophages stimulated with IL-4 and dexamethasone and is transported viastabilin-1 from biosynthetic to the secretory pathway. High levels of SI-CLP were detected in bronchoalveolar lavage of patients with chronic bronchitis. Purified SI-CLP has specific lectin properties and induces signal transduction in lungfibroblasts. We propose that tissue macrophages use stabilin-1 1) to coordinate ECM remodelling, angiogenesis, and tissue turnover via clearance of SPARC; 2) to regulate extracellular concentration of PL in placenta; 3) to regulate secretion of chitinase-like protein SI-CLP.

P076

The distribution of antimicrobial Psoriasin (S100A7) in healthy human skin depends on localization and age

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Recently we isolated the S100 protein Psoriasin as potent antimicrobial protein (AMP) from healthy human stratum corneum extracts. The antimicrobial activity of Psoriasin is mainly directed against *E. coli* and it was shown that this AMP is inducible by proinflammatory cytokines, bacteria, differentiation, and after superficial skin injuries. Initial immunohistochemical analyses showed variable staining results depending on the body localization. Therefore, aim of this study was to perform a standardized and comparative analysis of Psoriasin expression in healthy human skin of different localization and age. Formalin fixed and paraffin embedded tissue samples ($n = 105$) of healthy human skin derived from surgery of benign melanocytic neavi was stained with a Psoriasin specific monoclonal antibody. Five samples per three age groups (<20, 20–60, >60years) derived from seven different localizations (periorbital region, neck, breast, upper arm, back, buttock and lower leg) were evaluated for Psoriasin immunoreactivity by two independent investigators according to a standardized scoring protocol. Highest Psoriasin staining intensity was reached in the periorbital region and the neck whereas the back and lower leg showed the lowest scoring. Within all Malpighian cell layers the immunoreactivity was mostly pronounced in the stratumspinosum and in the stratum granulosum. Variable differences in the staining intensity between the three age groups were observed. In summary, this study demonstrates that Psoriasin immunoreactivity is influenced by the body localization as well as by the age. This observation has to be considered when interpreting immunohistochemical analyses of Psoriasin and possibly other (antimicrobial) proteins. We therefore recommend the usage of age and localization matched controls for comparative immunohistochemical analyses.

P077

Murine psoriasis-like inflammation induced by IL-23 is dependent on CC-chemokine receptor 6 (CCR6) expression on skin homing T-cells and non Tcells as a source of IL-22

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IL-23 is a main survival and proliferation factor for Th17-cells and is expressed at elevated levels in human psoriatic skin. Intradermal injections of IL-23 into murine skin mediate inflammation and cutaneous changes reminiscent of key histological features of human psoriasis (i.e. hyper-parakeratosis, intracorneal pustules, acanthosis and dermal inflammatory infiltrates). Animal studies suggest that the Th17hallmark cytokine IL-22, in particular, acts as the key effector molecule down-stream of IL-23 in mediating psoriasiform cutaneous changes. Recently, CCR6 has been found to be expressed on human and murine Th17 cells and CCR6+ T cells as well as the CCR6 ligand CCL20 are abundant in lesional psoriatic skin. In this study we assess the role of CCR6 in psoriasiform changes after injection ofIL-23 into murine skin. Unlike wildtype (WT) mice, IL-23-injected ear skin ofCCR6-knockout (KO) mice displayed neither significant cutaneous changes, nor elevated levels of IL-22 mRNA vs. PBS-injected controls. Correlating with the diminished response to IL-23 injections CCR6KO mice failed to recruit CD4+ T cells and dendritic cells to the skin. However, injection of IL-22 resulted in equivalent psoriasi form changes in the ears of both, WT and CCR6KO mice. T cells from the ears of CCR6 KO mice did not differ from T cells from WT mice in their ability to produce IL-22 after stimulation *ex vivo*, excluding a possible inherent defect in IL-22production. Surprisingly, despite the relative lack of IL-22 expression in IL-23-injected CCR6KO mice, IL-23 injections yielded similar numbers of Th17 cells in the ears of WT and CCR6KO mice. Furthermore, in the complete absence of T cells, IL-23 injections initially induced cutaneous changes indistinguishable from WT mice in ears of Rag1-knockout mice, accompanied by WT- levels of IL-22 mRNA. Our studies demonstrate that CCR6 is essential for IL-23-induced, IL-22-mediatedpsoriasis-like dermatitis in mice, suggesting possible critical role(s) for this chemokine receptor in human psoriasis. Further, the data indicate that the production of IL-22 in this murine model is partly T-cell independent, implicating a significant involvement CCR6 in the recruitment and/or function of a non-T cell source of IL-22.

P078 (V21)

Production of adenosine by regulatory T cells through the ectonucleotidaseCD39 blocks adherence of effector T cell to vascular endothelium and thusabrogates contact hypersensitivity reactions

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We have shown that injection of regulatory T cells (Treg) into sensitized mice abrogates the elicitation phase of contact hypersensitivity (CHS) reactions by blocking the adherence of leukocytes to vascular endothelium. To analyze whether adenosine, a suppressive factor recently described to be produced by Treg, can account for the suppression of the effector T-cell (Teff) – endothelial cell (EC) interaction, we co-cultured isolated murine Teff on monolayers of activated EC in the presence of adenosine. After several washes with medium the remaining adherent Teff were counted. Here we show that adenosine suppressed the adhesion of Teff to EC *in vitro*. Similar results, i.e. suppression of adherence of Teff to EC, were obtained when EC were preincubated with Treg. Likewise *in vivo*, injection of adenosine abrogated the ear swelling response, and concomitant application of adenosine receptor antagonists neutralized these effects. Thus these data indicate a role of Treg-derived adenosine in mediating anti-inflammatory effects in CHS responses. As a possible source for Treg-derived adenosine we identified the ectonucleotidase CD39, as Treg isolated from CD39 deficient (CD39-/-) mice failed to prevent adhesion of leukocytes to the endothelium *in vivo*. When analysing the effects of adenosine on adhesion molecules involved in mediating adherence of Teff to EC we found, that the expression of E- and P-selectin by the vascular endothelium was down-regulated by adenosine *in vivo* and *in vitro*. Similar results were obtained when Treg were injected into mice. In aggregate our data indicate that CD39-drivenadenosine release by Treg down-regulates expression of adhesion molecules by EC, providing a novel mechanism by which Treg mediate suppression of CHS responses *in vivo*.

P079

In vivo activation of injected naïve regulatory T cell occurs at the site of antigen application in a murine model of contact hypersensitivity

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We previously demonstrated that adoptively transferred naïve CD4+CD25+Foxp3+regulatory T cells (Treg) suppress the sensitization- and the elicitation phase in a murine contact hypersensitivity (CHS) model. Since only activated Treg are able to suppress immune reactions, we asked at what site of the body the Treg become activated after injection. Therefore, we labelled freshly isolated naïve Treg with fluorescent dyes and injected the Treg either 2 h before sensitization or 15 min before challenging into recipient mice. Injection of CD4+ cells served as control. Twenty-four hour after sensitization or challenging, respectively, the re-isolated Treg showed a significantly increased expression of the characteristic activation markers CD69, Foxp3 and CD44 whereas CD62L expression decreased. Moreover, the activation of the Treg was dependent on the site of hapten treatment. That is, after sensitization Treg were most vigorously activated in the draining lymph nodes (LN), whereas the elicitation of the CHS reaction led to activation of blood-residing Treg. This data correlated with the site of action of the Treg, as only LN homing Treg are crucial to suppress the sensitization phase, while LN-residing Treg are dispensable for suppression of the elicitation phase. Therefore, during the course of activation of naïve Treg *in vivo*, Treg get first recruited to the site of action and then undergo activation as indicated by upregulation of characteristic markers. The underlying means of this activation are not clear yet; however, our preliminary data suggest that ATP, which we have shown to act as a potent activator of Treg *in vitro*, is also involved in this tissue specific activation of Treg *in vivo*.

P080

Application of skin antigens fused to a novel single chain fragment specific for murine DEC-205 to induce tolerance in a skin graft model

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Background: Patients suffering from geno dermatoses frequently have null-mutations in genes expressing structural proteins of the skin. *Ex vivo* skin gene therapy, applying the missing protein by genetic manipulation, may result in the rejection of the skin graft, which expresses a neo-antigen. As a model for this anti-graft response we grafted skin from a transgenic mouse strain that expresses human type XVIIcollagen as a transgene in the keratinocytes onto syngeneic wild type C57BL/6recipients. These grafts are rejected very quickly from wild type recipients.
Aim: The aim of this project is the induction of peripheral tolerance towards the antigen, human type XVII collagen, in order to achieve graft acceptance.
Methods and Results: TheDEC-205 receptor is expressed specifically by dendritic cells (DC) and greatly increases antigen presentation. Targeting of the DEC-205 receptor with specific antibodies results in antigen-loading of 'steady-state' DC, which induce regulatory Tcells and tolerance. In order to induce peripheral tolerance against human type XVIIcollagen in mice, we combined the mDEC-205-targeting ScFv with NC16A, the immuno-dominant domain of type XVII collagen. The fusion protein was expressed as a native protein in E. coli. Initial immuno-histochemical staining of cytospins from bone marrow derived DC displayed a positive staining of ScFv comparable to the monoclonal α-DEC-205 antibody. In further experiments, mice were subcutaneously injected with the ScFv-NC16A fusion protein or a non-binding control ScFv specific for β-galactosidase. Using immuno-histochemistry we showed that the mice injected with DEC-205-ScFv had positively stained DC in the draining lymph nodes, using a ScFv-specific anti c-myc antibody. Further analysis will reveal whether the novel method of DEC-205-targeting of a skin antigen to dendritic cells will be suitable to induce tolerance in the skin graft model.

P081

Dendritic cell loading and maturation with complexes of cationic, antigenic peptides and poly (I:C) dsRNA

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Dendritic cells (DCs) are popular candidates in cancer vaccine development because of their crucial role in inducing T-cell responses. We tested an intracellular delivery of peptide/poly (I:C) complexes for antigen loading and TLR-3 mediated maturation of human DCs in a single step using a cell-penetrating peptide (tat49-57:RKKRRQRRR) as delivery vector. In the present study a cationic Tat-sequence was fused with an antigenic, MHC-class I-binding melanoma epitope (Melan-A/Mart-1sequence: ELAGIGILTV) and then mixed with negatively charged poly (I:C) dsRNA to quantitatively form peptide/nucleic acid complexes. Poly (I:C) was shown to induce stable mature, Th1 response promoting, IL-12 secreting, clinically applicable DCs vialigation of Toll like receptor 3 (TLR3). Our analyses by flow cytometry and confocallaser scanning microscopy confirmed intracellular localization of TLR3 for monocyte-derived immature DCs (iDCs). Peptide/poly (I:C) complexes were readily internalized by iDCs without negatively affecting the viability. Peptide/poly (I:C)complexes induced a full DC maturation and bioactive IL-12p70 secretion as measured by a panel of surface markers and ELISA, respectively. When using peptide/poly(I:C) complex-loaded DCs for two cycles of autologous T cell stimulation a quantitatively superior epitope specific IFN-gamma secretion in comparison to DCs maturated by a cocktail of cytokines and loaded with peptide could be measured by ELISPOT assay. In conclusion, complexes of cationic, antigenic peptides and poly (I:C) might be used for a TLR-3 mediated DC maturation and intracellular peptide targeting in a single step. Resulting DCs induce a strong expansion/activation of antigen-specific T cells in the context of a sustained IL-12p70 secretion.

P082

Nanoparticles as carriers in transepidermal vaccination strategies

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Langerhans cells (LCs) play an important role for the generation of a potent cellular immune response in the context of transepidermal vaccination strategies. Recently, we have shown that these cells can be selectively targeted using nanoparticles. Upon skin treatment with cyanoacrylate stripping, particles of nanometer size can accumulate in hair follicles, penetrate the viable epidermis and be taken-up by LCs. We investigated the ability of different nanoparticles to penetrate in vellus hairfollicles (VHFs) and their uptake by LCs after application on human skin explants. Two types of fluorescent particles have been compared: commercially available poly-styrene (PS) particles and biodegradable poly-lactic-acid (PLA) particles. The follicular penetration profiles showed that both nanoparticles penetrate and accumulate in more than 50% of all observed VHFs. PS and PLA nanoparticles could be observed in the follicles orifices as well as in the infundibulum and in a significative percentage of VHF (15-20%) they were observed up to the entrance of the sebaceous gland. Both nanoparticles could be taken-up by isolated LCs in *in vitro* conditions. However, only PS nanoparticles were detected in LCs after topical application of nanoparticles on freshly excised human skin. The different uptake of NPs in our *ex-vivo* experiments is possibly due to the fact that PLA NPs had the tendency to form irreversible aggregates upon contact with the lipophilic environment of the skin while PS nanoparticles showed to be more stable, probably forming only reversible aggregates. These results show that the physicochemical nature of nanoparticles plays an important role with regard to their stability in physiological non-aqueous compartments like skin surface and follicular ducts. In fact, this has a direct influence on the particles mono-disperse state and consequently on their uptake by LCs. The understanding of the principle governing the stability of nanoparticles upon contact with the skin will open the possibility to design efficient and selective carriers systems for transepidermal drug delivery.

P083

Cytotoxicity of peripheral blood dendritic cells is subtype-specific and restricted to stimulation with intracellular TLR ligands

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We now know that dendritic cells (DCs) do not only exhibit the unique capacity to evoke primary immune responses by presenting antigens to naïve T cells, but may also acquire cytotoxic activity in response to Toll-like receptor (TLR) 7/8 stimulation. To determine whether cytotoxic activity in DCs is restricted to TLR7/8 stimulation, we incubated peripheral human blood DCs with various TLR ligands overnight and assessed their phenotype by FACS analysis. In response to TLR7/8 and TLR9stimulation, but not in response to other TLR ligands, virtually all plasmacytoid DCs (pDCs) expressed tumour necrosis factor-related apoptosis-inducing ligand (TRAIL).The vast majority of TLR7/8-stimulated myeloid DCs (mDCs), however, expressed perforin and granzyme B, but not TRAIL. In both pDCs and mDCs these events were accompanied by an up-regulation of co-stimulatory molecules. The expression of killer molecules on TLR7/8-stimulated pDCs, but not mDCs, was dependent on IFNαsecretion. Also, freshly isolated pDCs that had been stimulated with IFNα alone expressed TRAIL. At a functional level both TLR7/8- and IFNα-stimulated pDCs killed the tumour cell line Jurkat in a TRAIL-dependent fashion, while tumour cell lysis was abolished in the presence of neutralizing IFNα/β antibodies. In contrast, TLR7/8-stimulated mDCs lysed the MHC I low tumour cell line K562 and much less efficiently their HLA-A*0201-transfected counterpart in a granzyme B- and perforin-dependent fashion. Despite this killing pattern, TLR7/8-stimulated mDCs do not display the phenotypic profile of natural killer (NK) cells. In conclusion our data indicate that the mechanism by which DCs exhibit their cytotoxicity is subtype-specific and exclusively linked to the occupancy of cytoplasmic rather than membrane-bound TLRs, pointing to an as yet underappreciated powerful innate defence line in infectious and tumour immunity.

P084

Stage-dependent frequencies of regulatory T cells and T helper cell reactivity against recall antigens in melanoma patients

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Immunoregulatory mechanisms interfere in many immunological processes. While naturally occurring CD4+CD25+ regulatory T cells (Tregs) are important contributors to prevent autoimmunity and allergy, increased activity of Tregs are assumed to facilitate the development and progression of melanoma. Treg markers like CD25and Foxp3 cannot provide an unfailing determination of Treg frequencies in man, since these molecules are also expressed on activated effector T cells. Therefore, we used a panel of different markers including CD25, Foxp3, CD127 and HLA-DR to analyze the frequencies of Tregs in distinct stages of diseases. Using these mutiple staining technique we were able to show that the ratios of Tregs increased in melanoma patients during tumour progression. Remarkably, high Treg frequencies in the peripheral blood of progressed melanoma patients correlated with a general reduction of T cell responsiveness to different tumour-associated antigens as well as to recall antigens. In conclusion, our findings demonstrate that melanoma progression results in a general immunosuppression and is associated with an increase in Treg frequency, phenomena that possibly explain the disappointing success of immunotherapies in these patients.

P085

Plasmin is involved in generation of the linear IgA dermatosis antigenLABD97

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Collagen XVII (BP180) and its shed ectodomain represent major autoantigens in bullous autoimmune dermatoses of the pemphigoid group. The 120 kDa ectodomainis constitutively shed from the cell surface by disintegrin-metalloproteinases (ADAMs). Part of it is further processed to a 97 kDa fragment (LABD97), an autoantigen in linear IgA dermatosis (LAD), but the responsible proteinases remain elusive. In this study, we identified both the 120 and the 97 kDa ectodomain in blister fluids of bullous pemphigoid patients using new monoclonal antibodies. Since blister fluids contain significant plasmin-like serine protease activity, HaCaT keratinocytes or the purified 120 kDa ectodomain were incubated with several human serine proteases. Only plasmin generated a stable 97 kDa-fragment which was also targeted by LAD sera. Characterization with domain specific collagen XVII antibodies indicates thatLABD97 is derived from the ectodomain of collagen XVII through proteolysis and spans approximately the stretch from amino acid residues 520 to 1292. Interestingly, LABD97 was also generated in the presence of ADAM inhibitors and remained stable over more than 12 h incubation at 37°C, indicating that LABD97 can also arise in an ADAM independent manner through direct action by plasmin.

P086 (V23)

IL-10-treated (tolerogenic) allergen-pulsed dendritic cells induce production ofCCL18 in human CD4+ T cells from allergic donors which enhances the recruitment of regulatory T cells

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IL-10-treated (tolerogenic) dendritic cells (IL-10-DC) are able to inhibit T cell responses by induction of anergy and T cells with regulatory properties (iTreg). This study was set out to analyze the effect of IL-10-DC on T cells on a molecular level by gene expression profiling. CD4+ T cells from grass pollen allergic donors were stimulated with autologous monocyte-derived allergen-pulsed mature DC orIL-10-DC. After 24h, the transcriptional profile was analyzed in DC-depleted T cells using high density (Affymetrix) gene chips. As expected, IL-10-DC suppressed the expression of several genes such as IL-13, IL-5, and OX40. Interestingly, there was only one gene being significantly up-regulated in IL-10-DC-treated T cells at this time point, the chemokine CCL18, which was validated by quantitative real-time PCR and on the protein level. For functional analysis exogenous CCL18 was added to co-cultures of CD4+ T cells with regular allergen-pulsed DC. This resulted in a similar inhibition of the Th2 cytokines IL-4 and IL-5 as observed for CD4+ T cells cultured with allergen-pulsed IL-10-DC without exogenous CCL18, while the Th1 cytokine IFN-gamma, IL-10 and proliferation were not affected. Neutralization of CCL18 in co-cultures of CD4+ T cells with allergen-pulsed IL-10-DC did not restore Th1 or Th2 cytokine production. Chemotaxis assays revealed that CCL18 preferentially attracted Treg cells and less efficiently Th2 cells. These data demonstrate that tolerogenicIL-10-DC induce CCL18 production in T cells, which contributes to the recruitment of Treg. Thus, CCL18 may represent a molecule of significant importance inimmunoregulation and may have therapeutic potential to limit allergic inflammation.

P087

Water-soluble, non-allergenic factors from pollen regulate disparate signaling pathways in human dendritic cells resulting in a TH2 promoting phenotype

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Introduction: We recently expanded the view of allergenicity of pollen pointing to non-allergenic factors promoting a Th2 response. As one bioactive substance we identified E1-phytoprostanes (PPE1) that inhibit dendritic cell IL-12 production and drive TH2 polarization of naive T cells. However, not all observed effects induced by pollen can be attributed to PPE1 implying other pollen-associated factors adding to the effect of Th2-induction. Herein we aimed at defining possible signal transduction pathways induced by pollen.

Methods: For pharmacological receptor studies, human moDC were treated with inhibitors for PGE2 receptors EP2, EP4 (AH6809, AH23848), PPARgamma (GW9662), or H2R (Cimetidine), followed by LPS, alone or in combination with aqueos birch pollen extracts (APE), PPE1, PGE2 or histamine. IL-12p70 was measured in supernatants. cAMP levels were measured in lysates of cells stimulated with APE, PPE1 or PGE2 in the absence or presence of AH6809/AH23848. NF-kBp65 and IkBalpha were analyzed in nuclear and cytoplasmic extracts. RNA of DC treated with medium or LPS, alone or in combination with APE, PPE1 or PGE2 was subjected to real-time PCR using primers for Delta-1, -4 and Jagged-2.

Results: PPE1 inhibit IkBalpha degradation and p65 nuclear translocation.PPE1-mediated inhibition of LPS-induced IL-12 production is reversed by GW9662.In contrast, APE, but not PPE1 lead to an increase in intracellular cAMP, an effect partly blocked by EP2/EP4 antagonists. Furthermore, the inhibitory effect of APE on the LPS-induced IL-12 production is attenuated in the presence of Cimetidine. Finally, APE block the LPS-induced upregulation of Delta-1 and -4 while inducing Jagged-2.

Conclusion: Multiple signalling events elicited by different pollen-derived factors might integrate and program the DC to aquire a rubust Th2 promoting phenotype.

P088

Targeting of a MOG-single chain fusion protein to DEC205+ dendritic cells *in vivo* ameliorates experimental autoimmune encephalomyelitis in BL/6 mice

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For the purpose of Tolerance induction, we have recently established a novel method to load steady state dendritic cells (DC) *in vivo* with antigens, using biochemical coupling of antigens to DC-specific anti-DEC205 antibodies. The biochemical coupling is time consuming and not precise, as different conjugation products may occur. Thus we aimed at generating recombinant fusion proteins, containing a DEC205-specific single chain fragment variable (ScFv) fused to the autoantigen MOG. The respective DNA sequences were cloned into His-Tag and Myc-Tag containing vectors and the recombinant ScFv-MOG fusion protein was purified. To assess its targeting capabilities, cultivated DC were incubated with graded doses of ScFvMOG and stained thereafter with Myc-Tag specific antibodies. Examination via fluorescence microscopy revealed effective loading of MHC class II compartments with ScFvMOG. Likewise *in vivo* we show that after injection of ScFvMOG exclusively DC in the draining Lymph nodes (LN) stained positive for Myc-Tag antibodies. When analysing the T-cell populations of ScFvMOG injected mice we detected increasing numbers of CD4+CD25+FoxP3+ T cells in the LN, thus indicating that ScFvMOG targets selectively to steady state DC *in vivo*, which generate regulatory T cells. To test whether these Treg are able to protect mice from MOG-induced experimental autoimmune encephalomyelitis (EAE), a well studied model for multiple sclerosis, we injected ScFvMOG conjugates into the footpads of BL/6 mice, induced EAE 2 weeks later and assessed the course of the disease using standardized protocols. We found that in mice treated with ScFvMOG the onset of EAE was delayed and the severity of disease was reduced. Moreover only 20% of the ScFvMOG treated mice developed symptoms, where as in PBS-injected or peptide-injected groups, respectively, 80% of the animals developed clinical signs of EAE. Thus, our data show that specific targeting of steady state DC by DEC-specific ScFv fusion proteins, leads to induction of Treg and serves as a novel strategy to prevent autoimmunity.

P089 (V27)

Susceptibility to experimental epidermolysis bullosa acquisita (EBA) is correlated with the expression of Th1 cytokines

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Under normal conditions, the immune system recognizes and eliminates foreign antigens. However, B and T lymphocytes can also react against self-components which may then lead to an autoimmune disease. An example is the production of autoantibodies against type VII collagen, which are deposited at the dermal-epidermal junction and induce blistering of the skin, a disease called epidermolysis bullosa acquisita (EBA). We have previously established an active disease model of EBA by immunizing mice with recombinant murine type VIIcollagen. In this model, immunization of different mouse strains leads to different disease activities. In the present study we demonstrate that susceptible SJL mices how an increased T cell proliferation and primarily express Th1-specific cytokines-like IL-27 and IFN-γ in their draining lymph nodes. Resistant BALB/c mice, however, express lesser amounts of these cytokines, whereas IL-17 is significantly increased. In the serum of SJL mice, in line with these observations, we found significantly higher levels of IgG2b, which has previously been shown to be the most pathogenic antibody subclass in murine EBA because of its complement-fixing abilities. Binding of IgG2b to the dermal-epidermal junction was also significantly increased in SJL compared to BALB/c mice. Our results may provide a basis for novel strategies to treat EBA, i.e. by applying appropriate cytokines that shift the autoimmune response to the production of anti-type VII collagen antibodies which are non-pathogenic.

P090

TH17 deficiency in patients with chronic mucocutaneous candidiasis

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Chronic mucocutaneous candidiasis (CMC) constitutes a selective inability to clear infection with the yeast Candida albicans resulting in persistent debilitating inflammation of skin, nails, and mucous membranes. To date the underlying defect is unknown. In order to characterise cellular immunity in patients with CMC, we analysed chemotaxis and myeloperoxidase (MPO) release of neutrophils. In theT-cell compartment we focused on differentiation and effector-functions of Th17 cells only recently described to be involved in the clearance of candida infections. CMC patients ($n = 7$), healthy controls and immune competent patients with current candida infections, of same sex and similar age ($n = 20$) were enrolled into the study. Neutrophil chemotaxis was assessed by transwell migration assay, MPO release by ELISA. T-cell proliferation capacity was investigated by thymidine incorporation; cytokine production both quantified in supernatants by ELISA or intracellularly by flowcytometry. Neither neutrophil migration nor MPO release differed between CMC patients and healthy controls. CMC patients showed no difference in the relative lymphocyte stimulation index (SI Candida/SI PHA) compared to controls. In the T-cell compartment, Candida-specific IFN-gamma production was significantly reduced in CMC patients. Notably, Candida-specific T cell IL-10 production was markedly higher in CMC patients. Importantly, T cells from CMC patients produced significantly lower amounts of Th17-associated cytokines IL-17 and IL-22. Immune competent patients with Candida infections showed a much higher secretion of IL-17 than both CMC patients and matched controls. Production of IL-17 in CMC patients was also diminished after mitogen or CD3/CD28 stimulation. The inability to clear the yeast Candida albicans in CMC patients does not seem to be due to an impaired neutrophil function nor due to a reduced antigen specific proliferation of lymphocytes. In fact, T cells of CMC patients proliferate in response to Candida antigen. However, the impaired IL-17 cytokine production could play an important role in the pathogenesis of chronic mucocutaneous candidiasis.

P091

Intravenous immunoglobulin (IVIG) therapy acts independently of the neonatal Fc-receptor in experimental epidermolysis bullosa acquisita

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Type VII collagen, the main component of anchoring fibrils, is the autoantigen of epidermolysis bullosa acquisita (EBA), a subepidermal autoimmune bullous disease. We have recently shown that immunization of mice with a portion of the immunodominant NC1-domain (mCol7C) causes a subepidermal bullous skin disease duplicating the findings of EBA patients. Currently, response of EBA patients to treatment is often unsatisfactory and relies on immunosuppressive agents. Due to the low incidence of EBA, controlled clinical trials neither have been performed, nor are underway. Use of EBA animal models may allow evaluating different therapeutic options with regard to their effectiveness *in vivo*. To analyse the therapeutic efficacy of IVIG therapy, mice were immunized with mCol7C. Subsequently, if more than 2%of body surface area were affected by blisters/erosions, mice were randomly assigned to one of the following treatments: (1) methylprednisolone (daily 20 mg/kg i.p.), (2) IVIG 2g/kg (every second day) or (3) weekly, or (4) untreated. All treatment options significantly inhibited further disease progression, but it was most pronounced in both IVIG groups. In line, IVIG, but not MP, lead to a decrease of subepidermal autoantibody deposition, and IVIG had a more pronounced impact on neutrophil extravasation (MPO-assay). IVIG's mode of action is still a matter of debate. However, it is hypothesized, that IVIG acts through inhibition of the neonatal Fc-receptor (FcRn). By inhibition of IgG degradation, the FcRn is mainly responsible for the long half-live of IgG. Hence, blockade of the FcRn by IVIG, is believed to shorten the half-live of pathogenic autoantibodies. To challenge this hypothesis, FcRn deficient- and control mice were injected with pathogenic rabbit anti-mouse type VII collagen antibodies, and treated with IVIG or PBS. IVIG was effective in wildtype and FcRn deficient mice, indicating, that IVIG acts independently of FcRn expression. In summary, our data demonstrate that IVIG is an effective treatment for experimental EBA. Having excluded a role of the FcRn, further studies will aim at dissecting other mechanisms that mediate the effect of IVIG. Eventually, this may lead to a more targeted therapy in autoantibody-mediated diseases.

P092

The murine antimicrobial peptide beta defensin-14 induces Foxp3 expression in CD4+CD25-T cells

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Ultraviolet radiation (UV) suppresses the immune system in an antigen-specific fashion via induction of regulatory T cells (Treg). In contrast to the suppression of the adaptive immune response, the innate immune defence appears to be induced by UV since we could recently demonstrate that UV induces the release of antimicrobial peptides (AMPs), thereby fostering the defence against microbial attacks. In this scenario, T cells appear to be quite dispensable. In fact, T cells in the skin appear to be more harmful than beneficial since most of the inflammatory dermatoses are T cell-driven. This gives rise to the speculation that a certain degree of constant suppression of the adaptive immune response by ambient UV radiation might be beneficial. Hence, suppression of the adaptive and induction of the innate immune response by UV might reflect a kind of general protection mechanism. Since there is recent evidence that AMPs can also modulate the adaptive immune response, we asked whether AMPs which are induced by UV can influence UV-induced immunosuppression. To address this issue, C57BL/6 mice were irradiated with UVB (150 mJ/cm^2 per day for 4 days) and then sensitized with 2, 4-dinitrofluorobenzenethrough the UV-exposed skin. This procedure does not result in sensitization but immunotolerance mediated via CD4+CD25+ Treg. CD4+CD25+ Treg are characterized by the expression of the transcription factor forkhead box protein 3(Foxp3). Five days after sensitization CD4+CD25+ and CD4+CD25- T cells were isolated from lymph nodes and spleens and FoxP3 expression measured by FACS analysis. In contrast to CD4+CD25- T cells, CD4+CD25+ T cells expressed FoxpP3at significant levels. Upon incubation of CD4+CD25- T cells with the AMP murine beta defensin-14 for 24 hours a remarkable induction of FoxP3 was observed. This implies the beta defensin-14 might switch CD4+CD25- T cells into a regulatory phenotype. Taken together, these data for the first time indicate that the UV-induced release of AMPs might also contribute to the suppression of the adaptive immune response by UV.

P093

T cells reprogrammed by RNA Transfer to recognize HIV-1

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HIV-1 establishes a persistent infection, and abolishes the CD4+ immune cells, which ends in a paralyzed immune system that is unable to defend the body against opportunistic diseases. The highly active antiretroviral therapy (HAART) is only able to suppress but not to eliminate virus. However, high levels of HIV-1-recognizingcytotoxic T lymphocytes (CTL) with a widespread specificity, especially against conserved epitopes, play an important role in the control of HIV-1 replication. Unfortunately, most of the HIV-infected patients are incapable to form such a strong immune response. Therefore, a possible immunotherapy is the adoptive transfer of T cells, which are reprogrammed by introduction of an HIV-specific T cell receptor (TCR). This is done, up to now, through retroviral transfer. But this strategy harbours the threat of stable genetic alteration of autologous cells and the development of a life-long autoimmunity. Therefore, we investigated in TCR-RNA transfection intoCD8+ T cells using HIV-specific TCRs, which were able to recognize the HLA-A2restricted HIVpol-peptide ILKEPVHGV (IV9), and the HIVgag-peptide SLYNTVATL (SL9). These transfected T cells, gained the ability to produce the pro-inflammatory cytokines IL2, TNFa, and IFNg after stimulation with peptide-loaded target cells, and simultaneous expression of the latter two was observed. Moreover these cells showed an up-regulation of the activation marker CD25 and started to proliferate after stimulation with peptide-loaded target cells. More importantly, chromium-release assays proved that the reprogrammed CD8+ T cells, transfected with an HIV-specific TCR, were able to specifically lyse target cells loaded with the corresponding peptide, or presenting the natural processed epitope (SL9). The T-cells keep this lytic activity for at least three days. Furthermore, we compared the avidity of the parental CTL with the reprogrammed T cells and were able to show that the reprogrammed T cells were only one order of magnitude lower in avidity as the parental CTL. Taken together, functional virus-specific T-cells, which recognize and kill HIV-1 infected cells, can be generated by electroporation of TCR RNA. This technology represents an innovative, secure, and easy method to produce virus-specific T cells.

P094

Interleukin (IL)-31 induces pro-inflammatory cytokines in human macrophages and PBMCs upon stimulation with staphylococcal exotoxins

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Background: IL-31 is a newly characterized T cell-derived cytokine which is involved in atopic dermatitis (AD) and signals through a heterodimeric receptor complex composed of IL-31RA and OSMR which can be up-regulated upon stimulation with Staphylococcal enterotoxin B (SEB). AD is often complicated by an enhanced susceptibility to skin infections with *Staphylococcus aureus* (*S. aureus*). However, the regulation of the IL-31 receptor and functions of IL-31 in human monocytes and macrophages upon stimulation with staphylococcal components remain unknown.

Objective: We investigated the expression, regulation and function of the IL-31receptor in human macrophages and PBMCs upon stimulation with staphylococcal components.

Methods: Human PBMCs and macrophages were stimulated with SEB, α-toxin, IFN-γor IL-13 and IL-31RA expression and regulation were investigated both on mRNA and protein level. After up-regulation of the IL-31 receptor with SEB or α-toxin functional effects of IL-31 stimulation on cytokine secretion were measured on protein level.

Results: SEB as well as α-toxin could significantly up-regulate IL-31RA expression in monocytes, macrophages and macrophages co-cultured with T cells on both mRNA and protein level. Stimulation with IL-31 after up-regulation of the IL-31-receptor yielded in an enhanced secretion of IL-6, IL-1β and IL-18 in these cells. OSMR expression could not be found in monocytes and macrophages on both mRNA and protein level.

Conclusion: We provide new insights into IL-31 receptor regulation and functional effects of IL-31 in human monocytes and macrophages upon stimulation with staphylococcal components. Our study may have implications for the cutaneous inflammation in acute eczema where an overexpression of IL-31 has been described previously.

Clinical implication: Our findings provide a new link between staphylococcal colonization and pruritus induction via IL-31 which might be an interesting novel target for the development of antipruritic drugs.

P095

Alpha-toxin induces a higher T-cell proliferation in atopic dermatitis and is a strong inducer of Th1, Th2 and Th17 cytokines

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Background: Atopic dermatitis (AD) is a chronic inflammatory skin disease. One hallmark of AD is an enhanced susceptibility to bacterial skin infections, especially with *Staphylococcus aureus*. It has been shown previously that a subgroup of 34% AD patients are colonized with alpha-toxin producing *S. aureus in vivo* and sublytic concentrations of alpha-toxin can activate and polarize T cells intoTh1 cells *in vitro*. Therefore, alpha-toxin may have an influence on disease severity.

Objective: To explore further immunological effects of alpha-toxin on T-cell proliferation and cytokine secretion in peripheral blood mononuclear cells (PBMCs) from AD patients as well as healthy controls.

Methods: PBMCs from AD patients and non-atopic healthy controls were stimulated with sublytic alpha -toxin concentrations in a time and dose dependent manner. T-cell proliferation was investigated with CFSE-staining and cytokine secretion was measured on protein and mRNA level.

Results: Sublytic alpha-toxin concentrations induced a higher proliferation in T-cells from AD patients compared to healthy controls. Alpha-toxin was a strong inducer ofIL-2, IL-9, IL-10, IL-17, IFN-gamma and TNF-alpha. Interestingly, PBMCs from AD patients showed an enhanced production of IL-31 upon stimulation with alpha-toxin compared to healthy controls.

Conclusion: Our data show for the first time that besides pore forming and cell lytic functions sublytic alpha-toxin concentrations induce an enhanced T cell proliferation in AD patients compared to healthy controls as well as Th1, Th2 and Th17 cytokines. Therefore, staphylococcal alpha-toxin may play a pivotal role in the pathogenesis and maintenance of eczema flare-ups.

P096

Characterization of mast cell cultures from different origin

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For a long time, mast cells (MC) were primarily studied for their effects during type I immune reactions, later MC were identified to play a crucial role in innate and during the effector phase of adaptive immune responses, but only most recently, MC were identified as regulators of immunity. Much of this knowledge has been obtained by investigations carried out *in vitro* with MC generated from different organs or *in vivo* with MC deficient mice that are reconstituted with such MC. Using these experiments, the role for MC and MC genes could be characterized. The classical way to generate MC is from bone marrow (BMMC), alternatively MC can be obtained from foetal skin (FSMC) or from foetal liver (FLMC). However, the consequences of generating MC from different origin have never been characterized. We therefore established the side-by-side culture and generation of these MC types and analysed them in respect to morphology, surface marker expression, and release of mediators in immediate, intermediate, and late responses. All three cultures produced MC as depicted by electron microscopy and FACS analysis. However, *in vitro* expansion and lifespan of these MC was very different. Proliferation and expansion was highest for FLMC, while FSMC had the lowest proliferation rate but the longest lifespan. Moreover, beta-hexosaminidase release upon stimulation with PMA/Ionomycin was higher for FSMC compared to FLMC and BMMC. However, functional analyses with IgE-DNP revealed for all three MC types: (i) unequivocal immediate Ca2+-influx, (ii) identical sustained activation as detected by patch-clamp experiments for the K-channel SK4and (iii) the same levels of mediators. Next innate immune responses were analyzed. All three MC types expressed the same Toll-like receptors (TLR) and stimulation with several TLR ligands demonstrated comparable levels of different cytokines and chemokines on mRNA and protein level except for one cytokine: IL-10. High levels of IL-10 were only secreted by BMMC, much lower by FLMC while no IL-10 protein was detected in supernatants of stimulated FSMC. These investigations are most helpful to generate large quantities of well characterized MC, but also indicate that there maybe peculiar differences as seen for IL-10 that need to be addressed especially when MC functions as immune regulators are studied.

P097

Glucocorticoids promote resolution of inflammation by increasing survival of anti-inflammatory monocytes

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Active resolution of inflammation is a previously unrecognized process essential for tissue homeostasis. Monocytes play a pivotal role in the generation as well as resolution of inflammation. Glucocorticoids are widely used anti-inflammatory and immunosuppressive agents, acting on many cells of the immune system, including monocytes and macrophages. However, the exact mechanism of GC action on monocytes and macrophages is still not completely elucidated. We demonstrate that GCs exhibit anti-apoptotic effects in primary human monocytes resulting in differentiation to an anti-inflammatory phenotype. The molecular basis of this novel anti-apoptotic effect is a prolonged activation of the ERK/MAPK pathway resulting in inhibition of caspase activities and gene expression of anti-apoptotic genes via activation of c-Myc. We identifed up-regulation and activation of A3 adenosine receptor (A3AR) as the initial trigger of this anti-apoptotic pathway. Moreover, we demonstrate up-regulation of A3AR as well as enhanced resistance of monocytes to apoptosis after GC treatment in humans. This correlated with differentiation of an anti-inflammatory, pro-resolution phenotype *in vivo* as demonstrated by up-regulationof CD163 expression and an increased capacity to phagocytose pro-inflammatory stimuli. In summary, we deciphered a novel molecular pathway promoting survival of anti-inflammatory monocytes and provide evidence for its relevance *in vivo*. Specific activation of A3AR or its down-stream signalling pathways may thus be a novel therapeutic strategy to modulate undesirable inflammation in autoimmune disorders with fewer side effects via induction of inflammatory resolution rather than immunosuppression.

P098

Role of dermal fibroblasts in the regulation of dendritic cellmigration

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Dendritic cells (DC) are potent antigen presenting cells that capture antigen in peripheral tissue. Upon antigen capture DC mature and migrate to the lymph vessels and subsequently to the T-cell areas of draining lymph nodes. There they initiate the immune response by activating T lymphocytes. On their way from skin to the lymph vessel Langerhans cells or dermal dendritic cells have to cross the basement membrane as well as the dermal extracellular matrix. In the present study we show that dermal fibroblasts promote the migration of DC by stimulating the secretion of matrix degrading enzymes from DC. *In vitro* generated monocyte-derived DC were cocultured with dermal fibroblasts. In this coculture, the secretion of matrixmetalloproteinase-9 (MMP-9) from DC was significantly up-regulated within 6hcompared to DC alone or DC stimulated with lipopolysaccharide (LPS). In accordance, upon coculture with fibroblasts DC migrated significantly more effective through a collagen IV barrier than unstimulated DC or DC stimulated with LPS. Selective blocking of MMP-9 revealed the importance of MMP-9 for the migratory capacity of DC. Interleukin-6 was identified as one factor responsible for the fibroblast-stimulated MMP-9 secretion from DC. In summary, our results demonstrate that fibroblasts in the local dermal microenvironment potentiate the migratory capacity of DC, and thus might actively participate in the regulation of an immune response.

P099 (V19)

Regulatory T cells expanded in skin-draining lymph nodes via RANK-RANKL-activated Langerhans cells suppress cutaneous inflammation and colitis

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CD4+CD25+ regulatory T cells play an important role in suppressing immuneresponses but the requirements for peripheral maintenance of CD4+CD25+ T cells are incompletely understood. Previously, we demonstrated in a transgenic mouse model (K14-RANKL tg) that epidermal Langerhans cells (LC) stimulated via RANK-RANKL signalling can expand regulatory T cells. To identify the site of expansion we crossed K14-RANKL tg to DEREG mice, expressing a Foxp3-GFPfusion protein. Using a cutaneous contact hypersensitivity (CHS) model we were able to show that regulatory T cells are expanded in skin-draining lymph nodes by direct contact to RANK-expressing LC before elicitation of CHS. After elicitation of CHS, DEREG mice develop a normal ear swelling response and regulatory T cells are detectable in inflamed ears as well as in regional lymph nodes. In contrast, K14-RANKLxDEREG mice showed a significantly reduced ear swelling upon challenge and interestingly, the majority of regulatory T cells expressed increased levels of IL-10 but did not migrate to the inflamed ears suggesting that regulatory T cells in regional lymph nodes might inhibit the migration of effector T cells to inflamed tissues. Additionally, we investigated if non-cutaneous inflammation can be controlled by regulatory T cells expanded by cutaneous RANK-RANKL stimulated LC. Therefore, K14-RANKLxDEREG mice were fed with dextrane sulphate to induce colitis. Strikingly, all parameters pointing to a severe colitis such as weight loss or rectal bleeding were significantly reduced in K14-RANKLxDEREG mice compared to DEREG controls suggesting that regulatory T cells that have been expanded via cutaneous RANK-RANKL signalling are able to suppress non-cutaneous inflammation. Together, these results indicate that in K14-RANKL tg mice regulatory T cells are expanded in regional lymph nodes and seem to be activated but remain in draining lymph nodes upon cutaneous inflammation. Moreover, the suppressive activity of regulatory T cells in K14-RANKL tg mice is not tissue specific sinceCD4+CD25+ T cells expanded by epidermal LC down-regulate epithelial inflammation in the skin and the intestine.

P100

MAPK pathways regulate B7-H1 expression in dendritic cell (DC) subpopulations

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For the function of the immune system the tightly regulated balance between activating and suppressing mechanisms is crucial. Regulatory B7-H molecules, expressed by DCs, play an important role in upkeep of this balance but the regulation of their expression during DC maturation is not clear yet. We investigated the regulation of B7-H1 during DC maturation in monocyte derived DCs (MoDCs) and genuine DCs (gDC), isolated from blood of healthy donors. Stimulation of MoDCs as well as gDCs with a cytokine cocktail and TLR-ligands resulted in enhanced expression of classical costimulatory molecules like CD86 and increased T-cell stimulatory capacity. However this activation was also accompanied by up-regulationof B7-H1. While MoDC represent a homogenous myeloid population, gDCs consist of mixed populations of myeloid DCs (mDC) and plasmacytoid DCs (pDC) expressing different TLR receptor repertoires. To analyse the B7-H1 expression in these individual subpopulations we isolated and stimulated enriched mDC and pDC with TLR ligands and cytokines and performed FACS analysis and functional assays. LPS, Poly I:C and cytokines enhanced the expression of B7-H1 in mDCs. In contrast, in pDCs CpG and cytokines induced up-regulation of B7-H1 surface expression, although to a lesser extend as compared to mDC. The B7-H1 up-regulation was accompanied by the activation of the ERK and p38 MAPK-kinase pathways in DCs and specific inhibitors blocking those signalling pathways down-regulated B7-H1expression. To induce up-regulation of B7-H1 in gDCs simultaneous activation of both, ERK and p38 molecules, was necessary. Functionally the B7-H1 expression by DC was involved in silencing the IFN-γ production by T cells, since blockage ofB7-H1 on the DC in DC-T cells cocultures increased the amount of produced IFN-γ.Our result indicate that B7-H1 expression is up-regulated during maturation of MoDC and freshly isolated peripheral blood DC, involving the ERK/p38 MAPK kinase pathways. Functionally B7-H1 may serve to control IFN-γ cytokine production from T-cells and therefore our data may facilitate the development of new immunosuppressive therapies utilizing precise modulation of B7-H1 expression on DC.

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IL-27 cytokine affects phenotype and function of human DC

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Interleukin (IL-) IL-27, a new member of IL-12 family, was initially described as a proinflammatory cytokine. Meanwhile it is becoming clear that it also posses anti-inflammatory activity, since IL-27 down-regulates the differentiation of TH17 cells and induces inhibitory Tr1 cells. However until now nothing is known about the influence of IL-27 on dendritic cells (DC). We investigated the influence of IL-27 first on human monocyte derived DC (MoDC). We showed that treatment of MoDC withIL-27 led to specific up-regulation of B7-H1, B7-h (ICOS-L) and ICAM1 molecules on the surface of DC. This phenotypical change could be abrogated when DC were cultured additionally in the presence of a-IL-27 antibody. Otherwise IL-27 treated DC kept their immature phenotype. Moreover by coculture of IL-27 treated DC with allogeneic T cells we demonstrated that they have reduced capacity to stimulate allogeneic T-cells in proliferation assays as compared to control MoDC. In order to examine whether IL-27 exerts similar effects on freshly isolated DC, we isolated myeloid dendritic cells (mDC) directly from peripheral blood of healthy donors using paramagnetic beads, treated them with IL-27 and performed FACS analysis and functional assays. IL-27 treated mDC demonstrated similar phenotypic changes as MoDC, i.e., IL-27 kept mDC immature. In future studies we will further investigate, whether IL-27 treated MoDC and mDC, respectively, display a different cytokine profile and which molecule(s) of the B7-H family account for the reduced T-cell stimulatory capacity of IL-27 treated DC. In summary our first data suggest that, additionally to the dual role of IL-27 in the modulation of T-cell activation and differentiation, this cytokine is also involved in modulation of the DC compartment.

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Increased suppressor function of CD4+CD25+ regulatory T cells after interaction with endothelial cells

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Regulatory T cells play a critical role for the suppression of T-cell responses against innocuous antigens in the lymph nodes and at the site of inflammation. The recruitment of regulatory T cells into the inflamed tissue is highly dependent upon transmigration through the vascular endothelium. This process requires direct interactions between regulatory T cells (Treg) and endothelial cells (EC). Therefore, it might be possible that Treg - EC interactions have consequences for the Treg function. To test this, we cocultured activated CD4+CD25+ Treg and TNF-alpha/IFN-gamma stimulated EC over night and afterwards analyzed the Treg function. Treg cocultured with EC possessed an increased capacity to suppress proliferation of CD4+CD25- conventional T cells compared to Treg cultured in the absence of EC. In the supernatants of Treg - EC cocultures significantly increased amounts of IL-10 were detectable (130% increase) compared to the IL-10 levels in supernatants of Treg cultured alone. The observed IL-10 production was contact-dependent, since no increase in IL-10 production was determined in supernatants when Treg and EC were cocultured in a transwell-system without direct cellular contact. Intracellular staining for IL-10 revealed that Treg produced IL-10during coculture with EC in a cell contact-dependent manner. Furthermore, IL-10-producing Treg expressed high amounts of PD-1 on their cell surface. Further analysis of the PD-1 expression on Treg showed an up-regulation of PD-1 expression by 60-70% after coculture of Treg with EC compared to Treg cultured in medium alone. These data indicate that CD4+CD25+ Treg are able to functionally interact with endothelium. Since EC-conditioned Treg produce high amounts of IL-10 and up-regulate PD-1 expression these might be mechanisms by which Treg augment their suppressive capacity at the site of inflammation *in vivo*.

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Murine model of skin infection for *Staphylococcus aureus* to monitor innate and adoptive immune response

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Skin infections in humans due to *S. aureus* are clinically relevant and cause a variety of serious problems that can progress to sepsis and systemic shock. Therefore, a comprehensive understanding of the pathogenic pathways exerted by *S. aureus* is urgently needed to develop new therapeutic and preventive treatments.
Colonizing *S. aureus* can cause skin infection when the pathogen gains access to the subepidermal space. The decision, if infection is locally controlled or if it spreads in the dermis depends not only on virulence factors of *S. aureus*, but also on the host-specific innate immune response. Using a murine model of subcutaneous infection with *S. aureus*, we wanted to elaborate the particular leukocytic response pattern. To determine host-specific differences which lead to local containment or dissemination of *S. aureus*, different inbred strains of mice were infected subcutaneously with *S. aureus* strain SH1000 and their immune response was monitored by measuring (i) bacterial loads in systemic organs, (ii) cytokine release into the blood and (iii) histopathological analysis of inflamed tissue. To verify whether Staphylococcal infection lead to systemic dissemination we determine the bacterial loads in several organs. Although a comparable bacterial seeding per organ was observed, the immune response differed between different inbred strains of mice. Analysis of serum cytokine level revealed differences in production of proinflammatory cytokines, such as IL-6 and MRP8/14, among different inbred strains of mice. Furthermore, we identified the cells which are involved in this cytokine production. As expected, histopathological analysis showed that *S. aureus* infection induces an influx of monocytes and granulocytes. Therefore, the mouse model provides an excellent tool to study the molecular mechanisms of *S. aureus* induced skin infection and subcutaneous spread.

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STAT1-dependent dendritic cells education by T helper 1lymphocytes

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CD4-positive T cells (Th) critically control the outcome of immune responses to mycobacterial or viral infections, in autoimmune diseases, allergy or cancer. In this context, the effects of antigen-presenting cells (APC) on the differentiation of naive T helper cells into Th1, Th2 or Th17 lymphocytes is intensively studied. Surprisingly little is known about the reverse signalling of T helper cells on APC. Yet, this, reverse action is of key importance, as Th cells are not capable to rcognized their target cells and Th cell immune responses seem to be mediated by APC, including DC. To address this question, we started analyzing effects of differentiated Th1 lymphocytes on APC, and more specifically on DC during APC-T cell interactions. Indeed, Th1lymphocytes strongly modulated a series of central signalling molecules such as cMyc and signalling molecules, like the chemokines CXCL9 or CXCL10. In order to get more solid information on specifically Th1 cell-mediated signals, we performed gene arrays and compared DC from wild-type mice with DC from mice deficient in Signal Transducer and Activator of Transcription 1(STAT1) that is criticaly involved in theIFN- signalling cascade. Besides a number of chemokines, arrays revealed that Th1 cells strongly induced cell death-associated genes. Correlating these findings with functional outcome, we performed that Th1 cells severely impaired DC proliferation and induced apoptosis in up to 50% of wild-type DC but not STAT1-/-DC. Simultaneously, Th1 cells also abrogated the capacity to stimulate CD4 T cells in wild-type DC but not in STAT1-/- DC. Thus, Th1 cells exert a strong, STAT1-mediated negative feedback signalling on DC that severely impairs DC survival and their capacity to stimulate proliferation by CD4 T cells.

P105 (V18)

IL-12/IL-23p40 small interfering RNA (siRNA) establish protective immunity against autoimmune disease *in vivo*

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Experimental autoimmune encephalomyelitis (EAE) is a prototypic organ-specific autoimmune disease, mediated by inflammatory myelin-specific T helper (Th) cells. Destructive inflammation within the central nervous system is initiated by the infiltration of IFN-γ-producing Th1 cells and IL-17-producing Th17 cells. CFA stimulates dendritic cells (DC) through Toll-like receptors (TLR), and induces inflammatory mediators such as IL-6, IL-23 and IL-12 that promote the differentiation of naïve Th cells into Th1 or Th17 cells. IL-12 and IL-23 are heterodimers sharing theIL-12/IL-23p40 subunit. Experiments with knockout mice or therapies with antibodies revealed that the IL-12/-23p40 subunit is crucial for the development of EAE and other autoimmune diseases. Antibodies against IL-12/IL-23p40 improve EAE and psoriasis but bear the risk of severe infections, as evidenced by animal experiments. In contrast to antibodies, siRNA is short lived and interferes with immune responses for very limited periods of time. We therefore asked, whether siRNA allows establishing protective immunity when applied at the time of DC activation. We synthesized various siRNA directed against IL-12/IL-23p40 and tested their capacity to inhibit IL-12/IL-23p40 production by DC *in vitro* after TLR-stimulation. Naïve SJL-mice were immunized with PLP in CFA and received either PBS or siRNA at the time of immunization. In the presence of IL-12/IL-23p40 siRNA IL-12/IL-23p40 production by DC was impaired *in vitro* and *in vivo* and induced Th2 immune response *in vivo*. Therapy of mice with IL-12/IL-23p40 siRNA during immunization significantly improved the clinical course of EAE (20% with clinical symptoms in the siRNA group vs 100% diseased mice in the control group). This is the first report on the therapeutic use of an IL-12/IL-23p40 siRNA *in vivo*. Our results may provide the basis for new vaccination strategies with IL-12/IL-23p40 siRNA establishing protective immunity against organ-specific inflammatory autoimmune diseases.

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ICOS-ICOSL interaction: A single costimulatory pathway responsible for the tolerogenic function of human myeloid immature dendritic cells

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Dendritic cell (DC) based therapy of melanoma is an attractive immunotherapeutic strategy used by several groups world wide in the last 15 years. However, success of treatment is limited and improvement of therapeutic protocols is mandatory. It is well known that the functional activity of DC is strictly dependent on their state of maturation and differentiation. Whereas terminal differentiated DC efficiently induce T effector cell responses, immature DC are tolerogenic and are able to induce regulatory T cells (Tregs) that prevent an effective anti-tumour immune response. Nevertheless, the molecular basis of tolerogenic DC is widely unknown. Here we show that the inducible costimulator (ICOS) is an essential molecule for the immunosuppressive function of immature DC. ICOS-/- CD4+ T cells from rare ICOS-deficient CVID patients are resistant to anergy induction by immature DC and cannot be converted into suppressor T cells. Furthermore, blockade of ICOS-ICOS-L interaction between immature DC and resting CD4+ T cells by antagonistic antibodies or siRNA mediated knockdown of ICOS expression also inhibits the tolerance induction completely. Detailed analysis of this phenomenon showed that ICOS-ICOSL crosstalk stabilizes the IL-10R expression on activated T cells, which renders them susceptible for the well known suppressive effects of IL-10. These data show that a single costimulatory pathway between myeloid DC and T cells is able to direct the quality of the out coming immune response. Further investigations of the potential role of ICOS-ICOSL interaction in the maintenance of tumour tolerance will help to improve the efficacy of DC-based melanoma therapies in the future.

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A Janus-like role of activating transcription factor 3 (ATF3) critically determines susceptibility to systemic bacterial infections

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Systemic bacterial infections (e.g. cellulitis) activate the innate immune system and may result in severe shock (fever, tachycardia hypotension). These symptoms result from TLR-signals like lipopolisaccharide (LPS) released during bacterial infections. Recent data revealed that activation of the transcription factor ATF3 provides protection against the toxicity resulting from innate immune activation by LPS and protects from LPS-induced death, by initiating a negative feedback loop in the NFκB signal pathway. Here, we found that stimulation of dendritic cells (DC) or peritoneal macrophages (PM) with LPS increased expression of ATF3. Moreover, stress signals such as reactive oxygen species (ROS) or glutathione depletion as they occur during sepsis induce ATF3, suppress the *in vivo* release of inflammatory cytokines, namelyIL-6, and protect from LPS-induced shock and mortality. In consequence, glutathione depletion, as it is occurs during sepsis, enhanced LPS-induced ATF3 4fold, significantly inhibited IL-6 mRNA and protein > 90% *in vivo*, and significantly decreases the risk of LPS-induced lethality. Comparing ATF3.WT with ATF3.KOmice, we found that glutathione-depletion regulates IL-6 through a strictlyATF3-dependent signalling cascade, as cells from ATF3.KO mice produced normal amounts of IL-6, even at sub-lethally low glutathione levels and mice remained fully susceptible to LPS-induced lethality. Next we addressed, whether the increased awareness of ATF3.KO mice to innate signals such as LPS might, inversely, promote the protection against systemic infection. We therefore compared bacterial infection after coecal perforation in ATF3.WT and ATF3.KO mice. In WT mice bacterial infection resulted in glutathione depletion, high ATF-3 mRNA levels, low IL-6 and high mortality. In sharp contrast in ATF3.KO mice infection-induced glutathione depletion did not suppress the IL-6-mediated protection from sepsis-induced mortality. As ATF3 is induced within minutes following innate immune activation, it abrogates susceptibility to LPS-induced immune activation. Thus, induction of ATF3 induces an immune paralysis protective against bacterial toxins, while ATF3 dramatically increases susceptibility to bacteria through inhibition of IL-6.

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Glutathione concentrations regulate innate immunity *in vitro* and in septic patients via modulation of ATF3 (activating transcription factor 3)

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ATF3 a stress-regulated transcription factor, is activated in response to TLR-mediated signalling and initiates a central negative feedback loop in the NFκB signal pathway. As we found that glutathione depletion, as it occurs during systemic infectious diseases, increases susceptibility to bacterial infections in mice, we analyzed the effects of ATF3 on human immune cells and the production of IL-6 under conditions of sepsis. Stimulation of peripheral blood mononuclear cell (PBMC) and CD14+ monocytes with LPS slightly increases ATF3; glutathione depletion, as it occurs during sepsis condition increased expression of ATF3 5fold. This increase inATF-3 resulted in significant inhibition of IL-6, IL-12p40, IL-1β and TNF mRNA and protein expression by human PBMC and monocytes. Interestingly, restoration of glutathione with N-acetly-cysteine impaired ATF3-induction and restored cytokine secretion to control values. Inhibition of cytokine secretion was AFT-3-dependent, as blocking of ATF3 induction with specific siRNA fully restored cytokine production at sub-lethally low glutathione levels. To correlate these findings with the *in vivo* situation, as it occurs during sepsis in humans, we analyzed ATF3, IL-6, TNF expression and glutathione levels in the blood of septic patients around day 4 and at the time of recovery. We compared the data to those of healthy controls. Surprisingly, both glutathione and ATF3 expression were decreased at the beginning of sepsis. Yet, further analysis revealed that in the course of disease glutathione continued to decrease, while the ATF3 mRNA was restored to normal levels. IL-6 levels in the serum correlated negatively with ATF3 expression: being high at the beginning they declined with increasing ATF3 levels, down to levels found in healthy controls. Thus, glutathione levels regulate innate immunity during sepsis, by determining IL-6production through the induction of ATF3. The findings explain why therapeutic restoration of glutathione during early phases of sepsis improve the outcome of sepsis; they also predict that exaggerated glutathione restoration may increases the susceptibility to bacterial toxins, such as LPS.

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Functional link for a psoriasis candidate gene: S100A7 mediates leukocyte chemotaxis directly through the pattern recognition receptor RAGE

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Psoriasis is a widespread chronic hyperproliferative disease that is characterized by a steady influx of inflammatory cells into the skin and disturbed epidermal differentiation. Calcium-binding proteins of the S100 family are genetically linked to the disease, where S100A7 is abundantly expressed and secreted by the inflamed psoriatic epidermis. Little is known about the function and mechanism of action ofS100A7. *In vitro* and when injected into mice, recombinant S100A7 is chemotactic for leukocytes attracting mainly neutrophils and mononuclear cells. S100A7-mediatedchemotaxis is pertussis-toxin independent and can be blocked by silencing the pattern recognition receptor RAGE (receptor of advanced glycated end products) using neutralizing antibodies and homologous desensitization. The S100A7 provoked inflammatory response is attenuated when the recombinant protein is injected into RAGE-/- mice. *In vitro*, S100A7 binds to cells when RAGE is reconstituted and stimulates the phosphorylation of the mitogen activated kinase ERK, which is linked to activation of migration pathways. The corresponding mouse ancestor also binds RAGE and induces chemotaxis. These data indicate that S100A7 directly mediates leukocyte chemotaxis through RAGE. The S100A7-RAGE interaction could provide the basis for novel therapeutic applications in the treatment of psoriasis and other inflammatory diseases of the skin.

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Mast cell proliferation and differentiation is promoted by adhesion to fibroblasts

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The homeostasis of tissue resident cells in general is a complex interoperation of numerous processes, including proliferation, survival, and immigration and subsequent differentiation of precursors. The underlying mechanisms of mast cell (MC) homeostasis in peripheral tissues are largely unknown. Bone marrow derived cultured MCs (BMCMCs) have the capability to differentiate into both connective tissue MCs and mucosal MCs after transfer to genetically MC-deficient W/Wv mice. Further, BMCMCs exhibit increased proliferation and a change of their phenotype towards CTMCs when co-cultured with fibroblasts (Fbs). Interestingly, we observed that BMCMCs exhibited spontaneously a strong adhesion to Fbs. Thus, we investigated here the regulation of proliferation and differentiation of BMCMCs initiated by co-culture with Swiss albino 3T3 Fbs with focus on the impact of direct cell-to-cell crosstalk. As expected, the proliferation of BMCMCs co-cultured with Fbs was markedly increased as compared to those cultured alone. Surprisingly, this effect disappeared, if the direct BMCMC/Fb cell-to-cell contact was interrupted by micropore cell culture inserts. Furthermore, the histamine content of BMCMCs was strongly increased in BMCMC/Fb co-cultures but only if BMCMC could adhere to Fbs. Furthermore, we could show the same impact of direct crosstalk when assessing the expression of mast cell protease 4 mRNA, which is a late phase differentiation marker for murine MCs. Thus, our data show that the enhanced proliferation of BMCMCs and the differentiation towards connective tissue type previously demonstrated in this BMCMC/Fb co-culture system requires the firm adhesion to Fbs. This indicates membrane bound receptors as key elements in MC proliferation and differentiation processes and thus as interesting therapeutic targets.

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Selective targeting NF-kappaB in macrophages substantially alleviates skin inflammation in a mouse model of psoriasis

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Enhanced TNF-α production in pro-inflammatory macrophages (MΦ) has recently been identified to play a causal role in mouse models closely resembling human psoriasis and in human psoriasis itself. As TNF-α is a target gene of transcriptionfactor-kappaB (NF-κB) activation, and TNF-α itself activates NF-kB, it is very likely that enhanced NF-κB activation in MΦ is responsible for the pathogenic amplification of TNF-α production, thus driving psoriasis. However, so far NF-κB activation and its selective therapeutic inhibition in MΦ in human and murine psoriasis have not been studied. Using an immunohistochemical approach, we found a distinct co-localization of MΦ specific markers (CD163, F4/80) and markers for NF-κB activation (phosphorylated IkappaB alpha (IkBα) Ser32 and phosphorylated p65 Ser532) in biopsies of human plaque psoriasis and the murine CD18hypo psoriasis model. NF-κB activation was confirmed by western blot analysis from lysates of isolated MΦ.To further address whether activation of NF-κB in MΦ is causally involved in the pathogenesis of psoriasis, we selectively inhibited NF-κB in MΦ of the CD18hypomice by subcutaneous or intraperitoneal injection of the liposome-encapsulated naturally occurring NF-κB inhibitor, acetyl-11-keto-β-boswelic acid (AkβBA), which we have purified to chemical homogeneity from gum resins of Boswellia species (Th. S.).Notably, AkβBA-liposomes specifically inhibited phosphorylation of IkBα at Ser32 inF4/80 MΦ and also inhibited the NF-κB activation-dependent genes TNF-α, MCP-1, EGF and IL-20 24 h after injection, however, no inhibitory effect on T cells, neutrophils, mast cells or mature Langerhans/dendritic cells was observed. Importantly, treatment with AkβBA-liposomes, but not with PBS-liposomes, led to are markable and persistent improvement of the psoriasis form skin inflammation as assessed by an adapted PASI score. These data for the first time show that the NF-κB activation in MΦ is pivotal to the pathogenesis of psoriasis in the CD18hypomodel. Thus, selective targeting NF-κB activation of MΦ by specific delivery of AkβBA may represent a potential therapeutic strategy for psoriasis that combines therapeutic efficacy with reduced side effects.

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High-dose IFN-alpha therapy shifts the balance of T cell responses in stage III malignant melanoma patients: reduction of regulatory T cells and enhancement of effector/memory T cell subsets

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In stage III malignant melanoma patients IFN-alpha currently represents the only effective adjuvant therapeutic approach. IFN-alpha pleiotropically affects tumour cells as well as the immune system. In our study, we investigated the effect of IFN-alpha on the induction and activation of naturally occurring CD4+CD25+ regulatory T cells (Tregs) and effector/memory T cell subsets in stage III melanoma patients during adjuvant high-dose IFN-alpha therapy. Blood samples of 14 melanoma patients receiving high-dose IFN-alpha therapy (Kirkwood protocol) were obtained before and one month after treatment and the phenotype of T cell subpopulations was analyzed by three-colour flow cytometry. Here, we show that 4 weeks after IFN-alpha therapy, the frequency of CD4+CD25high T cells slightly increased from 1,1% to 1,5% in the peripheral blood ($P = 0.11$). However, detailed analyses showed that the majority ofCD4+CD25high T cells did not express Foxp3. In contrast, this Treg-associated transcription factor significantly diminished ($P = 0,059$) during treatment. Furthermore, expression of CTLA-4 and the IL-7 receptor, both important markers for homeostasis and function of Tregs, was unchanged 4 weeks after high-dose IFN-alpha therapy. However, expression of chemokine receptors important for migration and homing of T cells such as CCR5 and CCR7 significantly increased in the CD4+CD25high T cell subset after therapy. Moreover, IFN-alpha treatment provoked a significantly enhanced expression of the early activation marker CD69 and the leukocyte adhesion molecule CD62L in CD4+CD25high T cell subset. Taken together, our data indicate that the amplified numbers of CD4+CD25high T cells did not represent Tregs but activated effector/memory CD4+ T cells mobilized or induced by high-dose IFN-alpha treatment. This effect may contribute to the benefit of IFN-alpha therapy for patients suffering from stage III malignant melanoma.

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Immunomodulatory role of IL-4 on human dendritic cell phenotype and consequences on human Th cells polarization

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MDC1, MDC2 and slanDC form the three subsets of human myeloid conventional dendritic cells (cDC) in peripheral blood. These cells migrate into peripheral tissues where they are exposed to pathogens as one of the first part of the innate immunesystem. Finally these DC also activate T cells to initiate an adaptive immuneresponse. Interleukin 4 (IL-4) is an important cytokine present in inflammatory responses such as atopic diseases and its immunomodulatory role on these conventional human DC in peripheral blood has not been elucidated till now. Immature DC were isolated from peripheral blood from healthy donors. Toll-like receptor (TLR) expression was analyzed by RT-PCR analysis and DC were stimulated to mature with the appropriate TLR ligands in the presence or absence ofIL-4. Interestingly, IL-4 exposure changed dramatically the cytokine expression pattern of mature DC. The presence of IL-4 led to an increase of IL-12p70 secretion whereas the level of Interleukin-10 (IL-10) and other cytokines was strongly down-regulated. Furthermore co-cultures of IL-4 treated cDC with naïve T cells showed an increased level of Interferon-γ and a reduced level of secreted IL-4 upon T cell restimulation. To elucidate the mechanism of action of IL-4 on DC, a gene chip analysis was performed. IL-4 stimulation led to regulation of more than 200 genes. Via Western Blot the most important regulated proteins which are potentially involved in this regulation are about to be verified. Several transcription factors seem to be involved in this immunomodulatory effect of IL-4 on human DC. Analyses of tissue factors that regulate DC phenotypes are of great importance for the understanding of inflammatory diseases especially how these exacerbate and clear. Our study shows that local IL-4 concentrations ultimatively determine whether DC acquire an IL-10+ phenotype allowing Th2 cell differentiation or whether they become IL 12p70 producing DC that prime T cells to a Th1 phenotype. It may serve as a 'proof of principle' of how a tissue milieu in general can regulate DC phenotypes and complete immune responses.

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Systemic IL-4 therapy abrogates IL-23 secretion and Th17 responses in psoriasis

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Interleukin (IL)-17 and the Th17 driving cytokines IL-6, IL-23 and IL-1β are critically involved in the development of inflammatory autoimmune diseases associated with neutrophilic infiltrates like psoriasis, rheumatoid arthritis or inflammatory bowel disease. In consequence, therapies inhibiting either Th17 cells or the IL-12/23p40 subunit improve psoriasis. Beside T cell deletion or anti-cytokine therapies, systemic IL-4 therapy significantly improves psoriasis. IL-4 therapy induces intra-lesional IL-4 expression, but does not significantly suppress IFN-γ. We therefore addressed the *in vivo* effects of IL-4 therapy on the IL-23/Th17 pathway in humans. Prior to therapy, immunohistochemistry of psoriasis showed HLA-DR+ cells expressing IL-23, and CD3+IL-17+ cells. Following 6 weeks of IL-4 therapy, bothIL-23 and IL-17 became almost undetectable immunohistochemically. To quantify and characterize the underlying effect, we performed quantitative real time PCR: IL-4therapy dose dependently suppressed cutaneous IL-23p19 and IL-17 mRNA expression down to <10%, whereas IL-12p35 mRNA was rather increased. To uncover the mechanisms underlying the divergent effects of IL-4 therapy on eitherIL-12p35 or IL-23p19, we studied the effects of IL-4 on IL-12 and IL-23 production by human dendritic cells (DC). We stimulated DC with LPS either in the absence or presence of 100 ng/ml IL-4. As expected, IL-4 promoted increased IL-12p70. In sharpcontrast, IL-4 severely suppressed (≤ 90%) the production of the central Th17inducing cytokines: IL-1β IL-6, and IL-23. A similar suppression was observed in slanDC and conventional myeloid DC. Consequently, IL-4 treatment stimulated DC to prime Th cells for IFN-γ production, while it strongly inhibited their capacity to induce Th17 cells: Th cells stimulated with IL-4 treated DC selectively failed to produce IL-17 and IL-22, a second Th17 defining cytokine. Thus, we show *in vivo* and *in vitro* that IL-4 has opposing effects on either Th1 orTh17 differentiation. Thus, IL-4 therapy of humans provides the unique opportunity to suppress selectively Th17 responses mediating autoimmune disease while preserving the IL-12 needed for antimicrobial defence.

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Pregnane X receptor (PXR) signalling links xenobiotic metabolism to the cutaneous adaptive immune response

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The pregnane X receptor (PXR) is a ligand-activated transcription factor that regulates the xenobiotic response by modulating genes central to drug and hormone metabolism in the liver. The role of PXR in the skin and the skin immune system is unknown. We here report that expression of PXR and its well known target genes, i.e. CYP3A11 is markedly up-regulated upon diverse inflammatory stimuli in mouse T cells. *In vitro*, pharmacologic PXR activation inhibits T cell proliferation and anergizes T-lymphocytes by decreasing the expression of CD25, and the production of IFN-gamma. Conversely, T-lymphocytes isolated from spleen and lymph nodes of PXR deficient mice display an hyperproliferative response when co-cocultured with epidermal dendritic cells. Furthermore, in T-lymphocytes pharmacologic PXR activation decreases the active form of NF-kappaB and MEK1/2. *In vivo*, the modulation of T cell function by PXR activation has anti-inflammatory effects in mouse models of irritant dermatitis and allergic contact hypersensitivity. Together, these results reveal a novel immune-regulatory role of PXR in T-lymphocytes and in the cutaneous adaptive immune response.

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Active LFA-1 on dendritic cells prolongs APC-T cell contacts but inhibits T cell proliferation

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The antigen-specific interaction of T cells with professional antigen presenting cells (APC) such as dendritic cells (DC) is essential for the induction of an adaptive immune response. Beta-2-integrins are expressed by most leukocytes and play an important role in leukocyte migration and antigen presentation. To be functional, beta-2-integrins need to be activated by divalent cations. Normal murine T cells require active LFA-1 for adequate function, since LFA-1-deficient T cells are defective in antigen-specific proliferation. In contrast, although DC express all beta-2-integrins, beta-2-integrin deficient DC are fully capable to stimulate T cells. Indeed, as demonstrated previously, active Mac-1 (CD11b/CD18) expressed on DC inhibits the antigen presenting capacity of DC. In this study, we investigated the relevance of active LFA-1 (CD11a/CD18)expressed on DC for the antigen presenting capacity of DC and for the physicodynamics of the contact between T cells and DC during antigen presentation. Therefore, we used bone marrow derived DC that constitutively express the active form of LFA-1 (LFA-1d/d). In another approach, we blocked the expression of cytohesin-interacting protein (CYTIP), a molecule which is believed to down-regulateLFA-1 activity in DC, by siRNA transfection. Both approaches resulted in prolonged contact between DC and TCR transgenic T cells in a 3-D collagen matrix. To our surprise, the artificial elongation of DC–T cell interaction did not result in increased T cell activation but instead negatively interfered with antigen-specific T cell proliferation. Thus, we provide evidence that LFA-1 has divergent functions on DC and on T cells, and that prolonged adhesion of DC and T cells during antigen presentation does not necessarily correlate with better T cell activation.

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Expression of molecules involved in the innate immunity is strongly enhanced during lactation in mammary epithelial cells

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Mastitis of the mammary tissue in response to invading bacteria is a serious problem in breast-feeding women, and the innate immune system is of particular importance for rapid elimination of pathogens in this setting. Toll-like receptors (TLRs) have been shown to be important and sufficient to mediate the induction of microbial peptides (AMPs) which represent a crucial part of the innate immune response. In the present study we have investigated the expression and regulation of AMPs and TLRs in non-lactating versus lactating human mammary epithelial cells *in vitro* and *in vivo*. We could demonstrate that during lactation the production of S100A7 (psoriasin), human beta-defensin (HBD)-1 and calprotectin (S100A8 and S100A9) were strongly up-regulated in human mammary epithelial cells, whereas HBD-2, cathelicidin, RNase 5 and RNase 7 were not induced. We could further show that high amounts of S100A7 (up to 400 ng/ml), HBD1 (up to 150 ng/ml) and calprotectin (up to 15 ng/ml) were present in human breast milk. In addition, the exposure of human mammary epithelial cells in monolayer culture to a cocktail inducing lactation (consists of prolactin, dexametasone and insulin) sensitized these cells to TLR-mediated up-regulation of AMPs by both TLR-agonists and heat-inactivated *Escherichia coli* cultures. We therefore investigated whether TLRs were also regulated by lactation. Western blot analysis of human primary mammary epithelial cells *in vitro* and immuno-histochemical stainings of non-lactating and lactating breast biopsy specimens revealed that TLR-1, -3, -6, -7, -8 and -9 were significantly up-regulated during lactation. We conclude that an enhanced secretion of AMPs as well as an enhanced production of TLRs by lactating mammary glands might represent an important step for rapid elimination of invading pathogenic bacteria from the breast. Furthermore, the high concentrations of some AMPs in human breast milk might also represent an important protective factor for breastfed babies.

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Humanized mice: Effective models for preclinical testing of novel biologicalsagainst allergy and autoimmunity?

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Our immunologic knowledge is mainly based on analyses of human immune cells *in vitro* or on *in vivo* investigations in mouse models. However, complex biological processes are species specific and data from mouse models cannot be directly transferred to the human situation. The fatal failure during clinical testing of theanti-CD28 superagonistic antibody TGN1412 in 2006 approved this assumption. Therefore, the demand of so-called humanized mouse models, which allow systematic *in vivo* analyses of human immune reactions, is greater than ever. Here we provide detailed analyses and comparison of Rag2-/-γc-/- and NOD-scidγc-/-mice, humanized by an intrahepatically transfer of human CD34+ blood stem cells a few days after birth. Transfer of purified human stem cells led to an engraftment of human cells, thymus development and generation of various lymphocyte and leukocyte populations that repopulate the murine immune tissues. This includes not only a repopulated bone marrow with human CD34+ stem cells but also human T cell subsets in spleen, lymph notes and skin, B cell and NK cell populations as well as members of the innate immunity (e.g. monocytes, granulocytes) detectable in all tissues in the periphery. Thus, humanized mice emerge as a potentially powerful tool for preclinical studies of novel immuntherapeutic concepts and new biologicals, especially monoclonal antibodies to treat asthma, autoimmunity and melanoma diseases.

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Efalizumab induces T cell hyporesponsiveness *in vivo* and *in vitro*

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Targeting the lymphocyte function associated-molecule 1 (LFA-1; CD11a/CD18) with the anti α-chain (CD11a) binding monoclonal antibody Efalizumab (E) is an effective treatment regimen in moderate to severe psoriasis. Although LFA 1 is ubiquitously expressed on peripheral blood leukocytes, it is generally assumed that E exerts its effects mainly on T lymphocytes by blocking their migration and by interfering with the immunological synapse. To test the validity of the latter assumption, we asked whether E could interfere with T cell proliferation induced by qualitatively and quantitatively different stimuli. Using PBMC of both E treated patients and, after *in vitro* exposure to E, healthy individuals, we observed that anti-CD11a, while unable to interfere with T cell proliferation under optimal stimulation conditions (plate-boundanti-CD3, PHA), does effectively block the allogeneic mixed leukocyte reaction andanti-CD3-driven T cell proliferation induced under suboptimal conditions. To see whether reduced anti-CD3 proliferative responses, which we observed in PBMC of E treated patients, could also be induced *in vitro*, we exposed normal PBMC to E under various conditions. Cells were subsequently stimulated via CD3. Whereas addition of E to the medium alone did not modify the reactivity of PBMC to plate-boundanti-CD3, crosslinking via an anti-E antibody rendered T cells less reactive to a following anti-CD3 stimulus. Our findings suggest that binding of E to CD11a can induce molecular events that down-modulate T cell responsiveness/threshold to a T cell receptor-mediated stimulus. This may also provide an explanation as to why E is highly effective in patients with stable psoriasis, but often fails to control disease flares.

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The transcriptional program of naturally occurring human regulatory T cells

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Naturally occurring CD4+CD25+ Tregs (nTregs) are essential for T cell homeostasis and the maintenance of peripheral tolerance. They prevent the activation of auto aggressive T cells in the context of autoimmune diseases and suppress inadequate allergen specific T cells. On the opposite, nTregs inhibit also effective immune responses against tumours such as melanoma. A detailed understanding of molecular mechanisms that control the functional properties of human nTregs is mandatory for the development of novel immunotherapies against allergy, autoimmunity and cancer. Therefore, we initiated a genomic, proteomic and kinomeprofiling of human nTregs to identify key molecules in human nTregs associated with their functional activation which are responsible for their state of anergy and/or their suppressive activity. We started with large scale isolation of nTregs using whole leukapheresis products followed by polyclonal stimulation and analysis at different time points. As a result, we identified a distinct molecular activation pattern specific for the activation state of human nTregs. The impact of identified key molecules was tested in functional assays using specific inhibitors and siRNA mediated knockdown of these targets. A general transcriptional network analysis is currently under investigation and will be presented on the meeting. The main objective of our analysis is the identification of novel targets for the immunotherapeutic intervention of dysregulated immune responses in the near future.

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Large differences in the programming of naive T cells by slan (6-sulfoLacNAc) dendritic cells and CD1c+ dendritc cells from human blood

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Dendritic cells (DCs) play an important role in the immunopathogenesis of T-cell mediated inflammatory skin diseases. Distinct T-cell subsets can be found in lesional skin of psoriasis (Th17) and also of acute or chronic atopic dermatitis (Th2 versusTh1). It is assumed that individual DC subtypes differentially contribute to the predominance of either T-cell subset in a given disease, however, direct evidence for this is rather limited. A simple approach to study primary differences among DC subsets is the use of human blood leukocytes from healthy donors. We here present the first side-by-side comparison of human slanDCs (6-sulfo LacNAc-expressingDCs) and CD1c+ DCs (BDCA1+) in respect to their capacity to programme naïve allogeneic T cells. Initially we observed that slanDCs compared to CD1c+ DCs produced by far higher levels of proinflammatory cytokines (IL-6, IL-1β, IL-12p70- and TNF-α) when stimulated with various TLR-ligands. Interestingly, IL-23 production is differentially induced by TLR4- and TLR2-ligands within slanDCs and CD1c+ DCs and IFN-γhad opposing effects on the IL-23 production by these two cell types. Studies on the programming of T cells revealed a higher capacity to induce IL-10producing T cells by CD1c+ DCs. In contrast, slanDCs induced twice as many Th1cells compared to CD1c+ DCs. SlanDCs also displayed a strong capacity to programme IL-17 producing cells. Most of these IL-17 producing cells were also positive for IFN-gand TNF-a. Taken together slanDCs compared to CD1c+ DCs are by far superior in producing proinflammatory cytokines and in programming proinflammatory Th1/Th17effector T cells. In light of our recent findings that increased numbers of slanDCs are present in psoriasis, a disease in which high levels of IL-23 and IL-17 can be found, the data presented here support the proinflammatory role of slanDCs in the immunopathogenesis of psoriasis.

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Mice with heterozygous deficiency of manganese superoxide dismutase (sod2) display increased contact hypersensitivity (chs) response: a mechanism possibly involved in aging associated inflammation

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Dendritic cells (DC) are of central importance in the regulation of innate and adaptive immunity. It is well established that immunosenescence is associated with a chronic inflammatory state. However, to date little is known about the contribution of DC to this process. It has been proposed that production of mitochondrial reactive oxygen species is decisively linked with aging. Manganese superoxide dismutase (SOD2) protects against superoxide radicals by converting them into hydrogen peroxide. Here we investigated the function of partial SOD2 deficiency in an SOD2 heterozygous mouse model. When first investigating the number of Langerhans cells (LC) in old wt-mice we found that LC in 24 month old wt-C57/Bl6 mice were reduced to 1/3 the number found in 2 month old animals. Speculating that SOD2 deficiency may lead to an early reduction in LC numbers we investigated the number of LC in ear skin of 4 month old SOD2 ±mice and found that numbers were comparable to wt-litter mates. Immature bone marrow DC generated from SOD2 ±mice had a phenotype comparable to wt-DC. However, when stimulating these DC by LPS they were less efficient in upregulating IAb, CD44 and CD86. When inducing CHS in SOD2 deficient mice, we found that they had a significantly enhanced contact hypersensitivity response (CHS). Interestingly, when stimulating wt-T-cells with DC and staphylococcal enterotoxin A (SEA) that directly cross-links specific Vβregions on the TCR with the MHC II molecule on the antigen presenting cells, SOD2 ±DC were less potent activators. However, SOD2 heterozygous T-cells showed stronglyincreased proliferation, even when stimulated with SOD ±DC and SEA. Concluding our data in this model indicate that decreased SOD2 levels may be associated with impaired DC function, but increased T-cell proliferation in vivo leads to enhanced allergic inflammatory response.

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Antigen specific induction of the Th1 cytokine OPN is an important mechanism in the chronification of allergic contact dermatitis that may serve as a therapeutic target

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Allergic contact dermatitis (ACD) is a T-cell mediated immune response which in its relapsing chronic form is of high socioeconomic impact. The phosphoglycoprotein OPN has chemotactic and Th1 cytokine functions and in various models is essential for robust T-cell mediated immunity. In allergic contact dermatitis we found that memory T-cells are a major source of OPN. Upon NiSO4 stimulation T-cells from nickel allergic individuals highly secreted OPN. Similarly OPN was induced on them RNA and protein level by antigen specific CD4+ and CD8+ T-cells of TNCB sensitized mice. To characterize OPN regulation, T-cell clones from nickel allergic donors were established. Clones with an IFN-γsecreting Th1 phenotype secreted little OPN, while clones with predominant IL-4 secretion (Th2 phenotype) produced high amounts. OPN secretion by Th2 skewed T-cells may therefore contribute to Th1dominated switch towards chronification. To prove an *in vivo* relevance OPN WT and OPN null mice were compared in their chronic CHS response to TNCB. Indeed OPN null mice had reduced acute and chronic ear swelling response and fewer CD4 andCD8 effector cells entered the chronic CHS inflammatory site. To confirm that this was due to reduced OPN secretion by antigen specific activated T-cells, OPN WT or OPN null T-cells were transferred into OPN wild type RAG2 -/-mice. The mice that had received the OPN null T-cells showed impaired influx of CD4 and CD8 cells into the elicitation site, confirming that OPN from T-cells is important for attracting additional effectors. Finally, to demonstrate that OPN may be a therapeutic target, OPN antibodies were injected and partially suppressed the strong allergic ears welling in chronic CHS. In conclusion antigen specific modulation of OPN seems to be an important factor in the promotion of chronified T-cell mediated disease that may serve as a therapeutic target.

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Neuronal nitric oxide synthase is involved in maturation of human dendritic cells and induction of Th1 responses

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Dendritic cells (DCs) are the most potent antigen presenting cell of the immunesystem and crucial mediators of immune defence and tolerance. Their specific function within the immune system depends on their stage of maturation and differentiation induced by mediators of the immune system. The inducible nitric oxide synthase (iNOS) and its product nitric oxide (NO) is important for the function of murine DCs. In our study, we investigated the regulation of the arginase/NO-system by nitric oxide synthases (NOS) in monocyte-derived human DCs. The expression of arginase I and II and the major arginine transporter (CAT-1) was unaltered during maturation of human DCs. In contrast to the murine system, iNOS was not detected during DC maturation induced by various stimuli (cytokines, LPS + IFN-γ). However, the expression of endothelial NOS (eNOS) was slightly increased in immature DCs, whereas a pronounced expression of neuronal NOS (nNOS) was found in mature DCs by RT-PCR and westernblot analyses. In addition, reporter cell assays revealed relevant production of NO by mature DCs, but not immature human DCs. Functional analyses using specific inhibitors of NOS (L-NAME) or the NO target soluble guanylate cyclase (ODQ) in DCs resulted in a significant prevention of DC maturation(immature phenotype, diminished IL-12 production) associated with an inhibited T-cell proliferation and IFN-γproduction. In conclusion, in the human system, nNOS-relatedNO plays an important regulatory role for the maturation of human DCs and induction of Th1 responses.

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Live-cell imaging of primary DC versus a DC cell line - Interaction with naive Tcells and regulatory T cells within 3D collagen gels

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Dendritic cells (DCs), which play a central role in the initiation of immune reactions by activating naïve T cells, are an important target of suppression mediated by regulatory T cells (Tregs) in humans and mice. Previous studies from our group show that the duration of contact between mature DCs and Tregs is significantly longer than between mature DCs and naïve T cells. The myeloid DC line SP37A3 matches primary bone marrow-derived DC (BMDC) in phenotypical and functional properties, but show more homogenous differentiation stages. A further advantage of SP37A3cells in comparison with BMDC is that the former cells proliferate extensively in the immature differentiation state. In the present study we compared the physical interaction of antigen-presenting mature SP37A3 cells and BMDC of C57BL/6 origin with T cells from TCR-transgenic OT-II mice in 3D collagen matrices by time lapsevideomicroscopy with respect to contact duration and number of contacts. We show that the median contact time between CD4+ T cells and mature SP37A3 cells was comparable to that between CD4+ T cells and mature BMDC. Nevertheless, the number of long contacts exceeding 60 minutes was enhanced when using SP37A3as antigen presenting cells. With both DC populations the number of contacts with T cells was independent of the duration of contacts. Similar to our findings with mature BMDC, antigen-presenting mature SP37A3 cells developed more long contacts withCD4+ CD25+ Tregs than with CD4+ CD25- naïve T cells. Moreover, both BMDC andSP37A3 cells cultured with Tregs exhibited a decreased velocity and lower mobility than those cultured with naïve T cells. These results show that SP37A3 cells resemble BMDC in their interaction with naïve T cells and Tregs. SP37A3 cells will be usefull for studies analyzing molecules expressed on DCs, which may be involved in stabilising the DC–Treg interaction.

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Naturally occurring *S. aureus* derived peptidoglycan monomer is a NOD2ligand and significantly aggravates TLR-induced inflammation

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Atopic dermatitis (AD) is frequently colonized with *Staphylococcus aureus* and the extent of bacterial colonization correlates with disease severity implicating a role for *S. aureus* for AD inflammation. Important *S. aureus* cell wall components act as pathogen associated molecular pattern (PAMP) leading to inflammation and initiation of immune responses. It is believed that peptidoglycan (PGN), lipoteichoic acid (LTA) and lipoproteins are such *S. aureus* PAMP recognized by TLR 2. As the exact role and interactions of these different components has not been investigated, PGN preparations were purified from three *S. aureus* strains: wildtype *S. aureus* and *S.aureus* either deficient in lipoproteins or in LTA. The direct effect of PGN was minor since a reporter assay revealed impaired NFkB activation when residual lipoproteinsor LTA were absent. Therefore, investigations with pure preparations of single components followed. Thus, PGN was enzymatically digested into short fragments that are also naturally released from bacteria and from these fragments PG monomers were purified by HPLC. For the first time, natural PG monomers were analyzed in regard to immune sensing. Interestingly and in contrast to different pure TLR-ligands, dendritic cells (DC) incubated with PG monomer remained immature and produced no IL-12p40, IL-12p70 or TNFα. Consequently, experiments were performed to analyze a more complex innate immune sensing as observed *in vivo*. DC were stimulated with different TLR-ligands in the presence or absence of natural PG monomer. In this setting, PG monomer significantly enhanced IL-12p70production by all TLR ligands that signal via MyD88. To define the coactive pathway utilized by PG monomer, DC deficient in the intracellular receptor NOD2 were analyzed. Strikingly, amplification of IL-12p70 production by PG monomer was completely abolished in DC lacking NOD2 identifying PG monomer as a natural NOD2-ligand. These data demonstrate that PG monomer is an active and potent *S.aureus* PAMP. Moreover, our data disclose a new level of innate immune regulation that may also be of importance for inflammation in AD: Innate immune sensing remains mute in response to one single PAMP but the responses are markedly enhanced upon dual signalling.

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Cross-presentation of protein antigen by Langerhans Cells

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We recently demonstrated that murine Langerhans cells (LC) were capable of cross-presenting protein antigen to $CD8^+$ T cells *in vitro* and *in vivo* in skin-draining lymph nodes. This fact makes LC a promising target for immunisation strategies through the skin. Ovalbumin protein (OVA) was applied epicutaneously or injected intradermally and 6 h later epidermal explants were placed in culture. LC emigrated from epidermis proved to be potent stimulators for $CD4^+$ and $CD8^+$ T cells. OVA injected into the dermis was captured by migratory LC and presented more efficiently than topically applied protein antigen. Intravenous injection of Toll-like receptor (TLR) ligand LPS did not induce obvious phenotypical changes of LC in the epidermis, however, inhibited cross-presentation of topically applied OVA. LPS applied simultaneously with OVA on the epidermis had no effect on antigen presentation to $CD8^+$ T cells in the skin-draining lymph nodes.
Our findings indicate that application route of protein antigen into skin and systemic activation by TLR ligands can influence the efficiency of cross-presentation by skin dendritic cells. These findings will be important for the development of skin immunization strategies that target LC.

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The NFkappaB signaling pathway is involved in the LPS/IL-2 induced up-regulation of FoxP3 expression in human $CD4^+CD25$ high regulatory T cells

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Natural $CD4^+CD25$ high regulatory T cells (Treg) play a pivotal role in inhibiting organ-specific autoimmune diseases and allergy. However, the mechanisms leading to the activation of regulatory T cells within an inflammatory setting still remain unclear. Recent evidence pointed out the importance of the proinflammatory Endotoxin LPS for survival and suppressive function of Treg. The stimulation of the LPS receptor, TLR4, leads to the activation of the transcription factor NFkappaB. In this study, we demonstrate that TLR4 mRNA is expressed at significant higher levels in Treg compared to $CD4^+CD25$ low effector T cells (Teff). Furthermore, our results indicate that upon stimulation of purified Treg with LPS in combination with IL-2, expression of the Treg specific lineage marker FoxP3 is increased and the suppressive capacity is markedly improved compared to unstimulated Treg. TheLPS/IL-2 induced up-regulation of FoxP3 expression can be abrogated by a previous blockade of the NFkappaB signal transduction pathway by the IKK Inhibitor III. The results suggest an important role of the NFkappaB signalling pathway for the modulation of phenotype and suppressive function of regulatory T cells during inflammation.

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Platelet P-selectin as a biomarker in psoriasis

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Hemostasis-maintaining platelets are also recognised as important modulators in the regulation of immune response. Activated platelets expressing P-selectin (CD62P) increase cell interactions and are involved in the extravasation of leukocytes into the inflammatory skin. Considering adhesion molecule P-selectin as a possible general indicator of inflammation, we investigated its expression in psoriasis and other inflammatory and infectious skin diseases.
Platelet activation measured as P-selectin expression was assessed by flowcytometry in 43 patients suffering from psoriasis vulgaris, admitted for in-patient treatment to our dermatological department. Thirty-eight healthy volunteers served as controls. Increased median P-selectin expression on the platelets was observed in patients with psoriasis before treatment, compared to the control group of healthy persons (3.48% vs 1.41%, $P < 0.005$). The successful anti-psoriatic therapy was followed by decrease in the median PASI from 17 to 4.8, as well as in levels of median P-Selectin from 3.48% to 1.33%, $P < 0.005$. Changes in the PASI evaluated for psoriasis patients before and after the successful anti-psoriatic therapy correlated with changes in P-selectin expression, $r = 0.33$, $P < 0.05$. In addition we investigated P-Selectin correlation with those PASI components reflecting inflammation: erythema alone and erythema with induration. These analyses documented an even better correlation ($r = 0.39$, $P < 0.05$ and $r = 0.45$, $P < 0.02$ respectively). Successful therapy resulted also in a marked decrease of median P-selectin expression in cases of other inflammatory and infectious skin diseases ($n = 50$, from 5.02% to 1.8%, $P < 0.05$ and $n = 10$, from 3.22% to 0.96%, $P < 0.05$, respectively).
Taken together, patients with inflammatory and infectious skin diseases exhibit increased platelet activation as documented by elevated P-selectin expression. Successful anti-inflammatory treatment results in reduced P-selectin expression. In the case of psoriasis, the change in P-selectin values correlates with the PASI, particularly with erythema and in duration which reflect inflammation. Platelet P-selectin expression may therefore be a potential biomarker for the severity of psoriatic inflammation.

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Characterisation of a human chitinase as mediator of the innate immune system

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Chitin, uniformly composed of N-acetylglucosamine (GlcNAc) units, is the second most abundant biopolymers in the world and part of crustaceans exoskeleton and of fungal cell walls. Chitin contents of fungal cell walls depend on the species and ranged from less that 10% in yeast species such as Candida albicans to about 20% in filamentous fungi such as *Aspergillus fumigatus*. Once, chitin is synthesized it can be converted to chitosan by the action of chitin-deacetylases. Although the physiological role of chitosan is not yet revealed pure chitosan was found to support wound healing.
We have investigated the enzymatic degradation of chitosan by human chitotriosidase a functional chitinase with the ability to hydrolyse chitin.
We found that the degradability of chitosan was controlled by the degree of acetylation (DA) since chitosans with high DAs were strongly degraded where by chitosans with low DA were less affected. Applying matrix-assisted laser desorption/ionisation time of flight mass spectroscopy and a computer-based model of chitotriosidase activity we could prove that the enzymatic degradation requires only two GlcNAc-units per cleavage which proceeds in a mainly endo-mode of action. However, further analysis indicates an exo-like progression of chitotriosidase for large and highly acetylated chitosans which is declined as processivity. In contrast to a pure endo-mode of hydrolysis processivity enables immediate generation of small chitosan oligomers as soon as chitotriosidase is in contact to chitosan. In subsequent experiments we could show that exactly such small oligomers were able to activate human macrophages and to provoke an inflammatory response indicated by an increased expression of TNF-alpha, IL-6 and chitotriosidase. Strongest cell activation was observed for small oligomers with a degree of polymerisation (DP) of about 2–4and with a DA above 40%.
Our data suggest that chitotriosidase is able to generate small chitin- and chitosan oligomers that act as signal molecules to elicit an inflammatory response. Further studies will investigate the role of chitotriosidase during skin inflammation and especially during chitosan-controlled wound healing.

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Phänotypische und morphologische Charakterisierung von murinenknochenmarksgenerierten Dendritischen Zellen aus Syndecan knock out Mäusen

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Die antigeninduzierte Aktivierung und Migration von Dendritischen Zellen (DZ) ausperipheren Geweben (z.B. der Haut) ist eng an eine Interaktion mit derExtrazellulären Matrix (EZM) verknüpft. Syndecane (SDC) sind transmembranäreProteoglycane mit Heparansulfat-Seitenketten. SDC fungieren als Korezeptoren, dieextrazelluläre Signale, wie z.B. Zytokine, binden und sequestrieren und darüber dieZellmigration beeinflussen. Die Induktion einer Immunantwort durch DZ ist wesentlichabhängig von ihrem Migrationsverhalten und dem Zytokinmilieu, in dem schließlicheine T-Zell Interaktion stattfindet. Wir vergleichen die Differenzierung vonKnochenmarkszellen zu Dendritischen Zellen aus C57/BL6 Mäusen mit SDC1-/- undSDC4-/- Mäusen im C57/Bl6 Hintergrund.
Dendritische Zellen von SDC1-/- und SDC-4-/- Mäusen wiesen eine ähnlicheMorphologie auf. Bei der Lipopolysaccharid induzierten Maturation zeigten sich kaumUnterschiede der Expression klassischen Marker CD86 und IAb. Wildtypmäuse alsauch die SDC-1-/- Mäuse wiesen eine erhöhte Expression von SDC-4 nach LPSStimulation auf. SDC-4-/- Mäuse zeigten hingegen eine erhöhte Expression vonSDC-1, was möglicherweise auf eine kompensatorische Regulation hinweist.

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Homing receptor expression in a lymphocyte homing model to human skin xenografts

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Studies of lymphocyte homing performed *in vitro* or in mice are inherently limited in application to human disease. To address this issue, we have developed a homing model based on mice bearing both human skin xenografts as well as circulating human peripheral blood leukocytes.
We had shown earlier that mouse vessel penetration of human skin xenografts begins and human vessels within the grafts gained continuity with the mouse circulation between day 5 and day 10 after engraftment. From day 10 onward, the number of human vessels and the degree of continuity with murine circulation was continuously reduced.
Now we further explored vascular homing characteristics in the xenografts. Analysis of adhesion molecule expression in human skin xenotransplants revealed that the number of ICAM-1 and E-selectin positive vessels remained low after transplantation and that expression at 10–20 days posttransfer was similar to that found in pretransplant human skin. Local Injection of TNF-alpha induced fivefold upregulationof these adhesion molecules.
Leukocyte trafficking to human skin under various conditions was studied by adoptive transfer of human peripheral blood mononuclear cells labeled with Cell Tracker Orange into skin grafted mice. After 24 h, grafts were harvested and digested with collagenase D and the resultant cell preparation characterized by flow cytometry. Activation of human skin grafts with TNF-alpha in combination with the chemokineSDF-1 (CXCL12) or TARC (CCL17) resulted in a substantial (up to 300-fold) increase in cell accumulation within grafts compared to TNF-alpha treatment alone.
We conclude, therefore, that during the time frame of 10–20 days after engraftment adhesion molecule expression in human skin xenografts is similar to pretransplanted skin so that adhesion receptor manipulation and its effects can be studied in this model. Furthermore initial studies on the role of chemokines show that TNF-alpha alone stimulates endothelial adhesion molecule expression, but is not ready to induce a significant increase in lymphocyte homing to the skin without additional application of chemokines.

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Intradermal administration of a synthetic glycolipid antigen together with a tumor model antigen induces efficient T cell responses against melanoma

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Interaction of natural killer T (NKT) cells with dendritic cells (DC) presenting glycolipidantigens on CD1d molecules improves T cell responses. Cytokines released by NKT cells enhance maturation of DC which in turn boost T cell responses. Thus, glycolipid antigens are currently tested as adjuvants for immunotherapy. We wondered whether skin DC, Langerhans cells (LC) in the epidermis and dermal DC in the dermis, express CD1d and are capable of presenting lipid antigens to NKT cells. All skin DC subsets up-regulated CD1d upon migration to the draining lymph nodes and were able to present the synthetic glycolipid antigen alpha-Galactosylceramide(alpha-GalCer) to the NKT cell hybridoma DN32.D3. Intradermally injected alpha-GalCer was presented by migratory skin DC and lymph node DC to NK Thybridoma cells. After intradermal administration of alpha-GalCer more DC and more activated NK T cells were present in the lymph nodes. Co-application of alpha-GalCer with the tumor model antigen ovalbumin induced strong $CD8^+$effector T cell function that could be targeted against B16 melanoma cells expressing ovalbumin. We are currently testing if this effect is partly mediated by migratory skin DC. In conclusion, skin DC are able to interact with NK T cells and might be involved in anti-tumor responses after intradermal immunization.

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Intrathymic beta2 integrin deficiency generates unconventional memory-like $CD44^+$ TCR$\alpha\beta$CD4-CD8- (DN) T cells

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Thymus is the specialised organ where maturation of conventional and unconventional T cells occurs. But distinct sets of unconventional T cells develop extra thymically. We previously demonstrated that β2 integrin deficient (CD18-/-) mice accumulate unconventional TCR$\alpha\beta$CD4-CD8- (DN) T cells expressing a characteristic memory-like $CD44^+$ phenotype in their periphery driven by homeostatic expansion under lymphopenic conditions in CD18-/- mice.
We here investigate the differential contribution of selective β2 integrin deficiency on T progenitors and the intra- and extra-thymic hematopoietic environment (since β2integrin are only expressed on hematopoietic cells) in the primary generation of unconventional TCR$\alpha\beta$DN T cells before they undergo homeostatic expansion. We found that peripheral memory-like $CD44^+$ TCR$\alpha\beta$DN T cells physiologically occur also in spleens of CD18wt mice at low numbers, whereas they were not convincingly detectable in spleens from native a thymic nude mice. This suggested that their development required the presence of a functional thymus. However, functional TCR rearrangement was also required, since in thymus-competent RAG2-/- mice, $CD44^+$TCR$\alpha\beta$DN T cells were also not detectable. Further establishing a key role for thymus in the generation of $CD44^+$ TCR$\alpha\beta$DN T cells, we detected significantly higher numbers of these cells in thymi from CD18-/- as compared to CD18wt mice. Furthermore, the inability to recover CD18-/- TCR$\alpha\beta$DN T cells in bone marrow-reconstituted athymic nude mice showed that β2-integrin deficiency of hematopoietic progenitor cells was not the reason for their excessive production inCD18-/- mice. Collectively, these data reveal that absence of β2 integrins during intrathymic T cell development plays a critical role in the generation of memory-like$CD44^+$ TCR$\alpha\beta$DN T cells that accumulate in periphery of CD18-/- mice while they progress with age.

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Development of a highly effective adsorber matrix for immunoapheresis therapy of pemphigoid gestationis

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Pemphigoid gestationis (PG) is a subepidermal blistering autoimmune disease associated with pregnancy. Patients suffer from pruritic polymorphic skin lesions, blisters are not always present. Immunopathologically, PG is characterized by the linear deposition of C3 or less often of immunoglobulin (Ig)G at the dermal-epidermal junction. Autoantibodies in PG are directed against the 180 kDa bullous pemphigoidantigen 2 (BP180), also termed type XVII collagen, a hemidesmosomal transmembrane glycoprotein. In PG, the N-terminal portion of the extracellular 16th non-collagenous domain (NC16A1-3) has previously been identified as an immunodominant region of the molecule. In the present study, we screened a library of short peptides (12 amino acid residues) derived from the NC16A1-3 region ofBP180 for their ability to suppress the binding of autoantibodies from sera of PG patients ($n = 7$) to recombinant NC16A. The peptide showing the strongest inhibitory effect and some of its derivatives were characterized with respect to their relative binding affinities to PG autoantibodies using ELISA data. Based on these results, an affinity matrix was designed consisting of a peptide ligand coupled to sepharose via ahydrophilic spacer and a stable acid amide bond. Subsequently, the effectiveness of this matrix was analyzed using an *in vitro* system where cryosections of human skin were incubated with IgG from PG patients and neutrophils from healthy volunteers. While PG IgG resulted in dermal-epidermal separation of the cryosections, preadsorption of patients' IgG on the newly developed matrix completely abolished the pathogenic effect. Due to the straightforward design, the improved chemical stability compared to other matrices based on cyanogen bromide chemistry, and the selective absorption of PG autoantibodies, this matrix qualifies as a prototype for a specific immunoapheresis therapy for PG and possibly other pemphigoid diseases, including bullous pemphigoid, caused by autoantibodies to the BP180 NC16Adomain.

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AP-1 represses INK4-dependent and -independent tumor suppressor pathways in human melanoma

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In the past years, cell biologic analyses have uncovered serious gaps in our understanding of genes and pathways that function to constrain transformation of melanocytes into lethal melanomas. In melanoma, oncogenic BRAF/NRAS mutations induce activation of AP-1, a fundamental regulator of cell proliferation and oncogenic transformation. Though of fundamental importance, the effects of AP-1 activity are largely unknown in human melanoma.
We used the human melanoma cells LOXIMVI and UACC257 to establish line sallowing inducible expression of a c-Jun (AP-1) dominant negative mutant. Using these cell lines in cell cycle analyses, proliferation and colony formation assays *in vitro* and an *in vivo* model of the disease, we show that AP-1 activity critically controls cell cycle progression at G1 and accelerates proliferation/growth of melanoma cells. Western blot analysis and RNA interference of genes involved in cell cycle progression at G1 identified repression of p18INK4c by AP-1. A series of functional studies employing promoter reporter assays, ChIP, immunofluorescence, and (Co-)IP confirmed that p18ink4c is a direct target of AP-1. Co-transfection and RNA interference in Western blot and promoter reporter analyses revealed also indirect repression of p21Cip1 by AP-1 via Tbx2. Finally, in-silico analysis of mRNA expression profiles from human melanoma samples identified c-Jun and JunD as potential AP-1 heterodimerization partners, which was supported by functional studies as described above.
These results identify for the first time INK4 member p18INK4c as an important melanoma suppressor gene whose repression, in combination with p21Cip1, allows transduction of BRAF/NRAS-induced and AP-1-mediated mutagenic signals inhuman melanoma.

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Characterization of the inflammatory skin disease in CTLA-4 knockout mice

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CTLA-4 (CD152) and perforin (Perf) share functional similarities. Both are well known negative regulators of immune responses, they are stored in intracellular granules and, after activation of T cells, are released into the immunological synapse. We recently demonstrated a decisive role for Perf in IgE-control *in vivo* in mice and inpatients with extrinsic atopic dermatitis. In these, a highly significant Perf-granule reduction as well as a Perf-hyper releasability are well known. We now asked, if CTLA-4 may be involved as well in skin immunopathology. Therefore, the skin of CTLA-4 knockout (-/-) mice was investigated. 10–20% of these developed a heavily itching skin disease within the first 5 weeks of life. During disease progression the fur thinned out at the flanks and neck, an erosive alopecia with fuzzy boundaries developed. Punch biopsies were obtained from lesional and from clinically uninvolved appearing skin. Biopsies from wild type as well as from heterogeneous CTLA-4 +/-mice served as controls. Histology revealed a significant inflammatory infiltrate in allCTLA-4 -/- skin biopsies. In severe lesions, no follicles or only microfollicles were sporadically present. A dense lymphohistiocytic infiltrate with a dominance of large granule-containing cells filled the dermis. Several cells showed signs of atypical mitosis. Inflammatory cells encroached upon the akanthotic epidermis where apoptotic keratinocytes were found. The thick hyperkeratotic and parakeratotic stratum corneum was filled spot wise with neutrophilic granulocytes. Gram-, PAS- and Grocott-staining ruled out the presence of microbes. Taken together, knocking out CTLA-4 in mice unleashes within 5 weeks a tremendous storm of skin inflammation which has no identical parallel in human skin pathology. Our results underline the importance of CTLA-4-based immuno regulation for the homeostasis of the skin immune system. The CTLA-4 gene polymorphisms known to be associated with atopy may, therefore, be of direct functional relevance for AD.

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Immune monitoring of peptide specific $CD8^+$ T-cell responses

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Any effective vaccination protocol should ideally lead to the induction or enhancement of T-cell mediated immune responses. To establish such protocols animal models are indispensable and robust methods to determine vaccination efficiency are critical. To optimize monitoring of epitope specific $CD8^+$ T-cell responses in mice, we compared three different methods, namely *in vivo* cytotoxicity assay and ELISPOT, *ex vivo* or after *in vitro* re-stimulation. To this end, mice were vaccinated with TRP-2180-188 pulsed APCs in combination with CpGoligodeoxynucleotides (CpG ODN) and IL-2. High frequencies of epitope specific$CD8^+$ T-cell responses were reproducibly detected in vaccinated mice in comparison to untreated mice using the *ex vivo* ELISPOT. Similarily, highly specific lysis of epitope pulsed target cells, was detected in vaccinated mice performing an *in vivo* cytotoxicity assay. In contrast, however, after *in vitro* re-stimulation of cells of vaccinated and control mice, quite distinct results could be observed using ELISPOT. Therefore the *in vivo* cytotoxicity assay and the *ex vivo* ELISPOT are more reliable for monitoring epitope specific $CD8^+$ T-cell responses than the assays after *in vitro* stimulation.

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Integrative cGH and gene expression outlier analyses identify metastasis suppressor 1 (MTSS1) as a novel lineage-addicted cancer gene in human melanoma

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Genomes of human cancers, including human sporadic cutaneous melanoma, are characterized by numerous (epi) genetic aberrations resulting in distinct gene expression signatures. To unveil patterns of genetic alterations linked to genesis and spread of human melanoma, we integrated cGH arrays with a novel bioinformatic algorithm, so called gene expression outlier analysis, from human melanoma samples and identified metastasis suppressor 1 (MTSS1), a regulator of the HOG-GLI pathway, as a target of a novel focal chromosomal amplification on 8q24.13. MTSS1 over expression was more prevalent in metastatic disease and associated with decreased overall survival in primary melanoma patients. By a series of functional studies employing RNA interference and reconstitution approaches we could demonstrate that MTSS1 is essential for melanoma cell growth (particularly forG1/S transition), filopodia formation, cell motility, spreading, and invasion in to extracellular matrix *in vitro*. Reconstitution experiments with wild-type and an IRSp53/MIM domain (IMD)-mutant of Mtss1 suggested a role of membrane deformation and tubulation in cellular invasion. *In vivo*, MTSS1 silencing inhibited pulmonary metastasis of B16F1 cells in a mouse model of the disease. In addition to chromosomal amplification, MTSS1 over expression in normal human melanocytes can also be induced by Microphthalmia-associated transcription factor (MITF). By linking melanocyte master regulator MITF to cell adhesion, motility and invasiveness, MTSS1 represents a distinct class of lineage-addicted cancer genes required for tumor progression.

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Lipofection or electroporation of RNA – consequences of different antigen-distribution on immunogenicity

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RNA transfection has emerged as a standard method to load dendritic cells with antigen for subsequent use in therapeutic vaccination against cancer. In most cases, the cells are electroporated as this results in a high percentage and satisfying expression of the introduced protein. However, other methods of transfection, i.e. lipofection, can result in an expression pattern that resembles the natural situation more closely, as this approach results in a more uneven distribution, with few cells expressing very high amounts of the antigen and a large proportion expressing little or no antigen.
We compared antigen loading of DC by MelanA-RNA electroporation and lipofection side by side, and examined the influence of the difference in antigen quantities on the capacity of the DC to prime MelanA-specific $CD8^+$ T cells. DC, lipofected with Transmessenger, expanded MelanA/A2-tetramer binding T cells substantially better than DC that had been electroporated. Therefore, we conclude that few cells expressing high levels of antigen are better for T-cell priming than many cells expressing intermediate quantities. As the presence of cells that present antigen at high densities bears the threat of deletion of high avidity T cells, we determined the functional avidity of the expanded cells in an ELISPOT-based protocol: equal numbers of MHC tetramer-binding T cells were used on target cells which were loaded with titrated concentrations of MelanA/A2 peptide. This allowed us to determine the distribution of high, intermediate, and low avidity T cells after antigen-specific priming. We observed no substantial difference in avidity between T cells that had been stimulated with the differently loaded DC. Repeated re-stimulation, however, resulted in little to no further expansion of the specific T cells in our *in vitro* system.
Taken together our immunopotency data clearly favor a strategy of maximal antigen loading of a few DC over a homogenous loading with lower antigen density. It is still open whether this '*in vitro* rule' holds true *in vivo*.

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Identification of $CD4^+$ T cell epitopes from human melanoma associated tumor antigens TRP-1 and TRP-2 based upon immunization of HLA-class II transgenic mice with recombinant adenovirus and combinatorial peptide library screening *in vitro*

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The two melanoma differentiation antigens tyrosinase-related protein 1 (TRP-1) and tyrosinase-related protein 2 (TRP-2) are targets of spontaneous cytotoxic $CD8^+$ Tcell (CTL) responses in melanoma patients. Since induction and maintenance of tumor-specific $CD8^+$ T cell responses depend on the activity of specific $CD4^+$ T helper (Th) cells, we set out to establish a comprehensive screening strategy for the identification of novel Th epitopes that could be used as tools in immunotherapy approaches against malignant melanoma. In fact, upon immunization ofHLA-DRB1*03-transgenic mice with recombinant adenovirus encoding TRP-1 or TRP-2 (AdV/TRP-1, AdV/TRP-2) followed by combinatorial peptide library screening *in vitro*, four DRB1*03-restricted Th epitopes were determined (TRP-1 64-78, TRP-1284-298 and TRP-2 60-74, TRP-2 149-163). All epitopes except TRP-1 64-78 could also stimulate Th cells of DRB1*0301+ donors. Whereas TRP-260-74 represented aDRB1*0301-restricted Th epitope previously identified by us, TRP-2149-163 turned out as a new Th epitope as Th cells of a DRB1*0301+ donor stimulated with this peptide also recognized HLA-matched targets cells with endogenous TRP-2expression. Thus, vaccination of HLA-class II transgenic mice with a strong, global antigen, i.e. recombinant adenovirus expressing the relevant tumor antigen followed by combinatorial peptide library screening *in vitro* could offer a rapid strategy for comprehensive identification of Th epitopes also of other human tumor antigens pointing towards a broad applicability of this approach.

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IFN-γ impairs the NKG2D-mediated killing of melanoma cells by natural killer cells

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Human malignant melanoma in its metastatic disease stage is frequently characterized by a down-regulation or even an irreversible total loss of HLA class I expression. Therefore, such tumor cells should be recognized and eliminated by cytotoxic natural killer (NK) cells, which prompted us to study the melanoma-NK interaction. Indeed, all HLA class I-negative cell lines established from metastatic tumors of different melanoma patients expressed the surface molecule MICA, a ligand of the activating NK cell receptor NKG2D. Analysis of MICA expression in frozen tumor tissues revealed a more variable and often low expression level in situ. Therefore we asked if factors of the tumor micro-environment, such as the cytokine IFN-γ, might influence MICA expression on melanoma cells. Interestingly, melanoma cells reduced the surface expression of MICA when treated with IFN-γ. By quantitative RT-PCR we observed a down-regulation of MICA mRNA upon cytokine treatment and siRNA experiments suggested that STAT-1 is involved in this process. Importantly, IFN-γ-treated HLA class I-negative melanoma cells were less susceptible to NKG2D-mediated NK cell cytotoxicity. Thus our study demonstrates that IFN-γinterferes with an effective NKG2D-mediated killing of HLA class I-negative melanoma cells by NK cells which might facilitate melanoma immune escape also *in vivo*.

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Getting T cells to work EUR" transforming quantity into quality

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Antigen specific T cells present their various capacities such as production of cytokines or killing potential with broad heterogeneity. Whereas some T cells exert several functions simultaneously, others are only capable of one single effector mechanism. These functional subpopulations are often referred to as different EUROeflavoursEUR of T cells. In HIV vaccination trials poly functional T cells have been shown to elicit more effective immune responses. But can this finding be translated into cancer immunotherapy? Which EUROeflavoursEUR do we find among tumor-specific T cells, and how do these EURoeflavoursEUR change with different vaccination strategies?
To find answers for these questions we compared preexisting and vaccine-induced immune responses in stage IV melanoma patients of an ongoing vaccination trial with autologous DCs transfected with RNA encoding defined tumor antigens (Mage3,MelanA, Survivin) +/- E/L-selectin for improved lymph node homing. Using peptide libraries and ELIspot analysis patients PBMC were pre-screened for CD4- and CD8-T cell responses and individual sets of 4–8 peptides were chosen for further analysis. Different peptide specific T cell functions like degranulation and production of cytokines such as IFN$\gamma$and TNFα were then analysed *ex vivo* by eight-color flowcytometry. In general, preexisting immune-responses were rather mono-functional with dominating EUROeIFNγ-onlyEUR, EUROeTNFα-onlyEUR and EUROedegranulation-onlyEUR cells in the CD8+ compartment. Vaccination increased some of these monofunctional subpopulations but also induced occurrence of some tumor antigen specific poly functional subsets. The further course of our melanoma patients will show whether these vaccination induced new T-cellEURoeflavoursEUR as well as different patterns of homing receptors do translate into better clinical efficacy.
In summary, multiparameter flowcytometry has set up a new era of better understanding the functionality of T cells and will be of crucial importance to improve vaccination strategies in infectious diseases and cancer patients.

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The galectin disaccharide ligand LacNAc prevents loss of tetramer staining after T cell activation

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Antigenic stimulation of human cytolytic T lymphocyte (CTL) induces dissociation of the T cell receptor (TCR) and CD8 resulting in a decreased binding to human leukocyte antigen (HLA)-peptide tetramers. This phenomenon renders simultaneous staining for peptide tetramers and activation molecules such as intracellular cytokines difficult. Here we investigated the effect of the galectin disaccharide ligand N-acetyl lactosamine (LacNAc), with regard to intracellular cytokine and tetramer staining after antigenic stimulation.
Cells from a cytolytic T cell line specific for MelanA.A2 were stimulated with peptide (10 μg/ml, 4 h) in the absence or presence of LacNAc and stained with tetramer and cell surface marker antigens (CD3/CD8). For intracellular cytokine detection cells were fixed with 1% formaldehyde, permeabilized with Saponin (Sigma-Aldrich, Munich, Germany) and stained for interferon gamma. Peptide stimulation resulted in detectable intracellular secretion of interferon gamma paralleled by a rapidly vanishing surface tetramer and CD8 staining. This loss of tetramer/CD8 staining was efficiently prevented by treatment with LacNAc. Importantly, LacNAc (1 μmol) did not hamper T cell activation and interferon gamma secretion.
Taken together simultaneous detection of activation molecules and tetramer based identification of peptide specific T cells has become possible by preventing the dissociation between TCR and CD8 using the galectin disaccharide ligand N-acetyl lactosamine (LacNAc).

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Activation of a TGF-beta-specific multistep gene expression program in mature M2 macrophages requires glucocorticoid-mediated surface expression of TGF-beta receptor II

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Alternatively activated (M2) macrophages regulate steady state-, cancer- and inflammation-related tissue remodeling. They are induced by Th2-cytokines and glucocorticoids (GC). The responsiveness of mature macrophages to transforming growth factor (TGF)-beta a cytokine involved in inflammation, cancer and atherosclerosis is currently controversial. Recently, we demonstrated that interleukin-17 receptor B (IL17RB) is up-regulated in human monocyte derived macrophages differentiated in the presence of Th2 cytokine IL-4 and TGF-beta1.Here we show that mature human macrophages differentiated in the presence of IL-4 and dexamethasone (M2IL-4/GC) respond to TGF-beta by five-fold induction of IL17RB mRNA. Further TGF-betainduced a gene expression program comprising 111 genes in mature human M2IL-4/GC, but not M2IL-4 which includes transcriptional/signalling regulators (ID3, RGS1) as early response genes, and immune modulators (ALOX5AP, IL17RB) as well as atherosclerosis-related genes (ALOX5AP, ORL1, APOC1, APOC2, APOE) as late response genes. Analysis of molecular mechanism underlying GC/TGF-beta cooperation revealed that surface expression of TGF-beta RII was high in M2GC and M2IL-4/GC, but absent from M2IL-4, while the expression of TGF-beta RI/II mRNA, TGF-beta RII total protein and surface expression of TGF-beta RIII were unchanged. TGF-beta RII surface expression was dependent on GC dose in a range of physiological to therapeutic GC concentrations and determined the strength and duration of Smad2-phosphorylation-mediated signalling. In summary, mature human M2 macrophages made permissive to TGF-beta by GC-induced surface expression of TGF-beta RII activate in response to TGF-beta1 a multistep gene expression program featuring traits of macrophages found within an atherosclerotic lesion.

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Regulation of M-CSF and its receptor by IFNγ IL-4 and glucocorticoids inhuman macrophages

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Macrophages play a key role in regulation of inflammation and tissue regeneration. They develop from blood monocytes under the influence of colony stimulation factor (M-CSF or CSF-1) – a growth factor produced by tissue cells, that acts through CSF-1R. The ability of macrophages to produce M-CSF by themselves was reported, however the data on its regulation by Th1 and Th2 cytokines or hormones remains to be inconsistent. In this study we investigated the regulation of M-CSF production by primary human monocyte derived macrophages in response to Th1 and Th2 cytokines (IFN$\gamma$and IL-4) and anti-inflammatory drug – glucocorticoid (GC)dexamethasone. We show that IFNg and IL-4 efficiently induce production of M-CSF by macrophages, while GC inhibited it in a dose dependent manner. Similarly GCs inhibit production of inflammatory cytokines by macrophages in response to bacterial stimuli. Testing the hypothesis that this effect of GCs is based on the inhibition of M-CSF we show that addition of exogenous M-CSF to dexamethasone treated macrophages rescues their ability to produce TNF in response to LPS. This data indicate that GC treated macrophages retain the ability to respond to M-CSF. Analyzing the mechanism of this responsiveness, we show that dexamethasone strongly up-regulates surface expression of CSF-1R, while having a little or no effect on CSF-1R mRNA and total protein. We conclude that the ability of macrophages to produce M-CSF secures macrophage differentiation under both Th1 and Th2 conditions. Increase of surface CSF-1R may represent a compensatory mechanism that guarantees response to minute amounts of M-CSF.

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Correlation of total serum IgE-levels and perforin release velocity in patients with atopic dermatitis

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We recently demonstrated that the perforin (Perf)-granule system of cytotoxic lymphocytes is involved in IgE-control *in vivo* in mice and man. In patients with extrinsic atopic dermatitis (AD), a highly significant Perf-granule reduction is well known. This phenomenon is caused in part by a severe functional alteration of Perf-release mechanisms, i.e. an accelarated Perf-release velocity which we termed Perf-hyper releasability. We now asked for evidence suggesting a significant functional role of Perf-hyper releasability in AD-immunopathology. Total serum IgE-levels of 50 patients with exacerbated AD were determined using the Pharmacia Cap-system. In addition, various paraclincal and clinical parameters were obtained. The perforin+ T cell compartment was characterized in parallel by flow cytometry with a panel of monoclonal antibodies against CD3, CD4, CD8, CD16, CD56, CLA and Perf. Velocity of Perf-release from peripheral CD8hi+ lymphocytes was determined over a time interval of 120 minutes using an ionomycin/PMA-based Perf-degranulation assay. Statistical analysis (SPSS) revealed a significant negative correlation between total serum IgE-levels and Perf-release after 30 and 60 minutes (Pearson coeff. < -0.25, $P < 0.05$). The faster Perf was released, the higher was the total serum IgE-level. Suberythrodermic AD-patients released their Perf-granules significantly faster than less severe affected individuals (Pearson coeff. < -0.2, $P < 0.05$). Correlation between Perf-release velocity and eosinophil count, ECP-level, clinical AD-type and other para/clinical parameters investigated, however, did not reach significance ($P > 0.1$). Within the CD8hi+ subpopulation, Perf-release velocity correlated significantlywith the Perf+ portion of CLA+ CD8hi+ lymphocytes ($P < 0.05$). This relationship between Perf-hyper releasablility and total serum-IgE-level as well as AD-intensity suggests a functional role for Perf-granule relase in AD-immunopathology.

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Further evidence for regulation of IgE-production by CD8+ perforin-containing T cells in patients with atopic dermatitis

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AD-patients are characterized, among other phenomena, by elevated levels of total serum IgE and by a defect of CD8+ T cells, namely by perforin (Perf)-granule reduction and Perf-hyper releasability. We recently suggested a role for Perf in IgE-control of patients with extrinsic atopic dermatitis (AD). These studies were now extended. Total serum IgE-levels of 80 patients with exacerbated AD were determined using the Pharmacia Cap-system. The $CD8^+$ T cell compartment was characterized in parallel by flowcytometry. In addition, total and specific IgE-levels were measured over a time period of 10 days in culture supernatant of (i) ficoll-isolated peripheral mononuclear cells (PBMC), (ii) PBMC depleted of $CD8^+$ Tcells by Milteny beads and, as an additional control, (iii) CD8-depleted PBMC reconstituted with $CD8^+$ T cells. Cells were obtained from 12 AD patients (serum IgE levels 500–10000 U/ml). Using SPSS for statistical analysis, a significant negative correlation was found between total serum IgE levels and the $CD8^+$ portion and the Perf+ portion of leucocytes (Pearson coeff. < -0.25, $P < 0.05$, respecitvely). Depleting >90% of $CD8^+$ T cells *in vitro* resulted in higher specific and total IgE levels which was most significant at day 8–10 as compared to controls. Pretreatment of $CD8^+$ Tcells (condition iii, $n = 4$) with the Perf-inhibitor concanamycin-A resulted in the same IgE-elevation as removal of the entire $CD8^+$ population. *In vitro*, IgE levels correlated with the portion of Perf+ $CD8^+$ T cells (e.g. day 9, total IgE, $P < 0.05$). Taken together our data strengthen the hypothesis that Perf+ $CD8^+$ T cells are involved decisively in IgE-regulation in AD-patients.

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Autoantibodies from PNP sera preferentially recognize desmocollins and the C-terminal epitopes of the desmoglein 3 ectodomains

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Paraneoplastic pemphigus (PNP) is a mostly lethal autoimmune blistering disease characterized by severe polymorphous mucocutaneous lesions which is commonly associated with hematologic malignancies. In contrast to pemphigus vulgaris (PV), PNP sera target several autoantigens, including desmoglein (Dsg) 3 and Dsg 1, desmoplakin, envoplakin, periplakin, and bullous pemphigoid antigen 1 (BP230) and a not yet characterized 170 kD protein. Despite *in vitro* evidence for a pathogenicityof anti-Dsg 3 IgG, the relative pathogenetic contribution of the additional autoantibodies needs to be determined.

In the present study, the epitope specificity of Dsg3-reactive PNP sera was thoroughly investigated and compared with those of PV patients. Overall, sera from 12 of 14 PNP patients showed IgG reactivity with the Dsg3 ectodomain, specifically with epitopes located in the COOH-terminal EC4/EC5 domains, while IgG autoantibodies from PV sera preferentially recognized the NH2-terminus ($n = 11/19$). In addition, four of 14 PNP sera exhibited IgA reactivity against the Dsg 3 ectodomain. Furthermore, six of the 14 PNP showed IgG reactivity against desmocollins (Dsc)1 ($n = 1$), 2 ($n = 2$), and Dsc3 ($n = 6$). None of the PNP sera showed IgG against BP230.

In summary, these findings suggest that PNP sera preferentially target COOH-terminal regions of the Dsg 3 ectodomain, while sera from patients with acute PV preferentially target the NH2-terminus. Furthermore, Dsc are commonly identified autoantigens in PNP but not in PV. These immunoserological differences may help to establish the diagnosis of PNP based on the autoantibody profile.

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Deficiency of regulatory T cells in syphilitic skin lesions is associated with an enhanced cytotoxic T cell response in HIV-infected patients

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Introduction: Severe clinical manifestations and therapy failure are seen more often in HIV-infected than in HIV-seronegative patients with syphilis.

Methods: To better understand this phenomenon we characterized the cellular and molecular events in syphilitic skin lesions of HIV-infected and HIV-non-infected subjects by immunofluorescence staining and RT-PCR analysis.

Results: Our results show that the cellular immune response is dominated by T cells in both HIV-infected and non-infected patients. Depending on the HIV status, however, we observed quantitative and qualitative differences in the T cell subsets of syphilitic skin lesions: in HIV-infected patients (i) the epidermal and dermal T cell infiltrate was denser, and (ii) the CD8/CD4 T cell ratio was higher, whereas, interestingly, (iii) the number of $CD25^+$FoxP3+ regulatory T cells was reduced compared to HIV-seronegative patients. Consequently, we observed elevated inflammatory (IFN-γ, IL-23p19) and reduced regulatory cytokines (IL-10, TGF-β) in syphilitic skin lesions of HIV-infected patients. We detected hardly any dermaldendritic cells and plasmacytoid dendritic cells within the inflammatory infiltrate of either patient group, but we found sizable numbers of $CD14^+$CD11b+ macrophages in HIV-infected and, to a lesser extent, in HIV-negative patients. Interestingly enough Langerhans cells were greatly reduced in HIV-infected patients as compared to HIV-seronegative syphilitic patients. At the cytokine level syphilitic skin lesions were characterized by a Th1-polarized immune response, with IFN-γbeing the key cytokine in the absence of IL-4, IL-13, IL-17 and IL-22.

Conclusions: Taken together our data imply that HIV infection may dampen immune regulatory responses, thereby augmenting cytotoxic T cell responses and leading perhaps to more extensive tissue damage. Differences in the cellular and molecular composition of the inflammatory infiltrate may therefore explain the distinct clinical courses of HIV-infected and non-infected syphilis patients.

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TRAIL-expressing plasmacytoid dendritic cells from HIV-1 viremic patients induce CD4$^+$ T cell apoptosis

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Artificial Toll-like receptor (TLR) 7/8 ligands can endow plasmacytoid dendritic cells (pDCs) with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-dependent lytic properties. Keeping in mind that ssRNA serves as naturalTLR7/8 ligand, we searched for TRAIL-expressing cells in HIV-infected persons and identified TRAIL+ pDCs in HIV-1 viremic, but not in non-viremic and healthy individuals. TRAIL expression on pDCs was directly correlated with individual viral loads. Conversely, HIV-1 viremia and T cell stimulation was found to be associated with the up-regulation of the apoptosis-transmitting receptor TRAIL-R1 on activatedCD4$^+$ T cells. As a consequence, the latter became susceptible to TRAIL-dependent pDC-mediated killing. In contrast, initiation of antiretroviral therapy led to the up-regulation of apoptosis-inhibiting TRAIL-R4 on CD4$^+$ T cells, which subsequently became resistant against pDC-mediated cellular injury.

Definition of pDCs as killers of CD4$^+$ T cells implies a new mechanism of disease progression in HIV infection.

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Uptake of protein antigen into murine Langerhans cells in situ

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Langerhans cells (LC) acquire exogenous antigens by several different mechanisms and the help of different surface receptors. Ovalbumin (OVA) is a widely used model antigen. Its uptake into LC, particularly in situ, has not been studied in detail. We therefore performed experiments using OVA-Alexa conjugates to investigate incorporation and localisation in LC. LC were derived from skin explant cultures or freshly prepared by trypsinization. With immunofluorescence microscopy and flowcytometry technique we were capable to visualize and measure the uptake of antigen by LC in situ and in freshly isolated LC. LC in situ showed preferential uptake of OVA, emphasizing their specialization in antigen capture. Furthermore this 'antigen-loading' could be accomplished *in vivo* by topical application of the antigen onto the skin of mice. For this *in vivo* uptake by LC, the disruption of the skin barrier and the induction of an inflammation seemed to be necessary. Irrespective of the present discussion about the *in vivo* functions of LC, these data underscore that LC can efficiently take up protein antigens in situ, an important and indispensible step for their antigen presenting function. Moreover this work underlines the potential for use of LC in epicutaneous immunization strategies.

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Regulatory monocytes control T cell responses *in vitro* and *in vivo*

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Glucocorticoids (GC) are still the most widely used immunosuppressive agents in clinical medicine. Surprisingly little is known about the mechanisms of GC action on monocytes which play a central role in propagation as well as resolution of inflammation. In a murine model we show that GC-induce regulatory monocytes that display a distinct and stable phenotype. We further show that these monocytes are able to present antigens to T-cells thereby regulating their inflammatory properties. In addition we demonstrate that T-cell regulatory mechanisms are mediated via CD80 and CD124 that also has been shown to be used by myeloid-derived suppressor cells (MDSC). Thus, GC-treatment does not lead to global suppression of monocytic effector functions but rather induces differentiation of monocytes with regulatory properties. We also tested the anti-inflammatory capacity of regulatory monocytes *in vivo* using CHS (contact hypersensitivity) and transfer colitis models in mice. We show that monocytes are able to regulate T-cell mediated responses also in these *in vivo* models. In conclusion, GC treatment generates regulatory monocytes that are capable to control ongoing T cell responses *in vitro* and *in vivo*, and thus have a high potential to become valuable tools in treatment of inflammatory diseases.

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Development of a human T-Cell based *in vitro* assay for the identification of contact allergens

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The Cosmetics Directive (76/768/EEC) prohibits the selling of cosmetics tested on animals as of 2009. Furthermore the new EU legislation on chemicals (REACH) requires the re-evaluation of commercially available chemicals (about 30 000) concerning their allergenic potential and emphasizes the use of *in vitro* tests. Currently there are only three validated *in vivo* tests available but no *in vitro* assay. Thus there is an increased requirement for reliable, ethical and financially attractive *in vitro* test systems. We have started to identify optimal conditions for the development of a T-Cell based *in vitro* assay for the identification of contact allergens by using the known sensitizers DNCB, DNBS.

To assess how chemicals cause chemical modifications that can be delivered as antigens to T cells we analysed different conditions (pH) for the coupling of the strong contact allergens DNCB to human serum albumin (HSA) and direct modification of dendritic cells (DC) using 1-D gel electrophoresis. The optimal condition for an efficient coupling of DNCB to HSA was identified at physiological pH (pH 7.4). In subsequent T cell-based assays, we observed proliferative and IFN-gamma responses of magnetically sorted naïve T cells co-cultured with autologous DC either directly modified with DNBS or preincubated with DNCB-coupled HSA but not unmodified DC or uncoupled HSA. Additionally we identified increased frequencies of IFN-gamma producing CD4$^+$ as well as CD8$^+$ T cells in the DNBS stimulated samples after a re-stimulation. To assure the specificity of our system we performed re-stimulations of DNBS-primed T cells with TNBS-modified DC and observed an antigen-specific T-Cell response as well with IFN-gamma or proliferation assays.

Taken together we present a protocol for a T-Cell based assay for the specific identification of contact allergens and we provide evidence that a prerequisite for efficient T cell stimulation in such assays is hapten coupling to protein or cell surfaces.

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Inhibition of PI3K-AKT-mTOR but not of RAF-MEK-ERK signaling sensitizes melanoma cells to chemotherapy

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In melanoma, the RAF-MEK-ERK (MAPK) and PI3K-AKT-mTOR (AKT) signaling pathways are constitutively activated and appear to play a role in chemoresistance. We investigated the effects of pharmacological inhibitors of the MAPK and AKT pathways on chemosensitivity of melanoma cells to cisplatin and temozolomide. Chemosensitivity was tested by examining effects on growth, cell cycle, survival, expression of antiapoptotic proteins and invasive tumor growth of melanoma cells in monolayer and organotypic culture, respectively. Combinations of MAPK pathway inhibitors with chemotherapeutics did not achieve additional growth inhibition. Furthermore, BRAF depletion by BRAF siRNA did not efficiently enhance growth inhibition of chemotherapeutics. By contrast, AKT pathway inhibitors synergistically augmented growth inhibition of chemotherapeutics in all cell lines tested. Co-treatment of melanoma cells with AKT pathway inhibitors and chemotherapeutics led to a two–three-fold increase of apoptosis ($P < 0.05$, combination therapy versus monotherapy) and completely suppressed invasive tumor growth in organotypic culture. These effects were associated with decreased protein levels of the antiapoptotic Bcl-2 family protein Mcl-1. These data suggest that inhibition of the PI3K-AKT-mTOR pathway potently increases sensitivity of melanoma cells to chemotherapy.

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Generation of a skin equivalent on a collagen/elastin matrix with autologous human keratinocytes and fibroblasts for clinical application

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Isolation and cultivation of skin keratinocytes allows not only to study cell physiology under defined *in vitro* condition but also the expansion of cell material for transplantation purposes. The present study is dedicated to maximize the keratinocyte cell count and minimize the time span until transplantation. Particularly, the latter is important in the treatment of severe burn injuries.

Initially, a split skin sample of 1 cm ^{2} size was taken from the patient. After enzymatic separation by thermolysin over night, the epidermis was detached from dermis by forceps. The keratinocytes were isolated by trypsin (0.05%, 20 minutes) and 5×10 5 cells were seeded in collagen 1 coated dishes (175 cm^2). The dermis was digested with collagenase I (0.25%, 4 h) and the resulting cell suspension was seeded in regular plastic dishes (2×10 6 cells/175cm^2).

After 10 days fibroblasts were detached by trypsin and seeded on a collagen/elastin matrix (Matriderm, Skin & Health Care, Billerbeck, Germany, 1.5×10 5 cells/cm^2). After conditioning the matrix for 1 week keratinocytes (3×10 5 cells/cm^2) were seeded on top. After an additional week of submerse cultivation the matrix was lifted up to the air liquid interface to initiate epidermal cell differentiation. After 16 days the matrix was fixed and underwent immunhistochemical analysis.

We observed collagen IV and laminin 5, markers for the basement membrane, at the interface between epidermis and dermis. Ki-67, a marker for proliferation was restricted to basal epidermal cells. The differentiation markers desmoglein, involucrine and cytokeratin 10 were found in the suprabasal layers of the epidermis.

Our results demonstrate that a skin equivalent is possible to generate by using a collagen/elastin matrix. The formation of a dermis together with a stratified epidermis makes this model particular useful for covering of deep wounds. Preliminary results derived from treatment of a severe burn injury shows promising clinicaloutcome.

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Betulin-based triterpene extract promotes keratinocyte differentiation *in vitro* and *ex vivo* and displays anti-tumor effects *in vivo* via up-regulation of transient receptor potential channel 6 (TRPC6)

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Triterpenes are secondary plant substances that promote keratinocyte differentiation. A triterpene extract (TE) from the outer bark of birch has recently been described as an effective treatment for actinic keratoses (AK). Here, we have assessed the effect of TE on cell proliferation, apoptosis and differentiation of human primary keratinocytes (hPKs) *in vitro*, *ex vivo* and *in vivo*. hPKs treated with TE were analysed for cell proliferation, DNA fragmentation, expression of differentiation markers and transient receptor potential (TRP) channels by immunohistochemistry and RT-PCR. Additionally, calcium influx was studied and TRPC6 was knocked down to evaluate its role in TE-induced differentiation of hPK. The effect of TE *ex vivo* and *in vivo* was examined on short-term cultured skin explants and on punch biopsies obtained from patients with AK treated with TE for 3 months. Treatment of hPK with TE resulted in up-regulation of differentiation markers such as cytokeratin-10 (CK10).Furthermore TE treatment led to increased calcium influx. Staining of hPK for different TRP channels revealed that TRPC6 is specifically up-regulated by TE, eventually leading to increased CK10 expression. Knocking down TRPC6 inhibited TE-induced CK10 up-regulation in hPK. Short-term (24 h) incubation of skin explants with TE *ex vivo* resulted in significantly increased expression of Ki67 and TRPC6 in supra-basal hPKs, and increased DNA fragmentation of distal stratum granulosum keratinocytes. Treatment of AK with TE *in vivo* resulted in down-grading of dysplasia, normalization of aberrant Ki67 and increased CK10 expression. Taken together, we hypothesize that TE promotes differentiation of hPKs at least in part via up-regulation of TRPC6. This mode of action may explain the histological down-grading of dysplasia and the clinical improvement of AK observed *in vivo* after long-term treatment of lesional skin with TE.

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Evaluation of diagnostic criteria and recommendations for diagnosis of Adamantiades-Behet disease in Germany

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Adamantiades-Behet's Disease (ABD) is diagnosed on the basis of several clinical criteria sets, but signs and symptoms differ throughout the world. The mostly used criteria of the 'International Study Group' (ISG) were established in 1990. Other used diagnostic criteria are the ones by Mason and Barnes (1969), O'Duffy (1976), Dilsen (1986), Japanese criteria (1989) and the Classification-And-Regression-Tree (CART, 1993). The most recent 'International Criteria for Behet's Disease' (ICBD, 2006) were calculated on the basis of 2556 ABD patients and 1163 controls from 27 countries. The ICBD consist of a traditional point system and three diagnostic trees. In the following study we looked for the most exact diagnostic criteria for ABD in Germany. We investigated 86 confirmed ABD patient cases and 75 control patients, who exhibited 1–4 signs or symptoms belonging to other confirmed diseases but initially mimicking ABD (such as mucous pemphigoid, other forms of uveitis, diseases having at least one major ABD sign) during a 5 year period. High sensitivity, which is of particular importance for diagnosis, was reached by ICBD (95.3%), but specificity was lower (85.3%) than that of Mason and Barns' criteria (97.3% – highest specificity) and of ISG criteria, whereas ISG has low sensitivity (83.7%). The highest accuracy was assessed for O'Duffy (91.3%) in our patients' group. A second evaluation on the basis of the 633 German ABD Registry patients revealed sensitivity of the ISG criteria of 70.3 %, of the CART of 86.1 % and of the ICBD of 87.4%. In conclusion, the new ICBD seem to be superior to ISG for ABD diagnosis in countries with low occurrence of ABD, but should be applied only under exclusion of possible differential diagnoses.

Table 1: Evaluation of diagnosis criteria (ABD patients $n = 86$; controls $n = 75$)

Criteria: accuracy [%]	Sensitivity [%]	Specificty [%]
O'Duffy: 91.3	88.4	94.7
Mason and Barnes: 90.7	84.9	97.3
Dilsen: 90.7	88.4	93.3
ICBD		
Trad. format: 90.1	95.3	84.0
Tree 1: 90.1	94.2	85.3
Tree 2: 90.1	94.2	85.3
Tree 3: 89.4	93.0	85.3
CART: 90.1	94.2	85.3
Dilsen revised: 88.8	84.9	93.3
Japanese: 88.2	84.9	92.0
ISG criteria: 88.2	83.7	93.3
Korea: 88.2	90.7	85.3
Zhang: 85.7	96.5	73.3

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Certain filaggrin (FLG) mutations are likely to be associated with combined allergic and irritative chronic hand eczema

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The pathogenesis of chronic hand eczema (CHE) is multifactorial and involves endogenous risk or susceptibility factors and exogenous or environmental factors that promote disease development. The aim of this investigation was to analyze the association of different types of CHE with certain genetic variants in the filaggrin(FLG) gene (R501X, 2282del4), a structural protein of the cornified envelope important for the formation of the epidermal skin barrier. 122 German patients with CHE underwent detailed survey of medical history, clinical and allergologic examination and filaggrin genotyping. Genotyping results were compared with 95 healthy individuals. Overall, the comparison of allele frequencies and numbers of the mutation carriers showed no significant association with CHE, when compared to healthy controls. When differentiated by phenotype, however, CHE based on a combination of allergic and irritant contact dermatitis was more frequently associated with the variant alleles. In conclusion, mutations in the FLG gene may serve asco-factors that may promote disease development and maintenance of CHE, when both contact allergy and irritant mechanisms are involved.

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The value of molecular diagnosis and staging in primary cutaneous B-cell lymphomas

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Introduction: Primary cutaneous B-cell lymphomas (PCBCL) are classified according to the WHO-EORTC classification as primary cutaneous follicle center lymphoma (PCFCL),primary cutaneous marginal zone B-cell lymphoma (PCMZL), primary cutaneous diffuse large B-cell lymphoma, leg type (PCLBCL, LT), PCLBCL, other (PCLBCL, O) and intravascular large B-cell lymphoma.

The diagnosis is based on the clinical picture, histology and immunohistochemistry. Various molecular biological techniques have been applied to support the diagnosis by identifying the monoclonal nature of the lymphoma tumor cells. Attempts have been made to standardize molecular biological diagnosis of extra-cutaneous lymphomas by the BIOMED-2 protocol.

We wanted to investigate the applicability of the BIOMED-2 protocol in diagnosing PCBCL and the value of molecular staging in PCBCL, LT.

Methods: Therefore we analysed paraffin-embedded skin specimens from 17 PCBCL patients with the BIOMED-2 protocol for IgH, primers A–E. Additionally blood and lymph node samples of patients with PCLBCL, LT were analysed. To avoid pseudo-monoclonality each sample was analysed at least twice with the sequencer-based GeneScan®technique.

Results: Monoclonality could be detected in 3/5 PCFCL, 2/6 PCMZL and in 5/6 PCLBCL, LT patients. Interestingly, it was not possible to amplify any DNA with primers A and B even by repeated analysis of up to nine times in 6/8 available skin specimens of PCFCL or PCMZL. In one patient with PCLBCL, LT we found a circulating clone in the peripheral blood, which was identical to the one identified in the skin and another patient with PCLBCL, LT showed the same clone in the skin and lymph node. Twice a clone of undetermined significance could be detected and in two patients pseudo-monoclonality was identified by repeated analysis.

Conclusions: Studies with the BIOMED-2 IgH protocol must be seen in the context of clinical, histological and immunohistochemical data. Sensitivity seems to be low in paraffin-embedded specimens of PCFCL and PCMZL for tubes A and B. We speculate that freshly prepared specimens analysed with tubes C–E combined with Igк analyses is superior.

In PCBCL, LT the BIOMED-2 protocol for IgH can be readily applied and might even serve as a useful tool for molecular staging of extra-cutaneous compartments. However, each sample should be analysed at least twice to avoid detection of pseudo-monoclonality.

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Confocal laser microscopic capillaroscopy – A novel approach to the observation and analysis of skin capillaries *in vivo*

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Abstract: Modern day dermatology in the 21st century is becoming increasingly non-invasive. New techniques for diagnostics and therapy are undergoing this trend and one of the most prevalent by now is confocal laser-scanning microscopy (CLSM). It allows visualization of cellular structures of the skin up to a depth of approximately 300 μm *in vivo*. Until now, most of the studies were conducted on pathologically altered skin, mostly oncologic lesions.

We now present our observations on capillaries located in the dermal papillaries. The measurements were performed on 30 healthy volunteers of both genders and different ages. All data were collected under standard conditions (room temperature, body position, time of day) on the dorsal and ventral surface of the right forearm. We used the Vivascope 1500 (Lucid, Rochester, NY, USA.) under standard settings. Obtained pictures were analyzed using ImageJ with a self written macro and allowed us to measure the area, circumference and widest diameter of the capillaries *in vitro*. In this physiological study we can clearly demonstrate that by confocal laser microscopic capillaroscopy (CLMC) it is possible to visualize and measure skin capillaries in different parts of the human body. This approach offers a considerable advantage over (i) nailfold capillaroscopy, which can only be performed at the proximal nailsegment, and (ii) histological analysis, which can be interfered with fixation artifacts resulting in altered size and shape of the vessels to be analyzed. CLMC could allow for a more precise analysis of skin vasculature in systemic and proliferative diseases of the skin.

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MGMT gene promotor methylation does not predict temozolomide response in melanoma

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Background: Despite having limited influence on patients' survival treatment with dacarbazine or temozolomide remains without proven alternative in treatment of metastasized melanoma. In glioblastoma, promotor methylation of the counteracting DNA repair enzyme O6-methylguanine-DNA-methyltransferase (MGMT) correlates with survival of patients treated with temozolomide plus radiotherapy. For melanoma data are limited and controversial so far.

Methods: Biopsy samples of 122 patients with metastasized melanoma who were treated with temozolomide in two different multi-center studies from the DeCOG and the EORTC (18032) were investigated. We used the COBRA technique to determine aberrant methylation of CpG islands in small amounts of genomic DNA isolated from paraffin embedded tissue sections. To detect aberrant methylation bisulfit-treated DNA was amplified by PCR, enzyme restricted and visualized by gel electrophoresis.

Results: Of 116 evaluable patients 19.6% responded to temozolomide treatment (5.1% complete, 14.5% partial response), 26.5% showed stable disease and 53.9% progressed as best response to therapy. In the COBRA analysis 25% of samples revealed a promotor methylation of MGMT above 25%. Correlation with clinical data indicated no significant difference in the MGMT methylation status between responders (34.8% methylated) and non-responders (23.4% methylated) ($P = 0.29$) nor an advantage in overall survival ($P = 0.57$).

Conclusions: In patients with metastasized melanoma methylation status of the MGMT promotor in biopsies of melanoma metastasis seems not to be a significant marker for temozolomide response.

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Successful therapy of chronic actinic dermatitis with infliximab

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Chronic actinic dermatitis (CAD) is a rare sun light-evoked persistent eczema of uncovered skin affecting mostly older men during the summer. Treatment is notoriously difficult. We describe a 69-year-old patient with a history of CAD for 7 years. He had been treated with topical corticosteroids and PUVA, which had only slightly ameliorated his condition. Although cyclosporine did improve the clinical picture it had to be discontinued due to nephro toxicity. In addition, this patient also had also suffered from mild Crohn's disease for 12 years and was treated with 16 mg/day methylprednisone intermittently. This, however, failed to improve his CAD. We then began anti-TNF treatment with 5 mg/kg infliximab (IFX) intravenously at weeks0, 2, 6 and 10. CAD improved markedly, at week 18 affected ankles, hands, and neck and face completely cleared; the methylprednisone dose was tapered to 4 mg/day. One year later the CAD relapsed, which we treated with only one infusion of IFX leading to complete clearing at week 8. Topical treatment consisted of emollient sand sun blockers only. A skin biopsy from the lateral aspect of the upper neck at week 0 during the first course of IFX treatment exhibited spongiotic dermatitis with psoriasiform acanthosis and papillomatosis; there were dense dermal lymphocytic infiltrates consisting of $CD3^+$ and $CD8^+$ cells with very few $CD4^+$ or $CD19^+$ or $CD68^+$ cells. MIB-1 (Ki67) staining showed an increased labeling of epidermal cells in the basal layer; at week 18 all these changes tended to normalize. We conclude that the prominent dermal $CD8^+$ lymphocytic infiltrates and the increased epidermal cell proliferation in CAD are normalized by successful anti-TNF treatment within fliximab. This suggests that TNF is important in pathogenesis of CAD.

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Autoimmune phenomena during long-term anti-TNF treatment for psoriasis: effects on the psoriasis area-and severity index

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Autoimmune phenomena such as the occurrence of antinuclear antibodies (ANAs) or double-stranded DNA antibodies (anti-dsDNA) are well known to occur during anti-TNF treatment. We examined whether the occurrence of such autoantibodies exerted a negative effect on the psoriasis area-and severity index (PASI) in patients treated with either Infliximab or Adalimumab.

During the course of 7 months–7 years 23 patients were treated with 5 mg/kg Infliximab. Eighteen patients were treated with Adalimumab for 3 months to 2.5 years with a loading dose of 80 mg, after one week with 40 mg every other week.

Among the 23 patients treated with Infliximab, 13 patients (56%) developed ANAs with a mean titer of 1:320; in six patients (26%) dsDNA antibodies occurred up 200 units. In three patients treatment had to be stopped after the second or third infusion due to an allergic (anaphylactic) reaction. During therapy of 18 patients with Adalimumab, eight patients (44%) developed ANAs, the mean titer was 1:160, in two cases up to1:1280; two patients (11%) developed anti-dsDNA antibodies up to 200 U. The PASI decreased in all patients treated with either Infliximab or Adalimumab up to 75% or more regardless of the occurrence of these autoimmune phenomena.

We conclude that roughly every other patient treated with Infliximab or Adalimumab is likely to develop ANAs. Antibodies to dsDNA occur in 10–20% of patients treated. These autoimmune phenomena seem to have no discernible negative effect on treatment success.

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Seborrheic eczema responds more quickly to treatment with pimecrolimus cream than treatment with ketoconazole cream

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Seborrheic dermatitis is a chronic inflammatory skin disease characterized by mainly erythema, papules, and scaling. Although the face and scalp are the most commonly affected sites, sternal and vertebral regions are also sometimes affected. The etiology remains unclear, although malassezia species are often found in the lesions. Standard treatment for seborrheic dermatitis is, therefore, the topical application of antimycotic creams, most commonly 2% ketoconazole. Pimecrolimus cream is anascomycin macrolactam derivative and skin-selective calcineurin inhibitor, widely used for the treatment of atopic dermatitis. The mechanism of action of pimecrolimusin atopic dermatitis has been related to a reduction in inflammation and immunomodulation. In this study we examined whether pimecrolimus improves skin clinical symptoms and barrier function of seborrheic dermatitis. In a randomized, double-blinded study 32 patients were treated either with 1% pimecrolimus cream or2% ketoconazole cream twice daily for 4 weeks. Facial investigators global assessment (F-IGA) score, transepidermal water loss (TEWL), and hydration were investigated weekly. Skin biopsies were taken from the chest, if affected, before and after 4 weeks of treatment. Both treatments showed a strong improvement of F-IGA scoring, but the pimecrolimus group reached significance more quickly than the ketoconazole group compared to day 1. TEWL values were enhanced by a factor of three in lesional skin before treatment compared to non-lesional skin, indicating disturbed skin barrier function. TEWL improved in both treatment groups, but did not reach the levels seen in non-lesional skin. Hydration was reduced by 18% in lesional skin before treatment compared to non-lesional skin. Both treatments enhanced hydration in lesional skin. Epidermal hyperproliferation and thickness were only significantly reduced in the pimecrolimus group. These findings show that pimecrolimus cream improves the clinical symptoms of seborrheic dermatitis more quickly than ketoconazole and improves the associated biophysical parameters for skin barrier function and epidermal hydration at least to the same level as ketoconazole. Only pimecrolimus treatment results in a significant reduction of epidermal hyperproliferation and hyperplasia after 4 weeks of treatment.

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Epidemiological investigations on cutaneous leishmaniasis and Leishmania/HIV co-infection in the Mokolo focus, Far North Province, Cameroon*

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It is estimated that 350 million people are at risk of acquiring leishmaniasis in 88 countries of Africa, Asia, Europe, Central and South America. The spread of HIV coupled with human population migrations due to war and natural disasters have expanded significantly the endemicity of leishmaniasis. Leishmaniasis is widely reported as an opportunistic infection in HIV-infected individuals. In Africa, few studies have focused on Leishmania/HIV co-infection. In an attempt to evaluate the occurrence of such co-infection, we are conducting epidemiological studies on cutaneous leishmaniasis (CL) and Leishmania/HIV co-infection in the Mokolo focus of Northern Cameroon. Such studies are of great public health importance as both diseases occur in the region and successful control programs against HIV should integrate opportunistic infections such as leishmaniasis. A total of 83 subjects have been clinically and parasitologically diagnosed with CL. Clinically, the disease ranged from localized to disseminated CL with the number of lesions varying from 1 to 19 per individual. HIV serological testing was carried out on serum samples of all CL individuals and five of them (6%) were HIV positive. All five subjects showed antibodies to HIV-1 while only two were positive for HIV-2. It should be noted that both HIV-1 and HIV-2 are prevalent in Cameroon including the subtype O. The highest number of lesions was recorded among those who were HIV positive. Studies aiming at the identification of parasite strains isolated from both CL and co-infected individuals are underway. Also, the characterization of cellular and humoral immune mechanisms underlying susceptibility to Leishmania and HIV in this endemic focus will be the next step.

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The synergistic action of IFN-γand IL-17 increases cutaneous innate immunity by the induction of antimicrobial proteins

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Human skin is in permanent contact with microorganisms usually without getting infected. This is in part due to the antimicrobial proteins which are expressed by keratinocytes. The expression of most of these antimicrobial proteins in keratinocytes can be induced by stimulation with bacterial products or cytokines.

To gain more insight into the regulation of antimicrobial proteins we stimulated primary keratinocytes with several combinations of cytokines and analysed gene and protein expression of the principal skin-derived antimicrobial proteins (RNase-7, psoriasin, hBD-2 and hBD-3) using real-time PCR and ELISA. These experiments identified the synergistic action of IFN-γand IL-17 as strong inducer of gene and protein expression of all tested antimicrobial proteins. Even picogram amounts of the combination of IFN-γand IL-17 induced an expression of antimicrobial proteins in primary keratinocytes which revealed this cytokine combination among various other cytokines as one of the most powerful inducer of antimicrobial proteins in keratinocytes known so far.

To assess the functional significance of these findings we incubatedIFN-γ/IL-17-treated primary keratinocytes and skin explants with bacteria.IFN-γ/IL-17-treated keratinocytes exhibited higher bactericidal activity compared to the unstimulated control cells. In addition, the *ex vivo* induction of antimicrobial proteins in skin explants through IFN-gamma/IL-17 increased the bactericidal activity of these skin explants. Together, these results indicate an important role of the synergistic action of IFN-gamma/IL-17 for cutaneous defense through the induction of various antimicrobial proteins.

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XPG D1104H and XPD K751Q polymorphisms are independent prognostic factors for melanoma

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Sunlight is a major risk factor for melanoma. Since UV radiation causes DNA damage, it is not surprisingly, that genetic variants in DNA repair enzymes contribute to the susceptibility to cutaneous melanoma. To extend these studies, we analyzed whether common non-synonymous single-nucleotide polymorphism in different DNA repair enzymes would also impact overall survival of cutaneous melanoma patients. To this end, we evaluated in 743 patients eight SNPs of seven different DNA repair enzymes. The impact of these polymorphisms on overall survival was subsequently calculated by the Cox proportional hazard model. The univariate analyses revealed that XPG1104 His/His was significantly associated with impaired survival. Indeed, the univariate hazard ratio was 3.2 times higher for patients with XPG 1104 His/His ($P = 0.02$) compared to XPG 1104 Asp/Asp. Accordingly, 5 year survival rate was 60.9% for patients with XPG 1104 His/His compared to 83.5% for patients with XPG 1104 Asp/Asp. Importantly, multivariate analysis confirmed XPG 1104 His/His (multivariate HR = 5.1; $P < 0.001$) to be an independent factor affecting overall survival. This adjustment to the known risk factors, i.e. age, gender, and tumor thickness, further revealed polymorphisms in XPD codon 751 to be an additional independent risk factor; XPD 751 Lys/Gln and XPD Gln/Gln had both a 1.8 increased HR compared to XPD Lys/Lys ($P = 0.01$ or 0.04, respectively). In conclusion, XPG codon 1104 and XPD codon 751 polymorphisms are independent predictive parameters for survival outcome in patients with cutaneous melanoma.

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Occupational UV-exposure of Austrian farmers

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The designation of UV-induced skin tumors as occupational diseases is still a matter of debate although farmers are at an increased risk due to chronic ultraviolet (UV) exposure. (1)Epidemiologic study: assessment of knowledge about photo-damage/-protection and of the frequency of UV-induced skin/eye problems in a group of farmers (AG) compared to a control group of indoor workers (CG) aged 35–55 both. (2)Field test: dosimetry (X-2000 Gigahertz-Optik GmbH, Puchheim, Germany) of the occupational UV-exposure over 6 months in 12 farmers who daily had to complete a digital dairy (occupational activity, location, weather, photoprotective measures). 386 (f55%/m44%) randomly assigned full time farmers (med age 43 year) and 107 (f72%/m28%) indoor workers (med age 42 year) participated. We found three non-invasive squamous cell carcinomas and one basal cell carcinoma in the AG and in the CG 1 SSM. The difference was statistically not sign. Some photo-ageing parameters were more frequent in the AG than in the CG. Sign ($P < 0.05$) were: wrinkles, teleangiectasias, giant comedos, solar lentigines. The frequency of all UV-induced eye conditions except dryness was higher in the AG but only conjunctivitis and tumors of the lids were sign ($P < 0.05$) more frequent. Farmers had sign more sunburns, complained about a sign lower level of information on photo-damage/-protection but were sign less interested in these items than the CR. The answers to all other questions did not show any sign differences. Our field test produced 1 427 complete daily records. The cumulative doses showed a high variability (7 663– 75 751 J/m^2). Risk factors for high occupational UV exposure were: machines without closed safety cabin, manual work, female gender, inadequate operating logistics. The attitudes of farmers towards solar radiation and photoprotection are almost identical with indoor workers. In the investigated age group no sign difference in the frequency of UV-induced skin tumors could be found (this does not support the designation of UV-induced skin cancer as regular occupational disease) whereas photoageing of the skin and UV-induced eye damage were more pronounced in the AG than in the CG.

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Vitamin D levels in patients with Xeroderma pigmentosum

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The skin is capable of de novo vitamin D synthesis. Keratinocytes, macrophages and fibroblasts synthesise active vitamin D from cholesterol precursors by photochemical activation in the skin. Recently vitamin D levels have been implicated in skin carcinogenesis and it has been postulated that photoprotection may lead to clinically relevant reduction of vitamin D levels. In order to address this point vitamin D serum levels were investigated in 17 patients suffering from Xeroderma pigmentosum (XP).XP patients are required to apply stringent photoprotection to reduce the risk of developing skin cancer. This includes measures such as strict avoidance of direct sun-exposure, UV-protective screens on all windows, long sleeved protective clothing and application of sun protection with very high protection levels in the UVB as well as the UVA range. In order to assess stringency of UV-protective measures, questionnaires containing questions regarding age of diagnosis, types of UV-protection applied, self assessment of stringency (scale 1–10). Vitamin D serum levels (1,25-OH VitD) were normal in 76% (13/17), elevated in 6% (1/17) and reduced in 18% (3/17) of XP patients investigated. Effect of photoprotection type, self-estimation of stringency, and age of diagnosis on vitamin D levels were not statistically significant. Only the duration of photoprotection in years showed a significant effect on measured levels of vitamin D. Due to this, 30 years of photoprotection as applied by XP patients will lead to a 50% risk of reduced vitamin D serum levels. These data indicate that photoprotective measures as stringent as inpatients with XP do not lead to a strong reduction of serum vitamin D levels.

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Accidental partial body exposure to ionizing radiation ascertained after almost 40 years by M-FISH analysis of human cutaneous fibroblasts

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Accidental exposure to ionizing radiation is rarely homogeneous, which makes it difficult to use counts of chromosomal aberrations in blood lymphocytes or bone marrow cells as a quantitative proof of exposure.

Two years ago our group published a method to identify stable chromosomal aberrations in human dermal fibroblasts cultured and irradiated *in vitro*. In the presented case this method was used the first time to identify traces of radiation-induced chromosomal aberrations in a patient.

The 69-year-old physicist worked on experiments in nuclear fusion technology in the frame of his diploma and doctoral theses from 1969 to 1974. As only found out later, the experimental setup bore the risk for emission of X-rays as a side effect of the high-energy experiments. Though the risk was confirmed later on by official authorities, a real exposure of the affected individual could never be proven, though he claimed repeated itching sensations on his skin and a reduced UV tolerance in the consecutive years, an in situ melanoma was excised from his right breast in 1996.

From biopsies taken from his right upper chest (the area most probably exposed during the experiments) and the upper gluteal region (probably less exposed) fibroblasts were cultured, and, after division, chromosomes were prepared and stained with fluorescent markers and analysed with a computerized imaging system. With this technique, typical radiation-induced stable structural aberrations, namely clonal translocations from chromosomes 3 to 12 (four metaphases) and 6 to 15 (two metaphases) could be identified, whereas no such clonal aberrations were found in the fibroblasts biopsied from the gluteal region.

Thus, by means of this technique we could prove 39 years after exposure that the upper part of the physicist's trunk was exposed to ionizing radiation in the frame of his experiments.

We conclude that M-FISH analysis of biopsied human dermal fibroblasts is an appropriate method to identify accidental radiation exposure in questionable cases even several decades after exposure, when normally accident protocols and patients' files do not exist any more.

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Isolation and characterisation of human melanocytes from hair follicles for clinical use

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Despite significant progress in tissue engineering and cell biology there is still an unmet need for treating patients suffering from Vitiligo. Vitiligo (or leukoderma) is an acquired chronic skin disease that causes loss of pigment, resulting in irregular pale patches of skin. It occurs when the melanocytes die or are unable to function. The precise pathogenisis of Vitiligo is multifactorial and not fully understood. There is some evidence suggesting it is caused by a combination of auto-immune, genetic, and/or environmental factors. The population incidence worldwide is considered to be between 1% and 2%.

The hair follicle bulge area is an abundant source of actively growing pluripotent adult stem cells. These cells can be differentiated into various cell lineages, e.g. keratinocytes and melanocytes amongst others. One important advantage is that the hair follicles are easily accessible by just plucking the hair follicles from the scalp. Using hair follicles as source for melanocytic stem cells we are going to develop acausative, non-invasive and autologous cell therapy for patients suffering from Vitiligo. We are aiming to differentiate and propagate the hair follicle cells *in vitro*. This will enable us to treat larger areas of depigmented skin. However, a crucial prerequisite for use in clinical application is that the growth conditions have to fulfill current GMP conditions. Very recently, we succeeded in the cultivation of melanocytes isolated from hair follicles by using culture medium that does not contain any supplements from animal origin, and therefore fulfills GMP requirements.

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Das Patientenregister des Deutschen Netzwerks für Systemische Sklerodermie(DNSS): Daten zur prospektiven Erfassung der Organbeteiligung nach 1 und 2Jahren

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Die systemische Sklerodermie ist eine seltene, heterogene Multisystemerkrankung,die durch Manifestation an unterschiedlichen Organen gekennzeichnet ist und eineinterdisziplinäre Versorgung der Patienten voraussetzt.

Analysen des Datensatzes von aktuell mehr als 2000 Patienten ergaben bei 47%eine lSSc, bei 31% eine dSSc, bei 12% ein Overlap-Syndrom, 9% zeigten eineundifferenzierte Sklerodermie sowie 1% der Patienten eine Sclerosis sineScleroderma. Jüngste Verlaufsdaten zeigten, dass im Verlauf eines Jahres ($n = 789$) die Prävalenz von Organmanifestationen wie Gelenkkontrakturen (27% vs 35%), Hypertonie (23% vs 30%) und diastolischer Dysfunktion (12% vs 20%) signifikantansteigt. Hingegen konnte für eine Nierenbeteiligung, für digitale Ulzera undpulmonal arterielle Hypertonie keine signifikante Zunahme nachgewiesen werden. ImBeobachtungszeitraum von zwei Jahren ($n = 346$) stieg die Prävalenz der PAH (13% vs 21%), der kardialen Beteiligung (15% vs 24%) mit diastolischer Dysfunktion (12% vs 30%) und der Hypertonie (25% vs 36%) signifikant an. Anhand der Verlaufsdaten kanndie Progredienz der SSc bedingten Organmanifestationen beobachtet und inweiteren Analysen auch mit eingesetzten Therapiemaßnahmen korreliert werden.Diese im Register erhobenen Daten unterstreichen die Notwendigkeit regelmäßigerVerlaufsuntersuchungen, um rechtzeitig therapeutische Maßnahmen einleiten zukönnen. Das Patientenregister des DNSS wurde u.a. mit dem Ziel initiiert durchjährliche Verlaufskontrollen prospektiv die Organbeteiligung zu erfassen.

P174 (V32)

Anti-phospholipid antibodies opsonise L. major parasites to promote dendriticcell (DC) phagocytosis and induction of protective immunity

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We have previously shown that DC acquire L. major through Fc receptor (FcR)-mediated uptake of complexes comprising antibodies bound to parasites. Thus, both B cell- as well as Fcγ-deficient mice were more susceptible to leishmaniasis and susceptibility was directly attributable to a failure of DC to prime T cells efficiently and, consequently, to reduced production of IFNγ. We now addressed the question if and how the initial B cell response to the parasite itself develops. L. major parasites display large numbers of phospholipids on their surface. Now, parasites were opsonised *in vitro* by incubation with normal mouse serum (NMS), immune serum (IS) from infected mice or serum containing anti-phospholipid IgG (PhAk-S). Binding of IS and PhAk-S to Leishmania was detected by FACS by staining with anti-mouse IgG. Second, both IS as well as PhAk-S significantly enhanced phagocytosis of L. major by DC as compared to unopsonized controls or NMS (35 ± 5 and $27 \pm 5\%$ vs 13 ± 1 and $18 \pm 3\%$ infected DC, $n \geq 5$). Next, naïve mice were infected with parasites opsonised with NMS, IS or PhAk-S. Both the serum containing Leishmania-specific IgG (IS) as well as cross-reactive PhAk-S significantly improved disease outcome. Finally, genetically modified C57BL/6 mice engineered to produce membrane-only IgM+ B cells and further have no serum immunoglobulin('IgMi') displayed increased susceptibility as compared to wild type mice. Interestingly, the IgMi phenoptye was normalized to the level of wild types upon reconstitution with NMS, but if IS was used for adoptive transfer, the mice showed a significantly improved disease outcome (smaller lesions/faster resolution). In conclusion, our findings suggest that cross-reactive antibodies (e.g. anti-phospholipid Ab) are found in NMS which bind to pathogens to facilitate phagocytosis, which incase of L. major infections leads to induction of protective immunity via preferential DC infection and IL-12 release. Prior B cell-priming does not seem to be absolutely required to facilitate clearance of this important human pathogen *in vivo*.

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Mechanism of treatment of cutaneous Leishmania major infection by a two component gel developing nitric oxide

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Nitric oxide (NO) released by e.g. activated macrophages exerts a powerful cytostatic/cytotoxic effect against a variety of pathogens, including L. major. We have investigated whether a compound that generates NO could serve as therapeutic agent against cutaneous leishmaniasis. First, a two-component gel containing ascorbic acid 5% and sodium nitrite 5% was developed; once mixed the gel releases exogeneous NO. Next, Leishmania-resistant C57BL/6 and – susceptible BALB/c mice were infected intradermally with physiological doses of L. major (10E3 metacyclic promastigotes) mimicking the bite of a sand fly. Mice were treated early on between weeks 3 and 6, or – similar to the clinical situation where patients present with already established infections – between weeks 6 and 9 with fully developed lesions. Treatment was performed 2×/week by applying 200 mg of gel onto each infected ear; side effects were not obvious at any point of time. Lesion development in all groups was assessed weekly in three dimensions. Controls were left untreated or were treated with sham gel. Lesion sizes were significantly smaller in all treatment groups at several points of time reaching maximal differences starting 2 weeks posttreatment (e.g. C57BL/6 4.5 ± 0.8 early treatment groups vs 10.3 ± 1.2 mm^3 in controls, $n \geq 16$). Enumeration of parasites in the infected tissue revealed a significant reduction of the parasite load both after early and delayed treatment as compared to untreated controls (C57BL/6 early: 0.8 ± 0.2 vs 9 ± 2×10E4 parasites/week 6; late: 1 ± 1 vs 10 ± 3×10E4 parasites/week 9, $n \geq 8$). In addition, visceralisation of parasites into spleen was significantly inhibited in all treatment groups. Finally, antigen-specific cytokine release in draining lymph node cells revealed strongly decreased levels of IL-4 and increased amounts of IFNγassociated with higher IL-12p40 release in all treatment groups as compared to controls, whereas IL-10 levels remained unaltered. In conclusion, our data indicate that local application of NO donors may be useful in treating newly developed as well as established lesions of L. major infection with no severe side effects. Together with a direct parasite killing effect by exogeneous NO, the mechanism of action is an alteration of the cytokine profile towards Th1-associated protective immunity.

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Exploring the diversity of dermatophytes using SARAMIS@AXIMA

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In dermatological mycology most pathogens are considered to belong to the dermatophytes with species of the predominant genera *Trichophyton*, *Microsporum*, *Arthroderma* and *Epidermophyton*. However, the occurrence of other fungal species in dermatological samples has to be considered and rapid identification methods are required for reliable identification of these uncommon species for adequate treatment. We analysed fungal specimen from dermatological samples by MALDI-TOF MS, a newly emerging technology for microbiological identification. Mass spectra of samples were matched against the database of SARAMIS (Spectral Archiving and Microbial Identification System) containing mass spectral data of a high number of reference strains. Among 120 isolates, 87 could be identified as common dermatophyte species, mainly *Trichophyton rubrum* and *Trichophyton interdigitale*, consistent with results from morphological analyses. Nine isolates could be clearly assigned to a dermatophyte genus but not to a species. For most of these isolates, the mass spectral patterns showed similarities to those of two or more dermatophyte species, e.g. *T. rubrum* and *T. violaceum*. These isolates are considered as atypical specimen representing transitions between anamorph, clonal species. Of the remaining isolates, several showed no similarity to dermatophytes in mass spectral patterns when matched against the SARAMIS database and could subsequently be identifed as *Fusarium perforatum*, *Aspergillus versicolor*, *Myriodontium keratinopilum* and others, by molecular methods. Mass spectral data of respective reference strains have not been available at the time of the first analyses but were collected consecutively. This allowed, for example, the identification of two 2007 isolates as *Scopulariopsis brevicaulis* based on mass spectral data obtained from specimen isolated in 2006. MALDI-TOF MS has been successfully applied to the identification of the majority of isolates from dermatological samples and proved to be highly adaptive for the recognition and identification of rare fungal pathogens.

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Inducible depletion of Langerhans cells leads to ameliorated disease following Leishmania major infection

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In cutaneous leishmaniasis, dendritic cells (DC) function as the relevant antigen presenting cells (APC) initiating the primary immune response and play a crucial role in orchestrating T cell immunity and tolerance. Langerhans cells (LC) constitute a subset of DC localized in the epidermis and are characterized by the expression of MHCII, CD11c and Langerin. In addition to LC, the skin contains a population of Langerin+ dermal DC (dDC). Both epidermal LC and Langerin+ dDC migrate to skin draining lymph nodes under steady state and inflammatory conditions. However, the exact DC subtype responsible for the induction of protective immunity against L. major remains elusive. In this study, we analyzed the role of Langerin+ skin-derived DC in cutaneous leishmaniasis by their inducible ablation *in vivo*. In Langerin-DTR knock-in mice expressing the human diphtheria toxin (DT) receptor (DTR) cDNA under the control of the Langerin gene all Langerin+ skin DC were eliminated 48 h after a single injection of DT. Interestingly, in low-dose infections with L. major (1x10E3 parasites), DT-treated Langerin-DTR mice developed significantly smaller lesions, increased IFNγ/IL-4 ratios and decreased parasite loads compared to control mice. Selective depletion of only LC showed that LC, but not Langerin+ dDC, were responsible for this effect. The number of CD4+/Foxp3+ regulatory T cells (Treg) in infected ears was reduced in DT-treated Langerin-DTR mice in comparison to control mice. In re-infection experiments, on the other hand, no difference was observed between DT- and PBS treatment suggesting that Langerin+ skin-derived DC are not required for development of an intact memory response. In conclusion, our data uncover a suppressive role of epidermal LC in the course of L. major infection via the induction of Treg, as the depletion of LC leads to a better disease outcome. Thus, in humans, therapeutic alteration of the LC function may promote protective immunity against this important human pathogen.

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Identifying CD8+ T-cell epitopes as candidates for vaccination against cutaneous leishmaniasis

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Infection with the parasite Leishmania major leads either to self-healing (resistant phenotype; most humans and C57BL/6 mice) or systemic disease (susceptible; BALB/c mice) depending on the genetic background. Healing of Leishmania infections is based on Th1/Tc1 immunity, since IFNγ secretion of both CD4+ andCD8+ cells plays a critical role for protection by inducing parasite eliminating NO- in macrophages. To date, no effective vaccine against leishmaniasis exists. Since the complete 33.6 Mb haploid genome of L. major has been sequenced in 2005, novel insights into genome/proteome structure afford new methods for the identification of vaccine candidates. To characterize possible immunodominant CD8+ epitopes from the total proteome of the parasite, two different strategies were used. First, an epitope prediction approach based on a distinct number of the most abundant proteins of both, the promastigote and the intracellular amastigote, life forms of L. major was employed. Surprisingly, by mass spectrometry, we detected only 240promastigote and 97 amastigote proteins out of 8370 proteins being expressed. Subsequently, epitopes from these abundantly expressed proteins were predicted using publicly available epitope prediction algorithms (SYFPEITHI and NetMHC). To further reduce the large number of potential CD8 epitopes, we analysed the presentation of processed antigenic peptides presented on either MHC class I molecules H2-Db or H2-Kb. MHC I-presentation of infected DC was blocked using non-toxic mAb. In T-cell co-cultures, we observed a significant decrease in the proliferation of CD8+ T-cells upon blockage of H2-Db as compared to isotype controls or anti-H2-Kb treatment. To further analyse the antigenicity of the predicted peptides, all possible candidates will next be tested for their ability to induce proliferation and/or IFNγ release by CD8+ T-cells. Second, size fractionation of soluble Leishmania antigen (SLA) by HPLC led to a number of peptides/proteins (1–300 kDa), which will be analysed for their ability to induce IFNγ secretion by co-culturing with primed CD8 cells. In summary, identification of novel CD8+ (and CD4+) T-cell epitopes would aid vaccine development against this important human pathogen.

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A novel twist in the pathogenesis of Hidradenitis suppurativa: sweat gland cells attract *Staphylococcus aureus* adhesion by Cytokeratin 8

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Staphylococcus aureus is an important human pathogen that causes a variety of infections and toxinoses ranking from superficial skin infections to deep-seated infections. The pathogenic variety of *S. aureus* results in part from its great number of extracellular surface proteins, the so called 'microbial surface components recognizing adhesive matrix molecules' (MSCRAMM), as well as from different surface factors like Protein A, capsular polysaccharides and cell-wall teichoic acids. These virulence factors enable *S. aureus* to adhere to the extracellular matrix and plasma proteins, which results in colonization and infection of the host. Clumping factor b (Clf b), a surface protein of *S. aureus*, which is a known virulence factor, was used in a yeast two hybrid protein-protein interaction screen in a keratinocyte cDNA gene library, where we identified Cytokeratin 8 (CK 8) as a novel putative host interaction partner. Moreover we could demonstrate a high binding affinity (comparable to other virulence factor targets, like fibrinogen, collagen and elastin) of *S. aureus* to the recombinantly in *Escherichia coli* expressed CK 8 by preparing an *in vitro* adhesion assay. CK 8 is a protein of the simple epithelia, which is not expressed in the skin epidermis. We performed immunofluorescence investigations on skin sections, and were able to demonstrate high CK-8 protein expression in sweatglands. In a novel *S. aureus* – NCL – SG 3 sweat gland cell line adhesion assay we detected a strong binding of *S. aureus* cells to high level keratin protein expressing sweat gland cells. This may be a further hint for the clinical correlation between *S. aureus* and sweat glands in Hidradenitis suppurativa, a chronic inflammatory scarring skin disease of the apocrine sweat glands, whose exact cause is still unclear.

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Natural killer (NK) T cells modulate immunity against Leishmania major infection

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Leishmaniasis is a serious cause of disease with 12 million people affected worldwide and tens of thousands of deaths every year. Infection with the parasite Leishmania is also considered a classic immunological model with resistant mouse strains developing Th1 immunity and susceptible strains initiating a Th2 response leading to a failure to contain the infection, a generalized systemic infection and eventually death. Here, we decided to study the influence of an innate effector T cell subtype, NKT cells, on the development of immunity against L. major. We used amore physiological low dose infection model mimicking natural transmission by the bite of a sand fly as compared to the standard high dose infection used in the majority of experimental studies. Our initial results comparing CD1d- orJα18-deficient mice lacking NKT cells on a C57BL/6 background with wild type mice showed that NKT cell-deficient mice were better able to contain Leishmania infections than their wild type counterparts. Lesion sizes and parasite burdens inCD1d-/- mice were at least twofold smaller than in C57BL/6 mice (lesions week 3, 5 and 8, $P \leq 0.05$, parasite load week 5, $P \leq 0.05$). Application of a minute amount of the glycolipid alpha-Galactosyl-Ceramide (aGalCer, 100 ng)- a strong stimulating ligand of NKT cells – given at the time point of infection, led to a more severe course of disease in otherwise resistant C57BL/6 wild type mice associated with higher parasite burdens in infected lesions and spleens (≥ 10-fold, $P \leq 0.1$ ear, $P \leq 0.05$ spleen). Surprisingly and in contrast to resistant C57BL/6 mice, application of aGalCer to susceptible BALB/c wild types improved their ability to effectively contain Leishmania infections as measured by differences in lesion sizes and large differences in lesional parasite loads (less than or equal to fivefold in week 5 and 8, $P \leq 0.05$). In summary, our findings show that in low dose infections with L. major, NKT cells can significantly alter the immune response against Leishmania dependent on the gentic background of the individual. Modulation of the NKT cell response, for instance with glycolipid ligands such as aGalCer, could be a successful approach to develop a long awaited vaccine against this important human pathogen.

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PLA-nanoparticles as a drug delivery system for topical dermatotherapy

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Novel drug delivery systems (DDS) enable a sustained drug release, thus maintaining constant drug levels. The need for a biodegradable DDS for topical dermatotherapy and the treatment of hair follicle associated diseases set the focus on PLA-nanoparticles (PLA-NP) since increased and selective penetration of nanoparticles in the human hair follicle were observed in earlier investigations. In this study we investigated the release properties of a lipophilic dye incorporated in 228 nm (nile red) and 365 nm (coumarin 6) PLA-NP in excised human skin with the use of fluorescence microscopy. After penetration and accumulation of the investigated PLA-NP in the in fund ibulum of human vellus hair follicles the dye was partially released and stained not only the follicle but also the sebaceous gland. Interestingly, the latter remained stained for more than 24 h indicating a prolonged release and a partially selective targeting. Uptake of free dye by viable epidermis cells was confirmed using flow cytometric analysis of single cell suspensions from skin explants treated with PLA-NP. The use of a biphasic hydrophilic/lipophilic suspension enabled the *in vitro* simulation of the interaction of the PLA-NP suspension with the sebum in the hair follicle. A linear release within the first 8 h was detected and quantified by means of fluorescence spectrophotometry. Microscopic analysis of the nanoparticles revealed a structural change and the formation of conglomerates at the interface between water and organic solvent. These results suggest PLA-NP to be potential candidates for an optimal DDS for topical dermatotherapy. The prolonged release of the incorporated drug through structural changes of the nanoparticles and drug diffusion would enable to achieve constant drug levels within the hair follicle, which is useful for the treatment of hair related diseases such as alopecia or hypertrichosis. Furthermore, targeting of the sebaceous gland is of utmost importance for the treatment of sebaceous gland related disorders such as acne and rosacea.

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Down-regulation of VEGFR2 is a major molecular determinant of dimethyl-fumarate mediated anti-angiogenic action in endothelial cells

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The association between Angiogenesis and chronic inflammatory diseases such as psoriasis seems to be an important phenomenon implicated in the pathogenesis of these medical conditions. Recent studies provide evidence that Fumaric acid esters (FAEs) modulate adhesion molecule expression by blocking tumor necrosis factor (TNF)-α induced expression of VCAM-1, ICAM-1 and E-selectin in human endothelial cells. As signalling via the vascular endothelial growth factor receptor-2 (VEGFR2) pathway is critical for angiogenic responses during chronic inflammation, we explored whether known anti-inflammatory effects of dimethyl-fumarate are mediated in part through diminished VEGFR2 expression. In this study, dimethyl-fumarate (DMF) is found to inhibit endothelial VEGFR2expression, Time- and concentration-dependent inhibition is demonstrated both at the level of protein and mRNA VEGFR2 expression. This blockade was paralleled by the respective inhibition of the formation of capillary-like structures and endothelial cell migration. In contrast, neither neuropilin-1 nor VE-Cadherin expression was significantly affected by DMF treatment. The suppressive effects on VEGFR2expression were not conveyed by increased shedding or by a decrease in the protein half-life, suggesting that transcriptional mechanisms accounted for the observe defects. Inhibitory effects of DMF on transcriptional activity of the VEGFR2 promoter are conveyed by an element located between base pairs -60 and -37 that contain two adjacent consensus Sp1 transcription factor binding sites. ConstitutiveSp1-containing complex formation to this sequence is decreased by DMF treatment, indicating that VEGFR2 gene expression is inhibited by repressing Sp1site-dependent DNA binding and transactivation. In addition, we could demonstrate that DMF reduced VEGFR-2 mRNA stability. Hence, VEGFR-2 expression constitutes a critical molecular target of DMF that may mediate its anti-angiogenic effects *in vivo*.

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Insights into understanding the mechanisms of action of alitretinoin in chronich and eczema

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Chronic hand eczema represents an inflammatory skin disease with a high prevalence ranging 6–11% in Western industrialized countries. Recently, the novel retinoid receptor agonist alitretinoin (9-cis retinoic acid) given at well-tolerated doses induced substantial clearing of chronic hand eczema in adult patients who were unresponsive to potent topical corticosteroids. In contrast to isotretinoin, alitretinoin is not only an agonist for RAR but also for RXR nuclear retinoid receptors. This pattern of retinoid receptor activation may be the key to understand the unique efficacy of alitretinoin in a chronic inflammatory skin disease. Here, we show that alitretinoin is able to interfere with recruitment pathways of pathogenic leukocyte subsets by down-regulating keratinocyte-derived chemokine (CCL27, CXCL9, CXCL10, CXCL11 and CCL20) production. In contrast to isotretinoin, alitretinoin also markedly impairs mixed leukocyte reactions. Detailed flow cytometric analyses showed the dose dependent suppression of the very early activation antigen CD69on the surface of activated T, B and dendritic cells. Moreover, alitretinoin significantly suppressed the expression of co-stimulatory molecules such as CD80 and CD86 on the surface of antigen presenting cells. Taken together, findings of the present study show that alitretinoin modulates leukocyte recruitment pathways, interferes with antigen presentation and leads to impaired leukocyte activation. These first insights into the mechanisms of action of alitretinoin may help to understand its clinical efficacy and provide perspectives for future indications.

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PPAR delta agonists exert profound anti-angiogenic action in endothelial cells via VEGFR2 down-regulation

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Peroxisome proliferator-activated receptors (PPARs) are ligand-activatedtranscription factors, originally implicated in the regulation of lipid and glucosehomeostasis. In addition, natural and synthetic PPAR activators may control inflammatory processes by inhibition of distinct pro-inflammatory genes. As signalling via the vascular endothelial growth factor receptor-2 (VEGFR2) pathway is critical for angiogenic responses during chronic inflammation, we explored whether known anti-inflammatory effects of PPAR ligands are mediated in part through diminishedVEGFR2 expression. In this study, PPAR delta agonists (L-165041 and GW 501516) are found to inhibit endothelial VEGFR2 protein expression in a time- and concentration-dependent manner. This blockade was paralleled by the respective inhibition of the formation of capillary-like structures and endothelial cell migration. In contrast, neither tie-2 nor VEGFR1 expression was significantly affected by PPARdelta agonist treatment. The suppressive effects on VEGFR2 expression were not conveyed by a decrease in the protein half-life, suggesting that transcriptional mechanisms accounted for the observed effects. In line with this conclusion, PPARdelta agonists significantly suppressed VEGFR2 mRNA accumulation. In addition, we could demonstrate via promoter luciferase assays, that the inhibitory effects ofPPAR delta agonists are conveyed by the suppression of VEGFR2promoter activity. Hence, VEGFR-2 expression may constitute a critical molecular target of PPAR delta-agonists that may mediate their anti-angiogenic effects.

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ZK 245186, a novel, selective glucocorticoid receptor agonist (SEGRA) for the topical therapy of inflammatory skin diseases

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Glucocorticoids are highly effective in the therapy of inflammatory diseases. Their value, however, is limited by side effects. The discovery of the molecular mechanisms of the glucocorticoid receptor, and the recognition that activation and repression of gene expression could be addressed separately, opened the possibility to achieve improved safety profiles by the identification of ligands that predominantly induce repression. Here we report on ZK 245186, a novel non-steroidal, glucocorticoid receptor-selective low-molecular weight SEGRA compound. Strong anti-inflammatory activity of ZK 245186 was demonstrated in multiple *in vitro* assays for inhibition of cytokine secretion and T cell proliferation. *In vivo*, in irritant contact dermatitis and T cell-mediated contact allergy models in mice and rats, ZK 245186 showed similar anti-inflammatory efficacy after topical application compared to the classical glucocorticoids, mometasone furoate and methylprednisolone aceponate. ZK 245186, however, exhibits a superior safety profile. *In vitro* and *in vivo* results indicated a lower risk for induction of diabetes mellitus and thymus atrophy which correlates with reduced activation of gene expression. After long-term topical application skin atrophy was reduced and less effects on animal growth were observed. ZK 245186 represents a promising drug candidate currently in clinical trials.

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The differential anti-inflammatory potential of SEGRA ZK 216348 and Prednisolone relies on T cell apoptosis rather than modulation of dendritic cell activity

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ZK 216348 (ZK) is one member of the family of Selective Glucocorticoid Receptor (GR) Agonists (SEGRA) which represent non-steroidal GR ligands with anti-inflammatory activity similar to Glucocorticoids (GCs) and a favourable side effect profile. Yet, while Prednisolone (Pred) inhibited oxazolone-induced ear inflammation by treating both the sensitization phase as well as the challenge phase, ZK was efficient in the suppression of the challenge phase but was less active when applied before sensitization. This study therefore aimed to delineate the immunomodulatory profile of ZK alongside with Pred on dendritic cells (DCs) and T cells (TCs). *In vitro*, ZK and Pred reduced the number of CD11c-expressing cells comparably effective when applied to BMDC cultures during DC differentiation. Similarly, ZK and GC treatment of DCs caused equi-effective down-regulation of MHC class II, CD40 and CD86. Furthermore, a comparable increase in phagocytic activity of naïve and activated DCs was observed when DCs were co-cultured with GCs and ZK. In mice, migration of skin DCs to the draining lymph node LN, as assessed by FITC painting, was either affected by ZK and Prednisolone. Some pro-apoptotic effects on BMDCs were seen under both GC- and ZK-exposition. Moreover, DCs preincubated with either Pred or ZK did not induce functional differences regarding TC proliferation oraapoptosis in primary or secondary mixed lymphocyte reactions (MLRs). In contrast, Pred and ZK showed differential effects on TC activity, differed markedly when present during the primary MLR. Here, Pred was a strong inducer of TC apoptosis while ZK harmed naïve TC only moderately with low potency and efficacy. *In vivo*, this was reflected by a severe reduction of draining inguinal LN weights after 3× topical Pred treatment (-82%) before sensitization while LN weights were significantly less affected after 3× ZK (-38%). This observation may explain the initially described differential effects on CHS responses after treatment before the sensitization phase. We conclude that ZK 216348 is a potent inhibitor of acute inflammation but is less harmful to naïve T cells and might therefore display less unspecific immunosuppressive effects than GCs.

P187 (V25)

Activation of the aryl hydrocarbon (Ahr) receptor via resveratrol prevents UV-induced immunosuppression

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Ultraviolet radiation (UV) suppresses the immune system in an antigen-specific fashion via induction of regulatory T cells (Treg). The mechanisms underlying both the induction and the activity of UV-Treg are still unclear. Recently it was shown that activation of the aryl hydrocarbon receptor (AhR) in a ligand-specific fashion can either induce Treg or proinflammatory T cells producing IL-17 (Th17). In addition, the AhR was identified as a molecular target for UV and to participate in UV-induced signal transduction. Hence, we were interested whether the AhR is involved in UV-induced immunosuppression. To address this issue, the AhR antagonistic ligand resveratrol was used and tested in the murine contact hypersensitivity (CHS) model. Mice which were hapten sensitized through skin exposed to UVB (4×150 mJ/cm^2) did not respond with an ear swelling response upon ear challenge, indicating UV-induced immunosuppression. In contrast, mice, which were exposed to identical UV doses but were injected i.p. with resveratrol (150 μM, 200 μl) before sensitization, revealed a normal CHS response, when compared to positive control mice. Administration of resveratrol alone did not influence CHS reaction. To investigate the impact of resveratrol on the development of UV-Treg, adoptive transfer experiments were performed. Injection of lymphocytes from donors, which were hapten sensitized through UVB-exposed skin, rendered naive recipient mice unresponsive to the hapten. In contrast, recipients of T cells from mice, which were hapten sensitized through UVB exposed skin but in addition received resveratrol, were not suppressed in their CHS response. This indicates that in the presence of resveratrol UV-Treg did not develop. In addition, resveratrol appears to down-regulate the AhR. This was demonstrated by PCR analysis of RNA isolated from the lymph nodes of mice which were injected with resveratrol. Together, these data suggest that the AhR appears to be involved in mediating UV-induced immunosuppression. Thus AhR ligands might represent a suitable tool to modulate the impact of UVB on the immune system.

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Infrared radiation does not enhance the frequency of UV-induced skin tumors, but their growth behaviour in mice

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Infrared radiation (IR) accounts for more than 50% of the solar energy reaching the earth's surface. There is recent concern about the interaction between UV and IR with regard to carcinogenesis. This is based on the fact that prolonged natural solar exposure is associated with an increase of the cumulative load not only of UV but also of IR. In addition, there is concern that the incidence of skin cancer may be augmented by rising temperatures as a consequence of the climate change. Therefore, the addition of IR filters into sun protection products is propagated recently. We recently demonstrated that IR reduces UV-induced apoptotic cell death both *in vitro* and *in vivo*. This effect is mediated via reduction of UV-induced DNA damage and induction of anti-apoptotic proteins by IR. The anti-apoptotic effects of IR may support the survival of UV-damaged cells and thus carcinogenesis. Since, however, IR reduces UV-induced DNA damage, the balance between these two effects may be important. To further elucidate this phenomenon and its impact on *in vivo* carcinogenesis we initiated photo-carcinogenesis experiments. C57BL/6 mice were irradiated three times per week with IR-A (780–1400 nm, 135 J/cm^2) followed 2 h later by UVB (800 mJ/cm^2) over 25 weeks. Mice exposed to IR or UVB only were included as controls. Kaplan–Meier analysis revealed that the occurrence of UV-induced tumors was neither accelerated nor increased in IR-pretreated mice when compared to animals exposed to UV only. However, once tumors had occurred the growth rate of UV-induced tumors was remarkably increased in mice pretreated with IR. To characterize the tumors *in vitro*, cell lines were generated from excised tumors and the proliferative capacity was determined after 0, 3 and 7 days using a MTT assay. Tumor cells obtained from IR-pretreated mice grew significantly faster in comparison to cells from tumors induced by UV alone. Long term colony formation assays confirmed this observation. After 21 days tumor cells obtained from IR and UV irradiated mice formed much denser colonies than cells derived from UV only induced tumors. Together, this study indicates that IR neither accelerates the occurrence nor increases the frequency of UV-induced skin tumors. However, as soon as tumors have developed, their growth behaviour appears to be much more aggressive.

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Extracorporeal photopheresis augments the number and increases the function of regulatory T cells by triggering Adenosine production

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Extracorporeal Photopheresis (ECP) is a procedure commonly used to reduce transplant rejection. It is also applied in diseases like GvHD, cutaneous T cell lymphoma and other medical conditions involving overwhelming immune reactions. The mechanism by which ECP exerts its immunosuppressive properties remains elusive; however, regulatory T cells (Treg) are a major cellular component contributing to immunosuppressive mechanisms in the periphery of the body. In our study we investigated whether ECP affects the frequency and function of Treg in peripheral blood of patients suffering from graft versus host disease. ECP treatment was performed on 2 consecutive days and blood samples were taken before and after each session. We observed an increase of CD4+CD25+FoxP3+Treg directly after each ECP cycle and also in the general course of treatment (12 cycles, over 5 months analysed). This effect was observed in GvHD patients but not in patients with other T cell mediated diseases. Moreover, to study functional properties of this distinct population in the GvHD patients, we analysed the suppressive effect of isolated Treg before and after ECP using conventional suppression assays. These assays revealed that Treg before ECP showed a significantly reduced suppression of T cell proliferation, whereas the suppressive capacity of Treg after ECP equalled that of Treg isolated from healthy volunteers. Another functional property of Treg is the conversion of ATP to Adenosine and free Phosphate by the ectonucleotidase CD39 which is also expressed by Treg. Consequently Treg generated Adenosine leads to down regulation of proliferative factors in effector T cells. Therefore we analysed the Adenosine generation by Treg by measuring the Pi -release and ATP consumption of Treg before and after ECP. After ECP, both activated as well as resting Treg released higher amounts of Pi and consumed more ATP as compared to those isolated before ECP, indicating that ECP enhances generation of immunosuppressive Adenosine. In conclusion our data indicate that ECP stimulates conversion of ATP to adenosine by the ectonucleotidase CD39, which acts as a novel soluble immunosuppressive reagent mediating the function of Treg.

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UV-activated lipids derived from lower plants induce the expression of cellular antioxidants in skin cells

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Long wavelength Ultraviolet (UVA-1) radiation causes oxidative stress that leads to the formation of noxious substances within the skin. As defensive mechanism skin cells produce detoxifying enzymes and antioxidants when stressed. Recently, we have found that phospholipids from cellular membranes induce the antioxidant defence when oxidized by UVA-1. Only lipids like1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (PAPC) that contain polyunsaturated fatty acids (PUFA) residues can be efficiently activated by UVA-1.Since such lipids are promising lead compounds for skin photo-protection applications, we searched for novel, botanical sources rich in PUFA. Higher plants cannot produce PUFA, but lower plants, especially mosses are rich sources of these lipids. We hypothesized mosses would contain lipids which could be activated by UVA-1.Using mass spectrometry, we analyzed lipid extracts from Physcomitrella patens, and Sphagnum girgensohnii, which has been used since the 19th century in wound healing applications. Both mosses contained high levels of PAPC and other PUFA. Accordingly, UVA-1 treatment of the lipids led to their oxygenation. We treated cultured dermal fibroblasts, keratinocytes and HaCaT with native and irradiated lipidextracts from both mosses. Using qPCR and western blot we found that hemeoxygenase-1, glutathione metabolism genes, two aldo-keto reductases, thioredoxin, thioredoxin reductase and sulfiredoxin were induced by the activated but not the native lipid extracts. When we treated three dimensional skin equivalents with the extracts, we found corresponding regulation. The abovementioned genes are key players in stress response and detoxification. These data provide novel leads on mechanisms of traditional botanicals. Further, they suggest a role for moss lipids as inducers of skin repair mechanisms and members of the novel class of skin photo-adaptation inducers (SPA).

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Laser-stripping of stratum corneum to enhance drug penetration for PDT

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Topical photodynamic therapy (PDT) with 5-aminolevulinic acid (5-Ala) is a meanwhile established treatment modality for epithelial skin cancer. Of particular interest is the application of 5-Ala, which is metabolized to proto-porphyrin IX (PpIX), the actual photosensitizer (PS). A major goal in PDT is the enhancement of permeability of PS or their precursors across the stratum corneum to achieve a higher concentration of the PS within the target area. Stratum corneum is believed to be the primary barrier for transdermal permeation of drugs. We investigated skin penetration and fluorescence induction of PpIX after application of a 20% 5-Alacream on laser-stripped stratum corneum using an *ex vivo* full thickness porcine skin model. For stripping of stratum corneum two different laser systems were used: Er:YAG (2940 nm) and Nd:YAG (1440 nm) laser systems are frequently used indermatological laser medicine. Different laser doses (2–48 J/cm^2) were used to ablate the horny layer. A reproducible correlation between 'laser-stripping' and thickness of the stratum corneum was achieved. A reduction of 60 ± 5% of the stratum corneum was determined without loss of viability of underlying epidermis and the dermal compartment. An enhancement of PpIX fluorescence were detected after treatment of skin areas with the laser-stripping method versus intact skin and subsequent incubation with 5-Ala for 6 h. The highest PpIX fluorescence levels were achieved depending on the used laser light doses. Laser-treated skin areas showed an increase of PpIX fluorescence of +45% after incubation with 5-ALA versus untreated controls. No significant differences between both laser systems were detected regarding an enhanced fluorescence intensity of PpIX upon ablation of the stratum corneum. Histological evaluations of laser-treated skin (±5-Ala) showed no significant degree of necrosis and apoptosis determined by NBTC staining and TUNEL-assay indicating that the skin is still vital up to 24 h. In this study it was shown that *ex vivo* porcine skin is able to discriminate penetration depth and PpIX fluorescence intensities upon topical application of a 5-Ala cream. Ablation of stratum corneum layers by laser light of different quality demonstrate a fast and easy approach to enhance penetration of selected PS for clinical application used in PDT without damage of underlying tissue.

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A humanized mouse model to investigate immunomodulatory mechanisms of extracorporeal photopheresis

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Extracorporeal photopheresis (ECP) has demonstrated to be clinically effective for the treatment of steroid refractory graft-versus-host-disease (GvHD). It has been suggested that ECP efficacy depends on immune tolerance induction by combined effects of cell apoptosis, modification of cytokine secretion and enhanced T regulatory cell activity. However, the underlying mechanisms remain elusive. To further investigate the clinical effects of ECP, we used a well known xenogenic graft-versus host model in NOD/SCID mice that were injected intra peritoneally with xenogenic human peripheral blood mononuclear cells (PBMC) between 1 and 5 days after birth. As a model for ECP, we treated unprocessed PBMC with 200 ng/ml8-methoxypsoralen (8-MOP) for 30 min, followed by subsequent irradiation with 2 J/cm^2 ultraviolet-A (UVA). To assay the effect of ECP on GvHD, NOD/SCID mice were either reconstituted with untreated PBMC alone or with untreated PBMC together with UVA-irradiated and 8-MOP treated PBMC. During the following weeks a clinical score including weight loss, mobility, posture, fur texture and skin integrity (higher scores indicating higher disease severity), as well as liver transaminases were determined in both groups to assess GvHD. In addition, tissues were examined by histopathology and immunohistochemistry. Whereas mice injected with unprocessed PBMC developed an acute and lethal GvHD, the clinical score of mice transplanted with PBMC plus ECP-treated PBMC was significantly lower. Furthermore, these mice showed a substantially prolonged survival after xenogenic transplantation. The typical clinical signs of GvHD, infiltrating immune cells in liver, lung and skin, were observed in both groups but with higher density and morphological changes in non-ECP-treated mice. Whereas non-ECP treated mice succumbed to GvHD, some ECP-treated mice survived and showed symptoms of chronic GvHD, accompanied with an infiltration of CD11c+ human cells in the lungand CD1a+ human cells in the skin. In conclusion, our data demonstrate that this humanized mouse model provides an effective system to investigate the immunomodulating mechanisms of ECP treatment in a preclinical manner.

P193 (V11)

Anti-inflammatory action of UVA-1 oxidation is mediated by Nrf2

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Long wave ultraviolet (UVA) irradiation causes stress to the skin and results in oxidative modification of cellular biomolecules. This contributes to skin aging, disturbance of the immune system and the development of tumors. On the other hand, UVA-1 is successfully used for the treatment of inflammatory skin diseases. Recently, we discovered that oxidation by UVA-1 induces the antioxidant response genes, a potentially beneficial part of the UVA response. This 'adaptive response' toUVA-1 by dermal cells was dependent on Nrf2, a redox sensitive transcription factor.Nrf2 activation and several of its target genes (Heme oxygenase 1, glutathione synthesis genes) have well known anti-inflammatory properties. We thus hypothesized that UVA oxidation would inhibit inflammation *in vitro* and *in vivo*. To test this hypothesis, we induced inflammatory gene synthesis in dermal fibroblasts, keratinocytes and dendritic cells using TLR agonists and PMA. Protein and mRNA synthesis of inflammatory cytokines induced by the TLR4 agonist LPS and by PMA was significantly inhibited by UVA-1 oxidation. We further hypothesized that this inhibition would depend on induction of antioxidant response genes via Nrf2. To test this, we silenced Nrf2 expression with siRNA and used cells from Nrf2 deficient mice for the inhibition experiments. The induction of pro-inflammatory cytokines was mostly restored in Nrf2 deficient cells. Finally, we investigated the anti-inflammatory effect of the UVA-1 oxidation *in vivo* using a PMA based acute contact inflammation model. Pretreatment with UVA-1 inhibited PMA - induced ear swelling and cytokine production, which was mostly reverted in Nrf2 deficient mice. In summary, our data identify Nrf2 as key factor in the modulation of inflammation byUVA-1 and UV generated photoproducts.

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Lack of professional knowledge of tanning bed operators

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We assessed the professional knowledge of tanning bed operators (TBO) by an undercover investigation. In 2003 – shortly after the introduction of a standardized voluntary educational program for TBO – a mixed couple visited 25 randomly assigned tanning salons (TS). The investigators took a qualitative interview disguised as a sales talk. The female investigator (skin photo type I) pretended that she wanted to get quickly an appealing tan. Her male companion (skin photo type III), however, urged her to reconsider the decision to use a sun bed and pointed out clearly to the TBO the high UV sensitivity of his friend's skin (always sunburned, never tanned, polymorphic light eruption). The dialogue was tape-recorded and transcribed into a protocol. 5 years later we reassessed the expertise of TBO in 75 TS selected by random including 24 TS of the 2003 investigation. In 2003 the skin photo type of the client was assessed in three TS (12%) only one assessment being correct (4%). In 2008 the respective assessment was done in only 3/75 TS (4%) but correctly. In 2003 in all but 2 TS (98%) the TBO suggested a concrete irradiation plan; maximum number of tanning sessions (s) recommended: l–3 s/week in 12(48%) and > 3 s/w in 9(36%) TS. The respective values for the follow-up were: irradiation plan 99%, 1–3 s/w 95% and > 3 s/w in only 4% of TS. In 2003 no irradiation plan was suggested in only l TS (4%) and the number of sessions was not defined in three TS (12%). At follow-up only 1 TBO did not define an exact number of tanning s/w. 6 TBO (24%) agreed that a history of polymorphic light eruption was a contradiction to artificial tanning in 2003 but only 7TBO (9%) at follow-up. 60% of TBO sold a tanning accelerator or intended to do so in2003 and 75% in 2008. 17 TBO (68%) denied any health hazards of artificial tanning in 2003. At follow-up 64% of TBO admitted that artificial tanning might be a risk factor whereas 36% denied any hazards. In 2008 still only two TBO said that they had attended a certified educational programme. The lack of professional knowledge results in flawed information given by TBO to potential clients. During the 5 years interval the expertise of TBO did not improve significantly. There is urgent need for a compulsory educational programme.

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Fluorescence induction by two different topical formulations used for photodynamic therapy in a full thickness *ex vivo* pig skin model

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An important aspect of a successful photodynamic therapy is the satisfactory and rapid penetration of 5-aminolevulinic acid (5-ALA) through the stratum corneum of the skin to allow the induction of effects in the respective target cells deeper in the epidermis. Previous studies have shown that the penetration of 5-ALA is influenced by the nature of the formulation. Aim of this study was to analyze the penetration into the epidermis of BF-200 ALA, containing 10% 5-aminolevulinic acid hydrochloride as the active ingredient in a nanoemulsion-based formulation, in comparison to the penetration of the commercially available 16% aminolevulinate methyl ester in anointment (MAL). Protoporphyrin IX (PpIX) fluorescence measurements in an *ex vivo* full thickness porcine skin model were utilized to analyze the penetration. Freshly excised skin from the back of pigs was cleaned, shaved and transferred to a Petri-dish containing Hepes-Agar. The amount of formulation applied was adjusted such that the same amount of the active ingredient per cm2 was applied in each case for 3, 5, 8 and 12 h. Multiple freeze-sections were prepared from each sample at the different time points and their fluorescence measured by quantitative fluorescence microscopy. At all time points, mean fluorescence signals (MFI) of PpIXin pig skin treated with BF-200 ALA were stronger than those for MAL. This was particularly evident after longer incubation times (8 and 12 h). Thus, the PpIX fluorescence signals measured 8 and 12 h after application of BF-200 ALA were4.8 and 5.0 fold higher than those measured after MAL application (MFI 122.2 ± 52.1vs 25.6 ± 5.3 at 8 h and MFI 202.7 ± 54.2 vs 40.3 ± 15.9 at 12 h). Performing semi-quantitative image analysis, the depth of the penetration into the tissue was measured. At all time points, the fluorescence signals of PpIX after application of BF-200 ALA were detected in deeper tissue layers of the epidermis than after incubation with MAL (34.4 ± 6.4 μm vs 21.2 ± 6.3 μm at 3 h, 44.9 ± 3.5 μm vs 27.0 ± 2.1 μm at 5 h, 53.9 ± 10.2 μm vs 33.6 ± 3.8 μm at 8 h and 97.2 ± 5.7 μm vs 42.0 ± 4.2 μm at12 h). In summary, for all incubation times tested in this *ex vivo* model, PpIX fluorescence intensity induced after BF-200 ALA application was higher and reached deeper epidermal levels than fluorescence intensity induced by MAL.

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Dependence between UV-A radiation intensity and ability of singlet-oxygen production by fatty acids

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UVA radiation has been known to generate reactive oxygen species such as singlet oxygen in skin leading to oxidation of lipids and proteins. This influences cellular metabolism and can trigger cellular signalling cascades since cellular membranes as well as the stratum corneum contain a substantial amount of fatty acids and lipids. We aimed to investigate the interaction of UVA radiation with different fatty acids regarding the generation of singlet oxygen. We irradiated fatty acids in solution with laser light at 355 nm. Generation of singlet oxygen was measured by means of luminescence detection at 1270 nm. Fatty acids absorb UVA radiation (320–400 nm), which includes our excitation wavelength of 355 nm. This is presumably due to the small concentration of oxidized forms of fatty acids, which are unavoidable present prior to irradiation due to auto-oxidation. Irradiation of the fatty acids showed a clear time resolved signal at 1270 nm, which is attributed to singlet oxygen. To confirm this assumption, the luminescence signal was measured at different wavelengths in the range from 1150 to 1400 nm. There was a clear maximum at 1270 nm that is equivalent to singlet oxygen transition to its ground state. When extending the irradiation time up to 90 min, oxygen concentration in fatty acid solutions significantly decreased with irradiation time as measured by an oxygen sensor. The higher the number of double bonds in the fatty acids the faster was the oxygen consumption. However, while the oxygen concentration decreased, the intensity of singlet oxygen luminescence increased directly proportional to the number of double bonds in the fatty acids. After that, the luminescence signal decreased. In conclusion, UVA radiation of fatty acids with double bonds generates singlet oxygen possibly due to charge transfer, which leads to peroxidation of those fattyacids. This increase of oxidized products leads to an increase of singlet oxygen generation possibly due to the Russell mechanism, which additionally enhances the oxidation process. After a certain irradiation time, the generation of singlet oxygen decreases due to either lack of oxygen or decomposition of fatty acids to products like alcohols or ketones. This enhancement of UVA induced damage of fatty acids and lipids, must have an impact regarding the oxidative damage in cells.

P197 (V05)

Oxidatively induced mitochondrial CSA and CSB are associated to complexes binding to oxidative DNA damage: implications for participation of CSA and CSB in mitochondrial DNA repair

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Mitochondrial DNA is involved in cancer and aging. Among a wide variety of genotoxic stressors ROS as a by-product of cellular metabolism has high mutagenic potential. Nuclear DNA can be repaired by several repair systems after oxidative insult. Nucleotide excision repair (NER) is a versatile repair system which also removes oxidative damage. Dysfunctional NER causes severe diseases like Cockayne syndrome (CS) which is caused by dysfunctional CSA and CSB proteins and is characterized by premature aging and neuro-degeneration. Although there is increasing evidence that the CSB protein is especially important for removal of oxidative nuclear DNA damage, up to now mitochondria are considered to be free of NER systems. We have previously shown that CSA and CSB proteins get recruited to mitochondria in a ROS dependent way. To further investigate the functional role of CSA and CSB in mitochondria, we assessed mitochondrial repair of 8-OxoGuanosine. In normal fibroblasts the bulk of oxidative DNA lesions in mitochondria were removed within 5 h. Similar results were obtained in other NER deficient cells. In contrast, CSA and CSB deficient cells showed almost no removal of oxidative DNA lesion within this time. Gel-electrophoresis mobility shift assays revealed binding of CSA and CSB proteins to mtDNA. We present evidence that CSA and CSB proteins are involved in NER-independent repair of oxidative DNA damage in mitochondria and bind to DNA damage associated mitochondrial protein complexes.

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Intense pulsed light treatment leads to long term mitochondrial DNA mutations

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Mutations of mitochondrial DNA play a central role in aging and carcinogenesis. Thephotoaging-associated mitochondrial common deletion is a 4977 bp long deletion in the mitochondrial genome and it is considered to be a marker for the presence of other mtDNA mutations. This mutation can be induced by reactive oxygen species (ROS) and UV-radiation. However, it is unclear whether other wavelengths than UV are also capable to induce mtDNA mutations. Therefore, in this study we were interested in possible long term mutagenic effects of treatment with intense pulsed light (IPL) and lasers on mitochondrial DNA. We irradiated normal human melanocytes with single doses from an IPL device and a Ruby laser. These light sources are of special importance as they are used for therapeutically purposes in skin lesions and are increasingly applied in clinical practice. Lasers and IPL employed for the removal of pigmented skin lesions emit radiation within the interaction spectrum of melanin, thus inducing selective photothermolysis. For both sources, immediate DNA damage reached its peak 2 h after irradiation and disappeared 8 h after irradiation. Interestingly, 32 days after a single irradiation dose, a sudden increase of the common deletion was observed. With this study we could show that IPL and Ruby laser are capable to induce mitochondrial DNA mutations long time after direct DNA damage is repaired. Although the reason for this long term effect is unclear it should be considered for future therapeutic use of IPL and Ruby laser.

P199 (V35)

Cancer-retina antigens cGMP-phosphodiesterase 6 and transducin control cGMP metabolism and Ca^{2+} homeostasis in melanoma cells

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Malignant melanoma is a highly invasive tumor derived from neuroectodermal melanocytes, thus sharing the lineage with retinal cells. Recently, we have shown that the key photoreceptor proteins can function as cancer-retina antigens in melanoma. Among these, the aberrant expression of cGMP-phosphodiesterase 6(PDE6) has a higher frequency. Here, we present evidence that PDE6 is the key enzyme regulating the cGMP metabolism in melanoma cells. Decrease of the intracellular cGMP as a results of its hydrolysis by PDE6 leads to the calcium accumulation in melanoma cells. This effect can be blocked by administration of the specific PDE inhibitors zaprinast (ZAP) and dipiridamole (DIP). Interestingly, cultivation of melanoma cell lines with ZAP, DIP and other PDE inhibitors (sildenafiland vardenafil) leads to increased proliferation of the cancer cells. Besides, PDE6can be activated in melanoma cells by another cancer-retina antigen – transducin through Wnt5a-Frizzled-2 cascade, which leads to lowering of cGMP and increase of calcium concentrations. Pertussis toxin (a transducin inhibitor) and antibodies againstFrizzled-2 receptor abolish the effect of Wnt5a on the PDE6 activation. A cultivated keratinocyte cell line HaCat secretes Wnt5a during proliferation of the cells. Cultivation of some melanoma cells with the Wnt5a-containg leads to the decrease of the intracellular cGMP and accumulation of calcium in the cells. An antibody againstWnt5a blocks this effect. The data obtained allow us to suggest that in melanoma cells PDE6 (i) represents a functional enzyme; (ii) maintains the low cGMP and high calcium levels; and (iii) can be controlled via Wnt5a-Frizzled-2-transducincascade.

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Targeted therapy of melanoma: Novel kinase inhibitors with potent and specific anti-melanoma activity

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Background: Melanoma is the most aggressive form of skin cancer and is highly resistant to conventional chemotherapy, immunotherapy and targeted therapy. The findings that the mitogen-activated protein kinase (MAPK; Ras/Raf/MEK/ERK)pathway is constitutively active in melanoma and that 66% of melanomas harbour an activating Raf mutation (B-RafV600E) has raised expectations for targeting this pathway for therapy. In clinical trials monotherapy with the multikinase inhibitor sorafenib has shown little activity in melanoma, although sorafenib augmented the activity of chemotherapy. Therefore, novel approaches to melanoma therapy are urgently needed.
Methods: As preclinical studies *in vitro* often poorly predict the outcome of clinical studies we have developed a novel cell culture model which better compares to the *in vivo* situation: human melanoma cells grown as three-dimensional spheroids are implanted into collagen gels to mimic the tumour architecture and microenvironment. Here we discuss the anti-melanoma activity of the mitogen-activated protein/extracellular signal-regulated kinase kinase (MEK) inhibitor AZD6244 (ARRY-142886) and the B-RafV600E inhibitor PLX4720 in our novel 3D spheroid model, our 3D angiogenesis model, and an *in vivo* xenograft model.
Results: Inhibition of MEK with AZD6244 causes G1-phase cell cycle arrest associated with up-regulation of p27 expression and is cytostatic as a monotherapy in melanoma, but cytotoxic when combined with the mitotic inhibitor docetaxel *in vitro*. AZD6244 – as opposed to sorafenib – has a minor effect on angiogenesis, but a direct effect on melanoma cell proliferation. Furthermore, AZD6244 decreases phospho-ERK *in vivo* and fully inhibits tumour growth at well-tolerated doses and causes tumour regression when combined with docetaxel *in vivo*. Specific inhibition of B-RafV600E with PLX4720 blocks proliferation exclusively in melanoma cells harbouring the B-RafV600E mutation and leads to tumour regression *in vitro* and *in vivo*.
Conclusions: For the first time we show here inhibitors that directly target the MAPK pathway in melanoma to correlate *in vitro* and *in vivo* data. Given their better potency and specificity these novel drugs are important candidates as second generation small molecule therapeutics targeting the MAPK pathway.

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Expression of tight junction proteins in Merkel cell carcinoma

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Merkel cell carcinoma (MCC) is one of the most aggressive skin tumors. It is a rare tumor of the elderly that is characterized by frequent regional lymph node involvement, distant metastases and a high rate of recurrence. Tight junctions (TJs) play a role in compartmentalization in multicellular organisms by sealing the paracellular pathway of epithelial and endothelial cell sheets. In the skin, TJs play a role in barrier function of the epidermis. They are composed of various transmembrane (Claudins, Occludin, JAMs) and plaque proteins (e.g. Zonulaoccludens proteins 1-3 (ZO-1 1-3), Symplekin). Down- as well as up-regulation of TJ proteins was described in several tumors. By investigating 15 MCC we observed a heterogeneous staining pattern for TJ proteins except for JAM-A which is present in all tumors. This reflects the known heterogeneity of MCC. To investigate whether the heterogeneous expression of TJ proteins is correlated to biological behaviour concerning growth and migration of Merkel cell tumor cells we used cell lines established in our lab from MCC of four different patients. Growth of these cell lines is very variable concerning proliferation and morphology of the cells. Two of the four cell lines produce spontaneously spheroids while the others comprise single cells only. Interestingly, only cell lines producing spheroids are positive for occludin and to a very low extent Claudin 4. All cell lines express Claudin 1 and ZO-1 but at different levels. Because it is known that in melanoma cells invasiveness correlates with expression of ZO-1, we performed invasion experiments into collagen gels by using spheroids of cell lines with high or low levels of ZO-1 respectively. After 96 h cells from the high level ZO-1 cell line invaded into the collagen gel whereas there was no invasion of cells from the low level cell line. These results suggest that ZO-1 might be important for invasion of Merkel cell carcinoma cells. The expression of other tight junction proteins like occludin and claudins might result in functional tight junctions and therefore play a role in three dimensional architecture of the tumor and in the isolation of the tumor from its environment.

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Usability of Connexin 26, 30 and 43 as markers for diagnosis and prognosis of malignant melanoma

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Gap junctions (GJ) are cell–cell junctions important for direct communication between neighbouring cells. They are involved in cell proliferation, differentiation and migration. GJ are formed by connexins. Down- as well as up-regulation of connexins have been described in tumour progression depending on the kind of tumour and connexin. Previously we described a down-regulation of Cx43 in malignant melanoma (MM) as well as benign naevi compared to epidermal melanocytes. In addition, we showed an induction of Cx26 and Cx30 in epidermis adjacent to MM but not in MM itself. Here we investigated sensitivity and specificity of the induction ofCx26 and Cx30 in the epidermis adjacent to melanoma as well as its correlation to tumour depth and other parameters, e.g. proliferation, ulcer formation and metastasis. Therefore we investigated 41 cases of MM in various tumour stages as well as 14 benign naevi by immunofluorescence microscopy. Sensitivity and specificity of induction of Cx26 (98%/71%) and Cx30 (100%/50%) in the adjacent epidermis exceeds that of the standard markers MelanA, S100, and HMB45 (in melanoma) in single marker use. In addition, we found a significant correlation of the dissemination of Cx26 and Cx30 protein expression to tumour depth vertically (Cx26 and Cx30positive layers respectively) as well as for Cx26 horizontally (horizontal area of Cx26protein expression). Dissemination of Cx26 and Cx30 expression was correlated to the amount of proliferative cells in the adjacent epidermis but not to proliferative cells in the tumour. Cx43 did not prove usable as a diagnostic or prognostic marker. We conclude that the induction of Cx26 and Cx30 in the epidermis adjacent to malignant melanoma might be a good complementary marker for MM and that MM influences connexin protein expression in the adjacent epidermis in a size but not proliferative cell dependant manner.

P203

ZO-1 in malignant melanoma and adjacent epidermis

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Zonula occludens protein 1 (ZO-1) is a protein originally identified as a tight junction molecule. However, dependent on cell type and differentiation it can also be associated with adherens junctions or gap junctions. Moreover, ZO-1 can be located in the cell nucleus. Therefore ZO-1 is thought to be involved in several functions, e.g. barrier function and cell proliferation. By investigating malignant melanoma (MM) of various tumour stages as well as benign naevi (NZN) we observed ZO-1 expression in 94% MM and 79% NZN. Interestingly, most MM showed increased staining intensity in areas facing the dermis while this was not the case in NZN. Previously, we showed that in cultured melanoma cells ZO-1 is associated with N-Cadherin and is involved in invasiveness of these cells. In the epidermis adjacent to MM we observed a broadened expression of ZO-1 which correlated significantly with tumour size. In addition, patients with ZO-1 in all layers of the epidermis showed significantly higher risk for metastasis than patients with ZO-1restricted to the upper epidermal layers. However, the biological impact of this broadened expression is not yet clear. Due to the fact that we also observed a down-regulation of Claudin 1, the most prominent tight junction protein in the epidermis which is known to be important for epidermal barrier function, we assume that the up-regulation of ZO-1 might not be accompanied by increased barrier function. Analysis of the correlation between broadened expression of ZO1 in the epidermis adjacent to malignant melanoma and cell proliferation showed significantly more proliferative cells in the epidermis when ZO-1 was localized in all epidermal layers. Future experiments using three dimensional skin equivalents over expressing or lacking ZO-1 shall further elucidate the meaning of the broadened expression ofZO-1 for malignant melanoma.

P204

FOXP3+CD25-tumor cells with regulatory function in Sezary Syndrome

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Cutaneous T cell lymphoma (CTCL) has been suggested by *in vitro* experiments to represent a malignant CD4+ T cell proliferation with an inducible regulatory T cell(Treg) phenotype (CD4+CD25+FOXP3+). We investigated percentages of FOXP3+and CD25+ cells in the blood in 15 Sezary, 14 mycosis fungoides (MF), 10 psoriasis patients, and 20 normal healthy donors (NHD). We found similar numbers ofFOXP3+ cells in MF (10.4% of blood CD4+ cells), psoriasis (11.1%) and NHD (9.8%). In 8/15 (53%) Sezary patients significantly reduced percentages of FOXP3+cells were seen in the blood (2.9%) and skin (10.4%). Interestingly, 6/15 (40%) Sezary patients showed significantly increased percentages of FOXP3+ cells (39.7% [blood], 20.3% [skin]), however, these cells did not express CD25. In these latter patients clone-specific T cell receptor-(TCR-)Vβ-chain antibodies were used to demonstrate that these FOXP3+CD25- cells were the monoclonal CTCL tumor cells, but not bystander Treg. FOXP3+CD25- CTCL tumor cells showed a highly demethylated status of the foxp3 gene locus similar to Treg, and they were functionally able to suppress IL-2 mRNA induction in TCR-stimulated conventional T cells. Thus, FOXP3+CD25- CTCL tumor cells with functional features of Treg definea sub-group of Sezary patients which might influence prognosis and treatment.

P205 (V26)

Generation of myeloid derived suppressor cells and regulatory T cells during the progression of murine RET melanoma

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Myeloid derived suppressor cells (MDSC) comprise a phenotypically (CD11b+,Gr-1+, CD124+) heterogeneous population of cells, which can be found in tumor bearing mice and in patients with cancer. Likewise, immunsuppressive CD25+ regulatory T cells (Treg) are elevated in cancer patients and *in vivo* depletion of the Treg is thought to improve cancer therapy. To investigate the role of MDSC – Treg interaction during melanoma growth, murine RET melanoma cells were subcutaneously injected into the C57BL/6 mice and after tumors had grown to approx. 12 mm in diameter, the mice were sacrificed and single cell suspensions were prepared from the tumors. FACS analysis revealed significant numbers of CD124+MDSC within the CD11b+ cells in the tumor and after co-cultivation with syngeneicCD4+ T cells and anti-CD3 antibodies, we show that tumor-derived CD11b+ cells suppressed the proliferation of CD4+ T cells. Moreover, to assess the 'cross-talk' of Tregs and MDSC, we examined the MDSC from CD25-depleted or non-depleted tumor bearing mice. After depletion of the CD25+ regulatory T cells *in vivo*, the relative frequency of CD124+/CD11b+ cells was almost the same in all groups, but the production of IL-10 from the tumor-residing macrophages was up-regulated in theCD25 depleted host. Therefore our results suggest that MDSC together with tumor residing Tregs mediate suppression of anti-tumor immunity and depletion of Treg is not sufficient to improve anti-tumor vaccination.

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Serum amyloid A as a prognostic biomarker in melanoma identified by proteomic profiling

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Purpose: Currently known prognostic serum biomarkers of melanoma are useful in metastatic disease, but fail in early-stage patients. This study was aimed to identify new prognostic biomarkers of melanoma by serum mass spectrometry (MS) proteomic profiling, and to validate candidates compared to established markers.

Patients and methods: Two independent sets of serum samples from 596 melanoma patients were investigated. The first set (stage I = 102, stage IV = 95) was analyzed by matrix-assisted-laser-desorption-and-ionization time-of-flight (MALDI TOF) MS for biomarkers differentiating between stage I and IV. In the second set (stage I = 98, stage II = 91, stage III = 87, stage IV = 103) the serum concentrations of the candidate marker SAA and the known biomarkers S100B, LDH, and CRP were measured using immunoassays.

Results: MALDI TOF MS revealed a peak at m/z 11.680 differentiating between stage I and IV, which could be identified as serum amyloid A (SAA). High peak intensities atm/z 11.680 correlated with poor survival. Univariate analysis showed serum concentrations of S100B, LDH, CRP and SAA as prognostic markers in stage I–IV patients, with high serum values associated with poor survival; in stage I–III patients only S100B, CRP and SAA showed prognostic impact. SAA combined with CRP were strong prognostic classificators of stage I–IV ($P < 0.0000005$), and stage I–III ($P = 0.011$) patients. Multivariate data analysis revealed S100B, CRP, and SAA as independent prognostic factors, with an interaction between CRP and SAA.

Conclusion: SAA combined with CRP might be used as prognostic serological biomarkers in early-stage melanoma patients, helping to discriminate low-risk patients from high-risk patients needing adjuvant treatment.

P207

cFLIP isoforms block death receptor-induced NF-κB activation irrespective of caspase-8 or cFLIP processing

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Death receptors such as CD95 and TRAIL-R1/R2 induce apoptosis in many cells, but can also activate non-apoptotic signalling pathways (NF-κB as well as mitogen-activated protein kinases (JNK, p38)). Different isoforms of FLIP (cFLIPS and cFLIPL) inhibit different steps in death receptor (DR)-associated activation and maturation of procaspase-8. We reasoned that the cleavage of cFLIP, in turn, could differentially influence non-apoptotic DR signals. Thus, we established stable HaCaT cells expressing different cFLIP isoforms (cFLIPS, cFLIPL) or mutants of cFLIPL that are either uncleavable by caspase-8 (cFLIPD376N) or generated after stimulation by DISC-associated caspase-8-mediated cleavage (cFLIPp43). All isoforms/mutants of cFLIPL blocked death ligand (DL)-mediated apoptosis, whereas a distinct cleavage pattern of caspase-8 was detected in the DISC. Only cells expressing full length cFLIPL (irrespective of cFLIP cleavage) sufficiently induced proteolysis of caspase-8 to its p43/41 fragments. In contrast, cFLIPS or cFLIPp43 blocked procaspase-8cleavage. We next examined DR-induced non-apoptotic signals. TRAIL or CD95L activated JNK within 15 min. MAPK p38 was induced in a biphasic manner. Interestingly, all cFLIP isoforms/mutants completely inhibited the late DL-induced activation of p38 or JNK. Moreover, cFLIP isoforms or mutants blocked DL-mediated IκBα phosphorylation, NF-κB activation, and induction of the target gene IL-8. In summary, cFLIP isoforms are not only potent inhibitors of DL-mediated apoptosis, but also block DL-mediated non-apoptotic signalling pathways such as NF-κB or MAPK JNK or p38. This indicates that cleavage of cFLIPL or caspase-8 in the DISC is neither associated with increased NF-κB signalling nor necessary for the inhibitory function of cFLIP isoforms on DR-induced NF-κB or MAPK activation. Taken together, our data highlight the importance of cFLIP and its isoforms for the inhibition of DR-induced non-apoptotic signals that might be of crucial importance during tumorigenesis of keratinocyte skin cancer.

P208 (V22)

Methylthioadenosine phosphorylase is a predictive marker for response to adjuvant interferon therapy in patients with malignant melanoma

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Using tissue microarrays assembling 465 nevi, primary melanomas and metastases, we investigated whether expression of methylthioadenosine phosphorylase (MTAP), a recently suggested biomarker of malignant melanoma, has a prognostic significance and may predict responsiveness to adjuvant interferon therapy inmelanoma patients. Because of its association with MTAP activity and interferon signalling pathways, STAT1 immunoreactivity was analyzed, too. MTAP expression was significantly reduced in melanomas and metastases compared with nevi ($P < 0.001$); STAT1 expression significantly increased. In melanomas, loss of MTAP expression was significantly related to Clark level ($P < 0.028$), tumor thickness ($P < 0.008$) and nodal status ($P < 0.0033$); whereas STAT1immunoreactivity was only significantly related to Clark level ($P < 0.049$). Interestingly, subgroup analysis of patients with a tumor thickness of 1.5–4.0 mm revealed a significant survival benefit from adjuvant interferon treatment regarding recurrence free survival (RFS) ($P = 0.025$) if MTAP expression was positive in the primary melanoma. Patients with STAT1-positive melanomas also tended to benefit from interferon concerning RFS ($P = 0.074$) and showed a significant benefit concerning overall survival ($P = 0.045$). Moreover, according to Cox analysis, MTAP expression in contrast to STAT1 was an independent prognostic marker. In conclusion, MTAP represents a highly promising immunohistochemical marker for prognosis and interferon response of patients with malignant melanoma.

P209

The role of the chemokine CCL20 in tumor-associated angiogenesis

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The activation of the EGFR/Ras/ERK-signalling pathway is a crucial step in the malignant transformation of a wide variety of tumors. The activation of Ras regulate schemokine expression in a dichotomic manner with an inducible set demonstrating pro-tumor and a repressible set showing anti-tumor properties. Here, we demonstrate that tumors may enhance angiogenesis by up-regulating the expression of CCL20through the activation of the EGFR/Ras/ERK-signalling pathway. CCL20 directly acts on endothelial cells *in vitro* and *in vivo* through its specific corresponding receptor, CCR6. Activation of CCR6 signalling in endothelial cells induces cell migration and leads to enhanced vessel formation. In *in vivo* Matri gel plug assays, CCL20 induced vascularization of plugs. Furthermore, tumor growth and vascularization of B16F10-derived tumors was dramatically inhibited in CCR6-deficient C57BL/6-micecompared to wildtype C57BL/6 mice. Collectively, our data identify a novel mechanism of tumors to induce tumor angiogenesis.

P210 (V20)

Genome-wide RNAi loss-of-function screen identifies key signalling pathways for melanoma progression

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Melanoma growth and progression are multistep processes involving a plethora of molecules and signalling pathways. Yet the key mechanisms have not been defined so far since a complete picture of molecules and pathways involved in melanoma growth and progression is still lacking. Here we performed a genome-wide lentiviral RNA interference (RNAi) loss-of-function screen in metastatic melanoma cells. More than 100 000 different short hairpin (sh) RNAs of a shRNA library covering all known genes were tested by lentiviral transducton of melanoma cells. shRNAs of functional relevance for melanoma cell growth and survival were identified by positive and negative selection of melanoma cell clones with individual siRNAs by keeping melanoma cells under standard growth conditions for 10 days. Genomically integrated shRNAs were extracted from melanoma cells, PCR amplified, labelled and hybridized onto commercially available oligonuleotide microarrays with 45 000 gene probe sets. By this means, key signalling pathways/molecules for melanoma cell growth and survival were identified, some of which had already been described before such as BRAF kinase, phosphoinositide-3-kinase and integrin-linked kinase. However, some were indeed new such as mitogen-activated extracellular signal-regulated protein kinase kinase 1 (MEKK1), Janus kinase 1, cAMP-dependent protein kinase β and protein kinase C θ. In subsequent experiments, corresponding pathways were blocked with commercially available specific inhibitors and a series of specifically designed new kinase inhibitors (of the in dirubin family, which is known to block ATP binding of a variety of signalling kinases). By this means, melanoma cell proliferation and cell cycle progression could significantly be inhibited. Taken together, we were able to identify key signalling pathways relevant for melanoma cell growth and survival. Moreover, chemical inhibitors of signalling pathways were identified that might in future be candidates for melanoma treatment after appropriate preclinical testing.

P211

Induced synthesis of hyaluronic acid in stromal fibroblasts supports cell proliferation, colony growth and motility of melanoma cells

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Tumor–Stroma Interactions are thought to be important for tumor growth and metastasis. Preliminary data suggest that fibroblasts receive soluble signals from melanoma cells and further deposit large amounts of hyaluronic acid (HA) in the tumor stroma, thus supporting melanoma cell proliferation and motility. In the present paper we analyzed (i) the gene expression pattern of cultured fibroblasts and melanoma cells concerning HA-synthetases and HA degrading hyaluronidases by RT-qPCR and zymography; (ii) the resulting amount of deposited HA was measured by HA-ELISA; and (iii) cell proliferation on fibroblast-feeder layers, cell motility and clonogenic growth of melanoma cells were assessed depending on the activity of HA metabolism in the fibroblasts. Melanoma cells synthesize only little amounts of HA-synthetases and consequently their cell supernatants contain very low concentrations of HA. Fibroblasts expressHAS2 and HAS3 synthetases and secrete 1000 times more HA than MM cell lines. Medium transfer experiments showed that melanoma cells secrete soluble mediators that induce HAS1 and HAS2 expression and HA-secretion by fibroblasts. Melanoma cell lines differ in their capacity to degrade HA thus modifying the net amount of HA that is deposited in co-cultures. This model demonstrates that stroma cells produce HA found in many tumors under the control of melanoma-derived mediators. We also found that melanoma cells proliferate up to 100% more when growing on HA synthesizing fibroblast- feeder layers and that blocking the HA-synthesis in fibroblasts reduces this effect by 20–40%. Furthermore, the fibroblast-derived HA increases the random motility of melanoma cells measured by time-lapse video microscopy and supports colony forming in soft-agar assays. These experiments outlined herein not only establish a model to study tumor promoting tumor–stroma interactions but may also identify novel targets for anti-proliferative or anti-metastatic therapies in malignant melanoma.

P212

The transcription factor c-Jun is regulated by loss of active cell–cell contacts during development and progression of malignant melanoma

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The transcription factor c-Jun is a key player in the process of cell proliferation and tumor progression. It forms homodimers or heterodimers with other members of the transcription factor superfamily AP-1, influencing the expression of a multitude of regulators involved in tumor development and metastasis. We could show by Western Blot analysis that c-Jun protein is up-regulated in melanoma cells, whereas in melanocytes c-Jun protein is not expressed. Moreover, reporter gene assays revealed a strong AP-1 activity in melanoma cells. Gel shift assays confirmed a significant binding to the AP-1 consensus sequence in melanoma cells. Inhibition of c-Jun by a dominant negative form of c-Jun (Tam67) leads to loss of the transcriptional activity of AP-1 in melanoma cells. This indicates an essential role of c-Jun for AP-1 activity in melanoma. The cell–cell adhesion molecule E-Cadherin plays a key role during development and progression of malignant melanoma. Loss of E-Cadherin leads to proliferation and metastasis. Interestingly, there is a coincidence in the loss of E-Cadherin expression and up-regulation in c-Jun protein in malignant melanoma. Our data could show that loss of E-Cadherin expression during melanoma development induces c-Jun activity, whereas in melanocytes active cell–cell-contacts via E-cadherin have a negative impact on c-Jun, suggesting a direct link between E-Cadherin and c-Jun. Further experiments show that the cytoskeleton is involved in the cell–cell contact dependent regulation of c-Jun. Treatment of melanoma cells, which re-express E-Cadherin with the cytoskeleton disrupting agent Nocodazole reveals a strong induction of c-Jun protein in the nucleus. Reporter gene assays confirmed an up-regulation in AP-1activity and gel shift assays showed an increased binding to the AP-1 consensus binding sequence. Melanoma cells treated with Taxol, a cytoskeleton stabilizing agent, show the reverse effects. These experiments point to an involvement of the cytoskeleton in the E-Cadherin dependent regulation of c-Jun. Loss of E-Cadherin and the resulting loss of cell–cell contacts during melanoma development induces c-Jun activity, suggesting an important role of this regulation in melanoma development and progression.

P213

Integrin beta3 expression is regulated by let-7a miRNA in malignant melanoma

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Although integrin beta3 is known to play an important role in melanoma progression and invasion, regulation of integrin beta3 expression in melanoma has not been analysed in detail until today. As transcriptional regulation of integrin beta3 was ruled out by our analysis we concentrated on regulation by microRNAs (miRNAs).Comparing primary melanocytes and malignant melanoma cell lines, we found that one candidate miRNA, miR-let-7a, was lost in melanoma and sequence analysis suggested an interaction with the 3' untranslated region (3' UTR) of integrin beta3mRNA. Transfection of melanoma cells with let-7a pre-miR™ molecules resulted in down-regulation of integrin beta3 mRNA and protein expression. In addition, we cloned the 3' UTR of the integrin beta3 mRNA containing the let-7a target sequence into a reporter plasmid and revealed that let-7a negatively regulates reporter gene expression. The repressed expression of integrin beta3 accompanies with reduced invasive potential of melanoma cells transfected with synthetic let-7a molecules observed in Boyden chamber assays. On the other hand, induction of expression of integrin beta3 was achieved in melanocytes by transfection with let-7a anti-miRs resulting in invasive behaviour of transfected melanocytes. In summary, we determined miRNA let-7a to be an important regulator of integrin beta3 expression and showed that loss of let-7a expression is involved in development and progression of malignant melanoma.

P214

Tumor suppressor 14-3-3σ and melanoma cell senescence

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Recently, we were able to demonstrate that the 14-3-3σgene, which is epigenetically silenced by gene methylation in melanoma metastases, is a potent tumor suppressor for malignant melanoma. Here we extend and refine these analyses showing that demethylation of the 14-3-3σgene in melanoma cells by Aza-CdR induced 14-3-3σ re-expression followed by cell cycle arrest in G0-G1 and G2-M. Similar results were obtained in 14-3-3σstably over expressing cells. Synchronous 14-3-3σ over expressing melanoma cells did not progress through cell cycle but showed a high and invariable number of 4n DNA containing cells after release of double thymidine block. Because permanent cell cycle arrest in G0-G1 is a hallmark of oncogene-induced cellular senescence, we further tested whether 14-3-3 σ might be involved in this process. Indeed, stable overexpression of 14-3-3σin melanoma cells after lentiviral transduction resulted in a dramatic increase in the number of senescent cells (>40%) compared with control cells (<5%), as determined by analysis of senescence-associated β-galactosidase (SA-β-Gal) activity. In order to study the possible contribution of 14-3-3σ to cellular senescence induced by genotoxic stress, melanoma cells were treated with chemotherapeutic agent adriamycin. Adriamycin induced p53 and 14-3-3σexpression and a high number of senescent cells in normal control cultures and a significantly lower number in cultures of 14-3-3σknockdown cells, suggestive for an important role of 14-3-3σ in genotoxic stress-induced melanoma cell senescence. Taken together, these findings indicate that 14-3-3σ is involved in cell cycle checkpoint control and cellular senescence in melanoma cells. Loss of 14-3-3σexpression by gene methylation might negatively interfere with both processes, ultimately leading to melanoma progression.

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Regulation of TRPM2 expression in malignant melanoma by a tumor-enriched antisense transcript

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It is known that in cancer, where the bulk of the genome becomes hypomethylated, the generation of antisense transcripts could affect the function of key on co-suppressor genes. Using a software called Anti-Hunter for a genome-wide search for antisense transcripts expressed in human ESTs from melanoma, a tumor-enriched antisense transcript, TRPM2-AS, was identified. It mapped within the locus of TRPM2, an ion channel capable of mediating susceptibility to cell death. Analysis of the TRPM2-AS genomic region indicated another tumor-enriched TRPM2 transcript, TRPM2-TE, which is located across a CpG island shared with TRPM2-AS. Expression ofTRPM2-TE results in a short in-frame transcript of the C-terminal region of TRPM2.Quantitative expression analysis confirmed the up-regulation of both TRPM2-AS andTRPM2-TE transcripts and down-regulation of full length TRPM2 in malignant melanoma. Activation of the two new transcripts in melanoma was shown to be correlated with hypomethylation of the shared CpG is land. To investigate the functional relevance of TRPM2-TE, expression was down-regulated via stable antisense transfection. Because full length TRPM2 is known to mediate susceptibility to cell death we performed apoptose assays. Measurement of cell death showed that the knock-down of TRPM2-TE increased melanoma susceptibility to apoptosis and necrosis. We also generated melanoma cell clones with increased expression of full length TRPM2. Cells expressing higher levels of full length TRPM2 showed reduced proliferation and higher amounts of apoptotic cells. Transient transfection of full length TRPM2 over expressing cells with TRPM2-TE protected these cells from apoptosis. Therefore, we hypothesize a dominant-negative role of TRPM2-TE in melanoma. In summary, we determined that TRPM2-TE could contribute to the down-regulation of TRPM2 function in melanoma cells and that increased expression of full lengthTRPM2 in melanoma cells leads to increased susceptibility to cell death.

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Dual role of beta-catenin in primary melanocytes and malignant melanoma cells

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The oncogene beta-catenin is up-regulated in different types of cancer, such as colon cancer. It is part of the cell adhesion complex, is a signalling molecule in the Wnt pathway and upon translocation to the nucleus binds to the transcription factors TCF/LEF and activates target genes. Our own studies have shown that during melanoma progression there is an increase in beta-catenin expression and a translocation of beta-catenin into the nucleus. Down-regulation of beta-catenin expression in metastatic melanoma cells resulted in a significant decrease in proliferation and survival of melanoma cells. These data indicated that beta-catenin plays an important role in the survival and growth of metastatic melanoma cells. However, until now the role of beta-catenin in melanoma development is largely unknown. Therefore, we asked whether the expression of constitutive active beta-catenin in primary melanocytes or radial growth phase (RGP) melanoma cells can initiate their transformation to invasive melanoma cells. Upon beta-catenin overexpression in primary melanocytes and RGP melanoma cells we observed increased expression of beta-catenin and a translocation to the nucleus which was accompanied by the activation of TCF/LEF/beta-catenin mediated transcription of target genes. Interestingly, melanocytes and RGP-melanoma cells over expressing beta-catenin stopped to proliferate, changed their morphology and lost their adhesion to the culture plate and to neighbouring cells. This went along with loss of E-cadherin expression and re-expression of N-cadherin. Subsequently the cells died via non-apoptotic cell death, after detaching from the culture plate. Microarray analysis of primary melanocytes expressing the constitutive active beta-catenin revealed changes in expression of genes involved in tumor survival, cell adhesion and differentiation. Our data suggest that beta-catenin is not capable to transform primary melanocytes on its own, however that beta-catenin is critically involved in survival and aggressive growth of metastatic melanoma cells.

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DNA repair host factors modulate side effects of temozolomide or dacarbazinemelanoma treatment rather than treatment efficacy and are determined by promoter methylation

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The efficacy of temozolomide (TMZ) or dacarbazine (DTIC) melanoma treatment may depend on low O6-methylguanine-DNA-methyltranferase (MGMT) repair and on high mismatch repair (MMR). The aim of this study was to identify markers for hematologic side effects and treatment efficacy of TMZ or DTIC melanoma treatment. For this purpose we recruited 51 Caucasian patients with metastasized melanoma. In each patient the mRNA expression of MGMT and two essential MMR genes MLH1and MSH2 was measured in peripheral blood lymphocytes using real-time RT-qPCR. The entire coding gene regions including splice sides were sequenced in each patient to identify genetic variants and the promoter methylation status of the three repair genes was determined with methylation specific PCR. We found that inter-individual variations in mRNA expression of MGMT, MLH1, and MSH2 did not correlate with treatment efficacy (*P*: 0.36–0.82; Fisher's exact test). Interestingly, either low or high mRNA expressions of MGMT, MLH1, and MSH2 were significantly associated with reduced hematologic side effects (P: 0.008-0.019; Fisher's exact test). We identified a total of 5 variants in the MGMT gene, 13 variants in MLH1, and 7 variants in MSH2, including 5 novel genetic variants in the MLH1 gene (C36985867A, C36993642del, G37010441A, T37030079C and CTT37032185del). Only 1 variant in MLH1 (G37010414A) showed a tendency for reduced MLH1 gene expression whereas the methylation status of the gene promoters correlated well with gene expression in all 3 genes. Our results indicate that either low or high expression of MGMT, MLH1, and MSH2 in peripheral blood lymphocytes may serve as a marker for reduced hematologic side effects of TMZ or DTIC melanoma treatment but not as a marker for treatment efficacy. Furthermore, our results indicate that the expression of MGMT, MLH1 and MSH2 is dependent on promoter methylation rather than on gene polymorphisms.

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Processing of MIA protein during melanoma cell migration

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MIA (melanoma inhibitority activity) has been identified as a small 11 kDa protein highly expressed and secreted by malignant melanoma cells but not expressed in melanocytes. Previous studies revealed that MIA protein plays an important functional role in melanoma development and cell invasion and that MIA expression directly correlates with tumor progression *in vivo*. Recent data describe a direct interaction of MIA protein with cell adhesion receptors integrin alpha 4 beta 1 and integrin alpha 5 beta 1. Integrin recycling, a process where integrins are internalized at the cell rear and subsequent re-exocytosed at the cell front is known to regulate migration processes. By modulating integrin activity MIA protein induces a phenomenon we refer to as active detachment of melanoma cells from extracellular matrix molecules. This study concentrates on the contribution of MIA protein to invasion and migration processes after secretion by tumor cells. It was aimed to elucidate the mechanism by which MIA protein promotes cell detachment and thus influences formation of metastases. Therefore, the melanoma cell line MelIm was treated with fluorescently labelled MIA protein and MIA processing by the cell was carefully determined. We could show that extracellular MIA protein directly binds to integrin alpha 5 beta 1 and that MIA protein is internalized together with these cell adhesion receptors at the rear cell pole. The defined localization is in agreement with MIA function during migration. By inhibiting the PKC-dependent integrin internalization or using MIA-inhibiting peptides, the MIA protein uptake was almost completely blocked, which is a further proof for integrin-coupled uptake. We could also demonstrate that endocytosis is followed by dissociation of MIA-integrin complexes and by degradation of MIA protein in acidic vesicles while integrins are recycled. Since MIA protein has a major contribution to the aggressive characteristics of malignant melanoma in particular in formation of metastasis, it is in alienable important to elucidate the MIA effect on tumor cells to find a novel therapeutic strategy in the fight against skin cancer.

P219 (V16)

Inhibition of platelet gpIb(alpha) and promotion of melanoma metastasis

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Platelet glycoprotein Ib(alpha) [gpIb(alpha)] is a part of the receptor complex GPIb-V-IX which plays a critical role in the initialization of primary hemostasis especially through the interaction with subendothelial von Willebrand factor (vWF) at the sites of vascular injury. There is accumulating evidence for a contribution of platelet receptors in the process of hematogenous tumor metastasis, thus making GPIb(alpha) an interesting molecule to study in this context and a possible therapeutic target in the treatment of cancer. The effect of GPIb(alpha) inhibition on experimental pulmonary melanoma metastasis was tested in a syngeneic mouse model using C57BL/6 mice and B16F10 melanoma cells. GPIb(alpha) was blocked monovalently using Fab fragments of p0p/B, a monoclonal inhibitory antibody directed against the vWF binding site on murine GPIb(alpha). GPIb(alpha) inhibition by p0p/B Fab-fragments led to a significant increase of pulmonary metastasis (control: 359.4 metastases/lung, 95% CI = 210.7–508; p0p/BFab-fragments: 899.4 metastases/lung, 95% CI = 631.3–1,167.5; $P = 0.0047$).Assessment of the early fate of circulating GFP-labelled B16F10 showed improved survival and pulmonary arrest of the tumor cells as early as 30–300 min following gpIb(alpha) inhibition. In contrast, when GPIb(alpha) was blocked inP-selectin deficient mice, the enhancing effect of GPIb(alpha) blockade on metastasis was completely abrogated. This is the first experimental evidence showing that inhibition of platelet functions may not always impair tumor metastasis, but may in some cases result in the opposite effect, namely increased metastasis of a tumor. Our findings support the hypothesis that the extracellular domain of GPIb(alpha) contributes to the control of metastatic tumor progression, in addition to its role in hemostasis. This novel function of GPIb(alpha) is presumably involved in the promotion of the initial, P-selectin-dependent steps of metastasis.

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H-Cadherin expression reduces invasion of malignant melanoma

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Melanocytic behaviour, survival and proliferation is regulated through a complex system of cell–cell adhesion molecules. Pathological changes, leading to development of malignant melanoma, upset the delicate homeostatic balance between melanocytes and keratinocytes and can lead to altered expression of cell–cell adhesion and cell–cell communication molecules. Malignant transformation of melanocytes frequently coincides with loss of E-cadherin expression. We showed loss of another member of the superfamily of classical cadherins, H-cadherin (CDH-13), which may be involved in the development of malignant melanoma. The data showed that H-cadherin expression is lost in nearly 80% of the analyzed melanoma cell lines. Functional assays showed that the re-expression of H-cadherin decreases migration and invasion capacity, as well as anchorage-independent growth in comparison to control melanoma cells. Further, melanoma cells which re-express H-cadherin via stable transfection show a reduction in rate of tumor growth in a nu/nu mouse tumor model in comparison to the parental control transfected cell lines. H-cadherin, also known as T- (truncated) cadherin, is one of the most unusual members of the classical cadherin superfamily, it lacks the highly conserved cytoplasmic and transmembrane regions and instead is attached to the cytoplasmic membrane through a glycosyl-phosphatidylinositol anchor. Little is known about the biological role of H-cadherin in human cancers, the only physiological role established so far is its participation in the regulation of neuron growth during embryogenesis. Therefore we were also interested to investigate the signalling potential of H-cadherin during melanoma development. Our data give a hint for the involvement of H-cadherin in the regulation of the PI3-Kinase signalling pathway which has influence on anti-apoptotic signals and proliferation. The PI3-Kinasepathway and Akt deregulation are well known molecules responsible for development of malignant melanoma. Additionally, described molecules which are deregulated during melanoma are the transcription factors AP-1 and CREB. These two molecules are also regulated via H-cadherin which could be shown by reporter gene assays and western blot. Our study presents for the first time the down-regulation of H-cadherin in malignant melanoma.

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Transcriptional targeting of adenoviral lytic replication and therapeutic gene transfer for combined viro-/gene therapy of malignant melanoma

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Novel treatment modalities for malignant melanoma are highly sought for and should ideally implement both a high degree of tumor-selectivity and multimodality. Virotherapy, tumor-restricted virus infection, is a particularly interesting regimen towards this end, because viruses allow for incorporation of biological targeting mechanisms at multiple stages of their replication cycle and for the insertion of therapeutic genes into their genome ('armed' oncolytic viruses). These facilitate the combination of viral oncolysis with a second cancer-killing mechanism defined by the therapeutic gene (prodrug activation, anti-tumor immunity, others). We engineered melanoma-targeted oncolytic adenoviruses by replacing the promoter of the essential early viral gene E1A with an optimized tyrosinase promoter (AdTyr).In non-melanoma cells, these viruses showed a block to their replication cycle before the viral genome was amplified. We hypothesized that insertion of a therapeutic gene into the late viral transcription unit, which is only expressed after viral DNA replication, would indirectly restrict its expression to melanoma cells. We inserted the reporter gene luciferase into the late transcription unit of AdTyr by using either an internal ribosomal entry site, a 'self-cleaving' 2A peptide or a splice acceptor site. The resulting oncolytic adenoviruses showed similar cytotoxicity in melanoma cells. The splice acceptor construct (AdTyr_SALuc) showed the highest level of specificity for viral replication, cell lysis and luciferase expression. In contrast, the 2A sequence interfered with tyrosinase promoter regulation of viral and transgene expression. Presently, we investigate different combination therapies by replacing the luciferase gene of AdTyr_SALuc with therapeutic genes. We conclude that the mode of transgene expression/locale of transgene insertion into the virus genome critically determine the efficacy of 'armed' oncolytic viruses. Our work established with AdTyr_SATransgene a virus that combines a high degree of selectivity for both replication and transgene expression. Thus, this virus represents a novel targeted agent for combined gene therapy and viral oncolysis of malignant melanoma.

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Improved therapeutic viruses for oncolysis of malignant melanoma: combining rational mutagenesis and therapeutic gene expression

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Viral oncolysis is a promising new modality for the treatment of cancer by specific viral replication in tumor cells resulting in cell lysis and release of progeny viruses that spread in the tumor. Adenoviruses are leading oncolytic agents and have been investigated in several clinical trials. These have demonstrated proof of principle and a favourable safety profile, but intra-tumoral spread of viruses and therapeutic efficacy were unsatisfactory. We developed oncolytic adenoviruses for treatment of malignant melanoma, which feature enhanced cell entry and highly selective replication. Aiming at increased therapeutic potency of these viruses, we pursued and combined two strategies: to enhance lytic activity by mutations of viral genes and to insert therapeutic genes into the viral genome. The latter, generating 'armed' oncolytic adenoviruses, aims at the expression of therapeutic proteins to work in concert with viral oncolysis. Specifically, we investigated in a panel of tumor cell lines and low passage tumor cells how the deletion of the anti-apoptotic early viral E1B19K gene affects both oncolytic potency and transgene expression of 'armed' viruses. Our data showed that the E1B19Kdeletion dramatically increases oncolysis in some tumor cells, but reduces lytic activity in others. Cells infected with the mutant virus showed signs of apoptosis earlier during the replication cycle in all cultures analyzed. The reason for differences in cell lysis by E1B19K mutant viruses between cell types is currently under investigation, data will be presented. Importantly, in reporter gene assays we revealed that the E1B19K deletion, thus early induction of apoptosis, does not interfere with the expression of transgenes inserted into the late viral transcription unit of oncolytic adenoviruses. We conclude that the deletion of the E1B19K gene is a promising strategy to increase the potency of oncolytic adenoviruses, however, its feasibility needs to be assessed individually for each tumor. Further, we demonstrate that this mutation, when functional, can be combined with therapeutic gene expression facilitating a multimodal viro-gene therapy.

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Bone morphogenetic proteins induce expression of matrix metalloproteinases in melanoma cells and surrounding fibroblasts

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Bone morphogenetic proteins are secreted growth factors which belong to the TGFβ superfamily. In previous studies, we demonstrated an up-regulation of BMP-4 andBMP-7 expression in melanoma cells in comparison to normal human epithelial melanocytes. Moreover, BMPs promote cell migration and invasion as shown by functional analysis. To further investigate the role of BMPs in degradation and remodelling of the extracellular matrix, we generated stabile cell clones with reduced BMP activity either by transfection with an antisense BMP-4 construct or by stable transfection with the general BMP inhibitor chordin. Interestingly, we found that these cell clones showed reduced expression of matrix-metalloproteinases MMP-1,-2,-3and -9. As BMPs are secreted growth factors, we also examined the influence of BMPs on tumour surrounding fibroblasts. Treatment of stromal fibroblasts with recombinant BMP-2 or BMP-4 increased expression of MMP-1,-2,-3 and -13. To analyse direct effects of BMPs secreted by melanoma cells, we cultured dermalfibroblasts in conditioned cell culture supernatant from the antisense BMP-4, the chordin transfected and control transfected cell clones. Accordingly to our previous data, we observed a reduced expression of MMP-3 in fibroblasts cultured with media from cell clones with diminished BMP activity compared to supernatant from control transfected or parental cell lines. Our results revealed for the first time that BMPs not only exert autocrine effects on the secreting melanoma cells themselves, but also paracrine effects on stromal fibroblasts and thereby enhancing the progression of malignant melanoma. Inhibition of BMP activity could, therefore, be an accretive therapeutic target.

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Does interferon gamma-induced tumor dormancy depend on the induction of tumor cell senescence?

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The T antigen (Tag) of the simian virus 40 under control of the rat-insulin-promotor(RIP) leads to the development of endogenous β-cell tumors in transgenic RIP1-Tag2mice. In previous studies, we demonstrated that interferon-gamma (IFN-γ)-producing, antigen-specific Th1 cells strongly inhibit tumor development through mechanisms independent of CD8+ cytotoxic T cells or destruction of Tag-expressing islet cells. Moreover, we found no increased apoptosis *in vivo*. Instead, Th1 cells reduce angiogenesis and decrease proliferation of tumor cells in RIP1-Tag2 animals. Both effects are strictly IFN-γ-dependent. To elucidate the principles underlying the therapeutic efficacy of antigen-specific Th1 cells, we analyzed the influence of IFN-γ on β cell functioning, apoptosis and senescence in an *ex vivo* approach. We isolated islets and tumors from RIP1-Tag2 mice at different stages of tumor development. Primary β cells and islet adenoma and islet carcinoma cells were then treated with physiological doses of recombinant mouse IFN-γ for 72–96 h. Subsequently, we determined senescence-associated β-galactosidase as a marker of senescent cells and TUNEL staining as a measure of apoptosis. Furthermore, we immuno-stained the isolated cell populations with an anti-synaptophysin antibody to discriminate between β cells and fibroblasts. First results showed an IFN-γ-dependent induction of senescent cells in up to 40% of the cells. In sharp contrast, IFN-γ did not induce detectable signs of apoptosis *in vitro*. The rate of apoptotic cells was below 5% of total cells. Double-staining with β-galactosidase/synaptophysin revealed that senescence occurred in synaptophysin-positive β cells. Thus, antigen-specific Th1cells reduce the tumor size of endogenous insulinoma, presumably by IFN-γ-mediated inhibition of tumor growth, whereas tumor cell destruction plays a minor role. As Th1 cells prevented islet carcinomas without inducing any signs of cell destruction or apoptosis, induction of ß cell senescence in the absence of apoptosis is at least one central mechanism underlying the antitumoral effect of IFN-γ-producing Th1 cells.

P225 (V14)

Eradication of primary melanomas by combinatorial chemo-immunotherapy

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Several new strategies for the immunotherapy of cancer have been experimentally developed based on insights how transplanted mouse tumor cells are recognized and destroyed by the innate and adaptive immune system *in vivo*. However, the predictive value of tumor transplantation experiments in mice for successful clinical translation has been questioned because promising treatment approaches frequently failed in man. To mimic the expected clinical situation more closely we established a genetically engineered mouse model which faithfully portrays the molecular pathogenesis of human melanoma. We crossed mice over expressing the hepatocyte growth factor with mice carrying an oncogenic germline mutation in the cyclin dependent kinase 4 (Hgf/Cdk4R24C mice). Aberrant growth factor signalling and impaired cell cycle control synergized to promote the development of primary and metastatic melanomas in the skin selectively. To investigate the interaction between tumor cells and cytotoxic T cells *in vivo*, we established an experimental system involving the adoptive transfer of TCR-transgenic CD8+ pmel-1 T cells which recognize the melanocyte-specific self antigen gp100. Adoptively transferred CTL could be efficiently activated *in vivo* by simultaneous adenoviral vaccination with recombinant Ad-gp100 leading to expansion and effector cell differentiation in tumor-bearing mice. T cells were able to infiltrate primary cutaneous melanoma but did not lead to tumor regression, suggesting that their effector functions were inhibited in the tumor microenvironment. To overcome the barriers for immune attack we designed a treatment protocol where adoptive lymphocyte transfer and viral vaccination were preceded by chemotherapy (to enhance homeostatic T cell expansion and perturb neo-angiogenesis) and followed by injections of immunostimulatory nucleic acids (to imitate a viral infection and support T cell effector functions). This protocol strongly enhanced cytotoxic destruction of melanoma cells and promoted complete and long-term regression of large primary tumors with minimal autoimmune side effects. Our results in an experimental model which faithfully portrays the clinical situation can be directly translated for the treatment of patients with melanomas.

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Efficient and selective killing of melanoma cells by new oncolytic adenoviral vectors with doxycycline-inducible expression of CD95L/FasL

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The unbroken high mortality of cutaneous melanoma demands the development of new strategies for targeting metastatic melanoma cells. Overexpression of the death ligands CD95L/FasL for induction of apoptosis has been shown as highly efficient in melanoma cells expressing the respective death receptors. For specifically targeting of melanoma cells, the conditional replication-competent adenoviral vectors Ad5-FFE-02 and Ad5-FFE-01 were constructed, which drive the expression of CD95L/FasL by a tetracycline-inducible promoter. In FFE-02 adenoviral replication is restricted to melanoma cells due to the control of the adenoviral replication gene E1Aby a human-derived tyrosinase promoter. In Ad5-FFE-01 expression of E1A is controlled by a telomerase promoter with tumor-selective activity. For safety reasons, in both vectors E1B is deleted preferentially allowing replication in tumor cells within activated p53 pathway. Furthermore, a mutated E1A was used, which lacks the retinoblastoma binding site allowing replication in tumor cells with uncoupled cell cycle. After transduction of FFE-02, high expression of E1A and doxycycline-dependent induction of CD95L were characteristic for tyrosinase-positive melanoma cell lines (Mel-HO, Mel-2a, SK-Mel-19 and MeWo), but were not seen in non-melanoma control cell lines (HeLa, Hep-G2, MCF-7 and PFSK-1). In consequence, also FFE-02-dependent viro-oncolysis and induction of apoptosis were restricted to melanoma cells. The cell killing activity of FFE-01 with the telomerase promoter-E1A construct was at least as pronounced as with FFE-02. Thus the combination of adenoviral replication and induction of apoptosis in FFE-02 or FFE-01-transfected cells resulted in synergistic melanoma cell killing. Based on the high sensitivity of melanoma cells for CD95L, this new adenoviral vectors open a field for development of novel gene therapy approaches for melanoma.

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Activation of p38 MAPK drives dendritic cells to become tolerogenic during melanoma development in ret transgenic mouse model

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DC population in tumor bearing hosts is heterogeneous. While some DCs stimulate antitumor T-cell reactivity, others can induce immune tolerance and promote tumor growth. Using a ret transgenic murine spontaneous melanoma model, we showed a strong tumor infiltration with immature DCs and a reduction in numbers of mature DCs in lymphoid organs (including metastatic lymph nodes) during melanoma progression. Moreover, being *ex vivo* isolated or generated from precursors, DCs from melanoma bearing mice secreted significantly more IL-10 and less IL-12p70 and showed a decreased capacity to activate T cells *in vitro* compared to DCs from tumor free animals. Observed DC dysfunction was linked to considerably elevated expression of phosphorylated p38 mitogen-activated protein kinase (MAPK). Inhibition of its activity in spleen DCs (*ex vivo* isolated from melanoma bearing mice) led to normalization of their cytokine secretion pattern and T-cell stimulation capacity *in vitro*. Our data demonstrate a critical role of constitutively activated p38 MAPK in the acquirement of tolerogenic pattern by DCs in the process of melanoma progression that contributes to the suppression of antitumor T-cell immune responses. We suggest that new strategies of melanoma immunotherapy can include inhibitors of p38 MAPK activity in DCs.

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DMBA-induced epidermal hyperplasia and inflammation in the absence of keratinocyte-derived VEGF-A

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We have previously shown that the inactivation of Vascular Endothelial Growth Factor A (VEGF) in murine epidermal keratinocytes (KC) abrogates papilloma development induced by 9,12-dimethyl 1,2-benzanthracene (DMBA), applied as a complete carcinogen. Instead, the treated skin of mutant mice showed evidence of inflammation, epidermal hyperplasia and a parakeratotic stratum corneum. To investigate the mechanism(s) underlying these observations we have now focused on the early phases of carcinogen treatment. DMBA (50 μg/50 μl ethanol) was applied to the backs of 1 day old mice and then once a week for 4, 6, 10 and 15 weeks. Treated skin was harvested for histological analysis one week after the last treatment. After only four treatments, mutant mice had developed epidermal hyperplasia, with an inflammatory cell infiltrate in the dermis, containing significantly higher numbers of neutrophils, and higher levels of IL1 and IFN gamma compared to control dermis. CD31 stained vessels were situated on average further from the epidermal-dermal junction from 6 weeks of treatment onwards, and an increased number of FITC-avidin positive cells (mast cells) compared to controls was present at the later time points. Confirming our earlier results, mutant mice did not develop papillomas, but after 15 treatments their skin again displayed extreme follicular hyperplasia, parakeratosis, and hyperkeratotic cysts, with concomitant aberrant keratinocyte differentiation, and an increased invasion of I-A positive cells into the epidermis. In contrast, the control mice developed a total of 14 papillomas/20 mice, and their uninvolved skin appeared normal until week 15, when it merely displayed mild focal hyperplasia. Fluorescein isothiocyanate (FITC), a molecule of similar size to DMBA, and also a contact sensitizer, was cleared more efficiently from the dermis of control mice after a single application to the shaved dorsal skin. We suggest therefore that passive clearance of DMBA is also slower in the dermis of mutant mice, resulting in an increased local concentration, enhanced inflammation and a nearly and sustained regression of the sub-epidermal blood vessels. This loss of the upper vascular plexus, together with the increased presence of mast cells may prevent the development papillomas.

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Myeloid-derived suppressor cells: a new immune escape mechanism in melanoma

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Introduction: Recent studies have described the so-called myeloid-derived suppressor cells (MDSC) in various tumors. They suppress anti-tumor CD8 T cell responses and represent a newly detected important immune escape mechanism of tumors. In mice they can be phenotypically identified by cell surface expression ofCD11b, Gr-1 and IL-4 receptor alpha (CD124). Question: We wanted to know if MDSC could also be detected in (murine) melanoma, define their phenotype and analyse if they are able to down-regulate immune responses.
Methods: After injection of 5×10^5 B16 melanoma cells into C57/BL/6 mice, the presence of MDSC was analysed in blood, bone marrow and spleen.
Results: We could detect an increased fraction of CD11b+Gr-1+CD124+ cells in all above mentioned analysed materials of tumor-bearing mice. MDSC from melanoma-bearing mice were able to down-regulate T-cell proliferation. Furthermore, we detected an increase of reactive oxygen species from MDSC which could represent an important immunosuppressive mechanism.
Conclusion: 1) Our data show that the generation of MDSC represents a new immunosuppressive mechanism in melanoma.
2) Pharmaceutical inhibition of this mechanism could enlarge the rapeutical perspectives.

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Inhibition of NF-κB pathway potentiates imiquimod-induced apoptosis in melanoma cells

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Background: Metastatic melanoma is characterized by an intracellular signalling disruption that includes blockage of pro-apoptotic pathways and activation of survival signalling. This reflects its extraordinary resistance to current chemotherapy. Imiquimod is a topical immune response modifier (imidazoquinoline) with both antiviral and antitumor properties. However, the mechanism of imiquimod-induced cell death is currently only partly understood.
Material and methods: H&E staining, flow cytometry analysis, western blot, *in vitro* kinase assays, electrophoretic mobility shift assays (EMSA).
Results: We demonstrated that imiquimod induced apoptosis in cultured melanoma cell lines by H&E staining and annexin V staining. Imiquimod-induced apoptosis was found to occur via induction of mitochondrial damage as evidenced by the loss of mitochondrial membrane potential (m), cytochrome c release and subsequent PARP cleavage, and via endoplasmic reticulum stress as demonstrated by intracellularCa2+ release. Of note, the inhibition of the NF-kB pathway by its specific inhibitor(Bay11-7082) was found to block the expression of the apoptosis inhibitor protein XIAP, leading to a significant augmentation of imiquimod-induced apoptosis.
Conclusion: Our data suggest a synergistic effect of imiquimod and target therapies such as NF-kB inhibitors in the treatment of malignant melanoma that warrants further investigation.

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SOX10 and nestin are co-expressed in primary melanoma tissues

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The potential role of stem cells in melanoma is a subject of recent interest. Nestin is an intermediate filament expressed in proliferating neural progenitor cells and has been considered as a stem cell marker. Nestin has also been found in melanoma and we could recently demonstrate that its expression is regulated by the transcription factors SOX9 and -10, but not BRN2 as previously suggested. In this study protein expression of SOX9, SOX10 and nestin was analyzed by immunohistochemistry in primary melanoma ($n = 50$), melanoma metastasis ($n = 20$) and melanocytic nevi ($n = 26$). SOX9 was expressed in 75–80% of primary and metastatic melanomas, while SOX10 was found in 43–50%. Nestin was expressed in 56–57% of primary and metastatic melanomas. SOX9 and SOX10 were also detected in melanocytic nevi in 73% and 31% respectively, however, with much lower staining intensity than in melanomas. Nestin was present in 23% of the analyzed nevi. Significant co-expression of nestin and SOX10 was found in primary melanoma confirming our *in vitro* data. A correlation analysis with clincopathological data revealed that nestin and SOX9 were significantly associated with the presence of ulceration in primary tumors. In conclusion, SOX9 and SOX10 are highly expressed in melanoma and seem to have a regulatory role in nestin expression. The association with ulceration suggests that SOX transcription factors may be negative prognostic markers in melanoma.

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Cadherin switch in merkel cell carcinomas

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Cell–cell adhesion molecules of the cadherin superfamily play an important role in tumorigenesis, and a switch from E- to N-cadherin can contribute to the development and progression of several types of carcinomas. We have studied the patterns of cadherins in Merkel cells and Merkel cell carcinomas, rare but highly aggressive tumors affecting mainly elderly and immunosuppressed patients, which have recently been attributed to polyoma virus infections. Paraffin sections of human plantar epidermis and of 42 Merkel cell carcinomas, i.e. 21 primary tumors, four relapses and 17 metastases, were double immunolabelled with antibodies to the Merkel cell marker cytokeratin 20 in combination with antibodies to adherens junction-associated and desmosomal cadherins and analyzed by immunofluorescence and confocal laser scanning microscopy. In healthy plantar epidermis, Merkel cells were connected to the surrounding keratinocytes by E- and P-cadherin and by the desmosomal cadherin desmoglein 2. By contrast, in Merkel cell carcinomas these cadherins were often down-regulated and replaced by N-cadherin. Interestingly, absence of E- and P-cadherin was correlated with increased malignancy, as 61% of the primary tumors but only 31% of the metastases were E-cadherin-positive and 86% of the primaries versus 41% of the metastases contained P-cadherin. N-cadherin was detected in ca.85% of all tumors, both primaries and metastases. The only desmosomal cadherin noted in a major amount of Merkel cell carcinomas was desmoglein 2, which was present in 65% of the primaries and 53% of the metastases, whereas other desmosomal cadherins were absent or infrequent. Taken together, our results indicate that a switch from E- and P- to N-cadherin might contribute to the development of Merkel cell carcinomas. The diagnostic and prognostic significance of our findings will have to be confirmed in future studies.

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Solubilized oleanolic acid induces apoptotic and necrotic cell death of murine B16.F10 melanoma cells

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Oleanolic acid is an almost water insoluble (<0.02 μg/ml), naturally occurring pentacyclic triterpenoid of the oleanane type, which has a variety of biological effects (e.g. anti-cancer, anti-inflammatory and anti-viral effects). The European mistletoe (*Viscum album* L.), olive trees (*Olea europaea* L.) and cloves (*Syzygium aromaticum* L.) are natural sources for oleanolic acid. The toxic solvent DMSO is normally used for *in vitro* administration limited by both solubility of oleanolic acid in DMSO and toxicity of DMSO. By using 2-hydroxypropyl-beta-cyclodextrin as complexing agent for mistletoe derived oleanolic acid we were now able to increase the water solubility of oleanolic acid, which allows *in vitro* and prospective *in vivo* administration without toxic solvents. Apoptosis induction by oleanolic acid is reported for different non-melanoma cell lines via a yet unknown mechanism. It is known that the intrinsic mitochondrial apoptosis pathway, reactive oxygen species production, caspase activation and interaction with pro- and antiapoptotic Bcl-2 proteins are involved in oleanolic acid induced apoptosis. We show here by annexin-V/PI staining that oleanolic acid, solubilized with cyclodextrins, induces apoptosis of B16.F10 mouse melanoma cells. Increased oleanolic acid concentrations induce a shift from apoptotic to necrotic cell death. Apoptosis induction was also observed regarding DNA fragmentation by using an oligonucleosome ELISA. In summary we demonstrate that oleanolic acid from mistletoe, solubilized by complexation with cyclodextrins, is able to induce apoptotic and necrotic cell death of B16.F10 mouse melanoma cells. Further experiments will reveal whether solubilized oleanolic acid is an option for melanoma treatment in animal models and humans.

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Vitamin D receptor (VDR) polymorphisms in malignant melanoma

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Vitamin D deficiency is associated with various types of cancer and vitamin D has the potential to become an important cancer chemopreventive agent. Moreover, analogues of 1,25(OH)2D3, the biologically active vitamin D metabolite, may be effective for adjuvant cancer treatment to prevent recurrence and metastasis, or for palliative cancer treatment to prolong overall survival. Functional polymorphisms across the 105 kb vitamin D receptor (VDR) gene may have important implications for successful chemoprevention or response to therapy as the VDR mediates most actions of 1,25(OH)2D3. Consequently, it has been shown that VDR polymorphisms are associated with cancers of the breast, colon, prostate and malignant melanoma. These cancer associated genotypes were shown to be common in all racial groups, having a minor allele frequency >10% and on average double the risk of cancer. However, the relevance of VDR polymorphisms may be influenced by other epidemiological and clinical factors such as sun exposure, diet and skin type. Using a gene sequencing approach, we have analyzed the presence of several VDR polymorphisms (Apa1, Taq1, Bgl1) in malignant melanoma (MM, $n = 60$), acquired melanocytic nevi (MN, $n = 70$), and in metastases of MM (MMM, $n = 40$). Our findings add to the body of evidence that VDR polymorphisms may be of importance for pathogenesis and progression of MM.

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Vitamin D receptor (VDR) polymorphisms in basal cell carcinomas (BCC) and cutaneous squamous cell carcinomas (SCC)

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Absence of BRAF, FGFR3 and PIK3CA gene mutations discriminates lentigo simplex from melanocytic nevus and solar lentigo

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Lentigo simplex (LS) is a benign skin lesion presenting as a brown or black macule in young persons. Histopathologically a proliferation of melanocytes and keratinocytes can be observed resulting in elongated rete ridges and hyperpigmentation of the epidermis. It has been proposed that LS may be a precursor lesion subsequently evolving into melanocytic nevus. Because melanocytic nevi show frequently theV600E BRAF hotspot mutation, we investigated the presence of this mutation in LS. We used a highly sensitive allele-specific PCR for the detection of the V600E mutation. Furthermore, we analyzed FGFR3 and PIK3CA hotspot mutations by SNaP shot assays because these mutations have been recently reported in solar lentigo and seborrheic keratosis. After manual microdissection from formalin-fixed paraffin-embedded material, 66 LS were analyzed for FGFR3, 54 for PIK3CA and 58 for V600E BRAF. None of the LS revealed mutations at the investigated loci of BRAF, FGFR3 or PIK3CA. Our results indicate that the V600E BRAF hotspot mutation is not involved in the pathogenesis of LS. This further implicates that either LS is not a precursor lesion of melanocytic nevus or the BRAF mutation occurs later during progression of LS to melanocytic nevus. The absence of FGFR3 and PIK3CAmutations in LS discriminates this lesion also from solar lentigo.

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Selective inhibition of IKK-2 suppresses epithelial-mesenchymal transition and metastasis in models of tumor progression

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Activation of the transcription factor NF-κB occurs in a broad range of human tumors, and studies have shown that NF-κB can promote cell proliferation and oncogenesis, possibly by protecting cells from apoptosis. Recently, using a combined *in vitro*/*in vivo* model of mammary carcinogenesis, we could demonstrate that NF-κB is required for the induction and maintenance of epithelial-mesenchymal transition(EMT), a central process governing both morphogenesis and carcinoma progression in multicellular organisms. In line with the importance of EMT for invasion and metastasis, blocking of NF-κB abrogated the metastatic potential of Ras-transformed EpH4 (EpRas) cells. While these results suggested that therapeutic inhibition of NF-κB may constitute a useful strategy for the control of tumor progression, the detailed molecular mechanisms underlying these effects remain to be elucidated. Our aim of this study was to determine whether targeted disruption of classical NF-κB signalling with a small-molecule inhibitor of IKK-2 can block EMT and metastasis in a number of models for tumor progression. In our studies, we used the highly selective small-molecule inhibitor of IKK-2, termed BI605700. Using this compound, we could show that specific inhibition of IKK-2 abrogates TGFβ-induced EMT in EpRas cells. In a second model system, IKK-2 blockade suppressed EMT in the mouse mammary tumor model 4T1. Additionally, IKK-2 inhibition using a daily dose of 150 mg/kg BI605700 significantly limited tumor growth and markedly reduced the metastatic potential of 4T1 cells *in vivo* after injection into mouse mammary glands. Conversely, treatment with BI605700 caused mesenchymal–epithelial transition (MET) in mesenchymal colon carcinoma (CT26) cells. Collectively, our findings suggest that targeting IKK-2 may represent an attractive opportunity for developing novel therapeutics to counteract tumor progression in a broad range of tumor entities, possibly including metastatic melanomas.

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Intra-tumoral heterogeneity of DNA stemlines and tumor suppressor gene promoter hypermethylation in malignant melanoma

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We investigated the degree of intra-tumoral heterogeneity in malignant melanoma. Analysis of intra-tumoral DNA stem-line heterogeneity was performed in 196 samples of 54 primary superficial spreading melanomas (tumor thickness median 1.60 mm) by sectional DNA cytometry. Additionally, 339 samples of 34 melanomas (15 primaries, 19 metastases) were investigated regarding promoter hypermethylation and associated gene product expression of the following genes: RASSF1A, p16, DAPK, MGMT, Rb. We found high degree of intra-tumoral variability. Between 39 and 70% of the investigated tumors must be regarded intra-tumoral heterogeneous. Furthermore, we found significant spatial separation of tumor cell stem lines within the investigated melanomas. In consequence, recommendations for future studies are suggested: (1) Histological correlation of genetically investigated samples, (2) Report of intra-tumoral sampling methods in all publications, (3) Previous analysis and adequate consideration of the degree of intra-tumoral heterogeneity of the study subjects especially in tissue prognostic studies.

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Δ9-THC supports anti-tumor immune defense of B16 melanoma cells induced by adenoviral vaccination and innate immune stimulation

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Cannabis preparations have been used in traditional medicine for the treatment of various diseases. Recently it has been suggested that cannabinoids like Δ9-THC (tetrahydrocannabinol) might be effective in the treatment of cancer by inducing tumor cell apoptosis and inhibiting angiogenesis. However, the use of cannabinoids for the treatment of malignant diseases is discussed controversially because of their immunomodulatory effects which can suppress tumor-specific immune defense. We examined the effect of Δ9-THC on tumor growth and anti-tumor immune defense in the B16 melanoma model *in vivo*. B16 melanoma cells were injected s.c. Immuneresponses were induced by adenoviral vaccination with Ad-hTRP2 (V) and innate immune stimulation with peritumoral injections of CpG DNA and polyI:C (I). This results in significant inhibition of tumor growth. Some mice additionally received daily s.c. injections of Δ9-THC (T). Δ9-THC application alone does not significantly affect growth of B16 melanoma cells *in vivo*. When given in combination with adenoviral vaccination and innate immune stimulation (VI+T), Δ9-THC significantly inhibited tumor growth. Almost half of the VI+T-treated mice rejected B16 melanoma. qRT-PCR analyses of the tumor microenvironment showed increased expression of IFN-γ, T-bet, Granzyme B and Perforin and FACS analyses revealed increased infiltration of CD8+ T-lymphocytes when VI+T-treated mice were compared with VI-treated animals. Taken together, our results in the B16 melanoma model suggest that Δ9-THC can support anti-tumor immunity induced by adenoviral vaccination and innate immune stimulation.

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Immunosuppression affects CD4+ mRNA and induces Th2 dominance in the inflammatory microenvironment of cutaneous squamous cell carcinoma in organ transplant recipients

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Squamous cell carcinoma (SCC) represents the most frequent tumor associated with drug-induced immunosuppression in organ transplant recipients (OTRs). Compared to general population, OTRs are at a 35- up to 100-fold risk of SCC development. The immune system plays a major role in the fight against SCC, however little is known about the local inflammatory response in SCC at all. We analyzed quantity and quality of the peritumoral inflammatory SCC microenvironment in immunocompetent patients and OTRs. SCCs from 15 immunocompetent patients and 13 OTRs were analyzed by quantitative real-time RT-PCR for CD4, CD8, TBET, GATA-3, FOXP3, RORC, IFN-gamma, IL-4, TGF-beta, IL-10 and IL-17A mRNA expression characterizing Th1, Th2 regulatory T cell and Th17 response. Considerable inflammation was seen in both patient groups. CD4+ mRNA was diminished in immunosuppression ($P = 0.03$), while CD8+ did not vary ($P = 0.37$). T-BET mRNA expression, normalized to CD4 did not vary between immunocompetent patients and OTRs; however mRNA expression of INF-gamma was significantly decreased in OTR group. Although GATA-3 mRNA was markedly increased with immunosuppression, IL-4 expression did not differ significantly. Th17weighted inflammation, characterized by expression of RORC mRNA was significantly increased in OTR group, while IL-17A mRNA level was markedly decreased. Regulatory T-cell response, characterized by FOX-P3 mRNA level, was unchanged in OTRs. IL-10mRNA expression did not vary between the groups, while TGF-beta was decreased with immunosuppression. Overall, transcription factors' mRNA expression levels differ within and between the patient groups. T-BET expression was significantly lower than GATA-3 and FOX-P3 mRNA level in both groups. However difference between T-BET and RORC mRNA level was significant in OTRs only, where RORC was at increased expression. Also, only OTRs showed significant difference between GATA-3 and FOX-P3 expression. Cytokine mRNA expression pattern was similar in both groups. The inflammatory microenvironment of cutaneous squamous cell carcinoma in organ transplant recipients is characterized by an attenuated state of local immune response comprising a lesser quantity of helper T-cell response and a Th2 polarized quality. The dramatically increased cutaneous carcinogenesis and its more aggressive course in OTRs may be influenced by a decreased regulatory T cells response.

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The functional microsatellite polymorphism of the Heme oxygenase-1 gene promoter is a prognostic factor in melanoma

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Heme oxygenase-1 (HO-1) is known for its strong anti-apoptotic potential in various tumors. Only recently, we could demonstrate that individuals with the high-expression genotype at the polymorphic promoter region [i.e. short (GT)n repeats ($n < 25$)] are associated with an increased risk to develop cutaneous malignant melanoma (M) than individuals without such genotype. Here, we describe a significant association of the short (S) allele carriers ($n = 148$) with shorter overall survival with a hazard ratio of 2.5 (95% CI: 1.1–5.8, $P = 0.03$) compared to patients homozygous for the long (L) allele (i.e. $n > 25$ GT repeats, $n = 117$) after adjustment for possible confounders such as age, gender and Breslow thickness. The calculated risk for acquiring primary M in homozygous S-allele (S/S) carriers was again higher compared to with L-allele carriers (odds ratio 1.9, 95% CI: 1.2–3.0, $P = 0.004$). These data confirms that HO-1renders an increased risk for acquiring M in S/S genotype individuals. Moreover, our current study reveals that the S-allele is a negative prognostic marker and represents a novel independent risk factor for progression in M. Thus screening for the HO-1(GT)n repeat promoter polymorphism might be of value in the molecular staging of M. Additionally, our data corroborate previous studies which identified HO-1 as a potential target for anti tumor therapy.

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EGFR polymorphisms in melanoma progression

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Little is known about the genetic factors that mediate progression and represent a potent prognostic factor in melanoma. Two polymorphic regions of the EGFR gene have been reported to be biologically relevant for EGFR efficacy: a dinucleotide (CA)n repeat and a single nucleotide polymorphism (SNP) R497K. To determine if the EGFR polymorphisms have an impact on overall survival (OS) in melanoma we examined these polymorphisms in a cohort of 155 human melanoma cases with at least 9-year follow-up. Though none of the polymorphic regions was associated with survival, we found a trend for an effect modification for the R497KSNP by gender. We saw an association of females with the 497 G/G with shorter OS [HR = 1.38 (CI 0.45, 4.26)] when compared to A/A or A/G, whereas males with G/G had longer OS [HR = 0.65 (CI 0.31, 1.36)] similar to the results in colon cancer. However, this finding did not reach the level of significance ($P = 0.273$). Given the current OS periods a sample size calculation revealed that more than 600 individuals would be required to detect a statistically significant difference with a statistical power of 80% with similar follow up periode. The second polymorphism [(CA)n repeat] had no influence on OS ($P = 0.656$) in melanoma.

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Signalling via RAGE promotes carcinogenesis by sustaining inflammation andtumor angiogenesis

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A broad range of experimental and clinical evidence has highlighted the central role of chronic inflammation in promoting tumor development. However, the mechanisms that sustain a tumor-promoting microenvironment remain largely elusive. We have demonstrated that mice deficient for the receptor for advanced glycationend-products (RAGE) are resistant to DMBA/TPA-induced skin carcinogenesis. Furthermore, RAGE-deficient mice showed severely reduced inflammatory response to treatment with TPA accompanied by impaired infiltration with neutrophils, macrophages and mast cells and impaired upregulation of pro-inflammatory genes, such as Ptgs2, MIPs, S100a8 and S100a9 using confocal microscopy and gene expression analysis. S100a8 and S100a9, representing activating ligands of RAGE, were induced upon TPA treatment of mouse back skin and overexpressed in advanced stages of mouse and human skin tumors using RQ-PCR and tissue microarrays. Since ligand expression is RAGE-dependent, we propose the existence of an S100/RAGE-driven feed-forward-loop in chronic inflammation and tumor formation. Most importantly, TPA-induced dermal infiltration and epidermal hyperplasia was restored in wildtype bone marrow chimeric RAGE-deficient mice revealing that RAGE expression on immune cells is essential for sustaining inflammation. Moreover, we present novel data on a prominent endothelial-specific role of RAGE in driving tumor-angiogenesis that underlines the importance of a specific RAGE-dependent microenvironment for effective tumor promotion. In conclusion, we demonstrate the complex role of RAGE signalling in driving the strength and maintenance of an inflammatory reaction during tumor-promotion and sustaining tumor angiogenesis and thereby we provide direct genetic evidence for novel cell type-specific functions of RAGE in promoting inflammation-induced cancer.

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5′-triphosphate-siRNA: turning gene silencing and RIG-I activation against melanoma

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Genetic and epigenetic plasticity allows tumors to evade single-targeted treatment approaches. A combinatorial approach that suppresses tumor cell survival and at the same time increases immunogenicity of tumor cells may lead to more effective tumor treatment. Here we used short RNA oligonucleotides targeting the anti-apoptotic protein Bcl2, a key regulator of melanoma cell survival, through the mechanism of RNA interference for melanoma therapy. To simultaneously activate the innate immune system, we generated Bcl2-specific short interfering RNA with triphosphate groups at their 5′-end (3p-siRNA). 5′-triphosphate RNA is specifically recognized by the ubiquitously expressed helicase RIG-I, one of two immunoreceptors that signal the presence of viral RNA in the cytosol of cells. Bifunctional Bcl2-specific 3p-siRNA elicited strong anti-tumor activity against B16 melanoma lung metastases. Recognition of 5′-triphosphate by RIG-I activated innate immune cells such as dendritic cells and directly induced interferon and apoptosis in tumor cells. These RIG-I-mediated activities synergized with siRNA-mediated Bcl2 silencing to provoke apoptosis of tumor cells both *in vitro* and *in vivo*. The therapeutic activity required NK cells and interferon, as well as silencing of Bcl-2 as evidenced by rescue with a mutated Bcl2 target, by site-specific cleavage of Bcl-2 mRNA in lung metastases and downregulation of Bcl2 protein in tumor cells *in vivo*. Together, 3p-siRNA represents a single molecule-based approach in which RIG-I activation on both the immune- and tumor cell level corrects immune ignorance and in which gene silencing corrects key molecular events that govern tumor cell survival.

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Demethylation of Line-1 DNA in melanoma

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Forty-five percent of the human genome consist of retroelements, 20% of them are long interspersed nuclear elements (LINE) serving as autonomous retrotransposons. Line-1 represents the predominant interspersed nuclear element. Line-1 elements are approximately 6 kb long, with two open-reading frames, ORF1 and ORF2, and a terminal poly-A sequence. In somatic tissues and mature germ cells Line-1 is highly methylated, specifically in CpG-rich 5-ends of intact sequences, so that the transcriptional activity of retroelements are largely suppressed yielding a stable genome. We investigated the methylation status of Line-1 elements in biopsies of melanoma metastases, primary melanoma and benign nevi. We performed positional methylation analysis of Line-1 by Pyrosequencing as recently reported by our group (1). Depending of CpG position in the Line-1 DNA we observed a reduction of Line-1 DNA-methylation between 12.4% to 5.1% in primary melanoma versus benign nevi and 24% to 17.3% in melanoma metastases comparison to benign nevi, respectively. The melanoma cell lines A375 and BLM which also showed a reduction in Line-1 methylation compared to normal human melanocytes were treated with 5′Aza cytidine, a potent DNA methyl transferase inhibitor with antitumor properties. 5′Aza cytidine enhances the methylation of Line-1in A375 and BLM melanoma cell lines approximately between 27% and 22.7%, respectively. In conclusion, a reduction of Line-1 DNA methylation in melanoma may lead to chromosome instability, and may thus permit the formation of tumors. Treatment with 5′Aza cytidine can revert this process. It has to be shown, whether exogenous factors such as UVB exposure or oxidative stress are responsible for Line-1demethylation.

Reference: Mirmohammadsadegh A, Marini A, Nambiar S, et al. Epigenetic silencing of the PTEN gene in melanoma. Cancer Res 2006: 66: 6546–52.

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Primary melanomas in Hgf-Cdk4 mice are highly vascularized and grow progressively without prominent tumor-associated inflammation and immune cell recruitment

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Immune defense and limited blood supply are important barriers for progressive tumor growth. Understanding the complex interaction between neoplastic, immune and endothelial cells requires adequate preclinical models where tumors arise authochthonously in immunocompetent hosts. We established a genetically engineered mouse model of primary cutaneous melanoma which imitates key events in the molecular pathogenesis of this disease in man. Aberrant growth factor signalling due to transgenic overexpression of the hepatocyte growth factor (Hgf) and impaired cell cycle control due to an oncogenic germline mutation in the cyclin dependent kinase 4 (Cdk4R24C) selectively promote sporadic malignant transformation of melanocytes in the skin. Primary melanomas in the skin of Hgf-Cdk4R24C mice histomorphologically resemble a subset of nodular melanomas in man, indicating that this mouse model faithfully portrays the biologic evolution of the disease. Tumors are highly vascularized and grow progressively without prominent inflammation and immune cell recruitment in the tumor microenvironment. Quantitative RT-PCR analyses demonstrate elevated expression levels of the immunoregulatory cytokine TGF-β but very low levels of pro-inflammatory cytokines and chemokines including IL-4, IL-6, IL-10, IFN-γ, CXCL-2, CXCL-9 and CXCL-10 in the tumor microenvironment. Metastatic tumor cells spread to the draining lymphnodes, diffusely infiltrate the T cell-rich areas, express low levels of MHC class I molecules and do not activate dendritic antigen-presenting cells. Tumor-bearing mice show splenomegaly due to extramedullary hematopoeisis without expansion ofGr1+CD11b+ myeloid suppressor cells. Hgf-Cdk4R24C melanomas can be transplanted and grow with a similar phenotype. In conclusion, our results demonstrate that sporadic primary tumors can grow progressively in the skin, escape immune recognition and metastasize in the draining lymph nodes in the absence of prominent inflammation and immune cell infiltration.

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Hepatocyte growth factor promotes motility in human melanocytes through CD44v6 via NF-kappa B

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The expression of hepatocyte growth factor (HGF) is induced in human skin after UV-irradiation. In murine melanoma cells HGF leads to up-regulation of CD44v6, a variant of the CD44 family of adhesion molecules. CD44v6 forms a complex with c-Met and HGF and is required for HGF-induced c-Met activation. Whether HGF induces CD44v6 expression human melanocytes and the functional consequences thereof, are unknown. Melanocytes were probed for changes in CD44v6 protein levels by immunoblotting after treatment with recombinant HGF for 8 h. Immunohistochemistry of melanocytic lesions was performed. To investigate on the involved signalling pathways in CD44v6 activation, specific inhibitors Ly294002 (PI3K), PD98059 (MAPK) and BAY-11-7082 (NF-kappa B) were used. The effect of HGF on three transcription factors, which have potential binding sites in the CD44v6 promoter, was studied. We demonstrate that HGF induces expression of CD44v6 in human melanocytes. Immunostaining of melanocytic lesions revealed a low, cytoplasmic staining of CD44v6 in nevi, but high membranous expression in primary cutaneous melanomas, cutaneous- and lymph node metastases. NF-kappa B inhibitor antagonized HGF-induced enhancement of CD44v6 expression, whereas interference with MAPK or PI3K cascade did not. HGF increased protein levels of transcription factors Egr-1 and C/EBPnbeta but not GATA2. A blocking antibody to CD44v6 decreased HGF-induced c-Met phosphorylation as well as enhanced random- and site-directed migration of HGF stimulated human melanocytes. Our data suggest that HGF is crucial at initial steps in melanomagenesis, whereupon a sustained exposure of melanocytes leads to increased motility through CD44v6 via NF-kappa B.

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HLA-A2 restricted peptides derived from CD105 induce specific T cell responses in melanoma patients

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CD105 (endoglin), expressed primarily on endothelial cells, activated macrophages and fibroblasts, functions as an accessory protein for kinase receptor complexes of the TGF-β superfamily. It antagonises the inhibitory effects of TGF-β1, e.g. the suppression of growth, migration and capillary tube formation. In addition, CD105 has anti-apoptotic effects under hypoxic conditions. Its expression has been described for a multitude of solid tumors and is correlated with vascular density and poor prognosis. These properties render CD105 as an attractive target for therapeutic interventions. By reverse immunology we selected several peptides derived from CD105 and tested them for their capacity to bind to HLA-A2 complexes. The identified HLA-A2 restricted CD105 epitopes induced human T cell responses in PBMC of melanoma patients, measured by IFN-γ ELISPOT. Exchange of anchor amino acids in some low affinity peptides enhanced not only their binding affinity to HLA-A2 but also the induction of IFN-γ responses. To analyse the immunogenicity of the CD105 epitopes *in vivo* HLA-A2/kb transgenic mice were vaccinated with the CD105-derived peptides resulting in induction of CD105-specific immune responses. Due to the homology between human and murine CD105 two HLA-A2 restricted epitopes have identical amino acid sequences in both species; thus this approach also served to exclude major side effects of induced anti-CD105 immune responses, e.g. impaired wound healing, in a preclinical setting. In conclusion, a CD105 peptide vaccine is immunogenic and safe in preclinical models and thereby warrants testing in the human setting.

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Transfer of mRNA encoding recombinant immunoreceptors reprograms CD4+and CD8+ T cells for the use in adoptive immunotherapy of cancer

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An innovative and promising approach of treating malignancies is the adoptive transfer of bulk T cells with a tumor antigen-specific T cell receptor (TCR). One opportunity to overcome the requirement of MHC-restricted antigen presentation is the use of chimeric antigen receptors (CAR), which consist of a scFv directed against a cancer surface antigen and the signalling domains of the CD3-zeta and CD28molecules. To avoid persistent auto-aggression, a reported life threatening risk of retrovirally transduced T cells, we explored RNA electroporation for transient immunoreceptor expression. Two CAR, one specific for carcinoembryonic antigen (CEA) expressed on colon, rectal, pancreatic and other carcinomas, the other specific for ErbB2/Her2neu expressed on breast cancer cells and some melanoma cell lines, were efficiently transfected into CD4+ and CD8+ T cells, with half-maximal expression at day 2 and no detectable immunoreceptor expression at day 9 after electroporation. Upon specific stimulation with ErbB2+ or CEA+ tumor cells, transfected CD4+ and CD8+ T cells secreted the cytokines IL-2, TNF-alpha, and IFN-gamma. Moreover, the reprogrammed CD8+ T cells were capable of killing antigen-expressing target cells with a cytolytic activity similar to retrovirally transduced T cells. In aggregate, RNA electroporation of T cells provides a versatile tool for transient CAR expression with at least two important advantages over retroviral transduction: first, it avoids the persistence of unintended auto-aggression and second, RNA transfection classified as none gene therapy is best suited for translational research to complement immunotherapy of cancer.

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CD105 (endoglin) as immunotherapeutic target in a murine melanoma model

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Tumors do not only comprise tumor cells but also tumor stroma cells, such as tumor endothelial cells. Notably, most immune evasion mechanisms of tumor cells do not apply for tumor stroma cells. Stroma cells in the tumor microenvironment differ from their normal counterparts by upregulation or induction of various antigens, i.e. tumor stroma-associated antigens (TSAAs), which are not confined to one tumor type. Thus, therapies designed to target the tumor stroma are not restricted to a single tumor entity. A promising immunotherapeutic target is CD105 (endoglin), because it is primarily expressed on endothelial cells of newly formed blood vessels, e.g. during tumorangiogenesis. CD105 is a component of the TGF-β1 receptor complex and functions as an auxiliary receptor to bind TGF-β1, TGF-β3 and other members of the TGF-β superfamily. As tumor model we selected grm1 transgenic mice (TG-3 and EPV) that spontaneously develop melanoma. We analyzed the expression of CD105 in C57Bl/6 and grm1 transgenic mice by quantitative PCR and immunohistochemistry. Biopsies of ear, tail, eye lid, and upper and lower lips, e.g. organs in which tumors in grm1transgenic mice are preferentially localized, demonstrated a strong expression of CD105. However, as CD105 is expressed on a basal level in endothelial cells, an enhanced expression was also present in endothelial rich tissues as lung, heart, kidney and liver of healthy mice. Subsequently, we applied reverse immunology and tested CD105-derived peptide epitopes for their capacity to bind to H-2kb molecules. This analysis revealed H-2kb-restricted peptide epitopes with low (muCD105 68-75, muCD105 93-100, and muCD105 526-533) to strong (muCD105 310-317) binding affinity. Exchange of anchor amino acids 4 and/or 8 in some low affinity peptides [muCD105 68-75 (72Y, 75L), muCD105 605-612 (609F, 612L)] enhanced their binding affinity in comparison to the parental peptides. The immunogenicity and therapeutic efficacy of theseCD105-derived peptides are currently evaluated in the spontaneous murine grm1-melanoma model.

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The angiogenic activity in cutaneous metastases of malignant melanoma

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In the last years several studies have shown the importance of angiogenesis and lymph angiogenesis in tumor development and metastasis of malignant melanoma. However, in contrast to the abundance of data concerning the angiogenic environment in primary tumors and sentinel lymph nodes, vascular alterations incutaneous metastases have not been investigated.
We therefore quantified the extent of angiogenesis in 20 specimens of human cutaneous melanoma metastases and 23 primary melanomas. We analyzed paraffin-sections immuno stained for the panvascular marker CD31 and the lymphatic marker LYVE-1. In each section the area with the highest vascular density ('hot area') was evaluated at x100 magnification using the IP-Lab software to determine the vessel number per mm^2, the average vessel size and the relative tissue area occupied by vessels. The data obtained were compared to the vascularity of 20 benign naevi and the tumor free margins of primary melanomas respectively.
Immunohistochemical analysis of cutaneous metastases showed significantly higher vessel density in metastases (86.8 ± 15.1 mm^{-2}) than in benign naevi (56.7 ± 2.8 mm^{-2}). This increase in vessel density was similar to that in primary melanomas when compared to benign naevi. Analysis of the corresponding tumor free margins confirmed that angiogenesis was induced only in the direct vicinity of the tumors.
Our results reveal a strong proangiogenic activity not only in primary melanomas but also in cutaneous metastases, providing a sound rationale that angiogenesis inhibitors could prove beneficial in the treatment of patients with metastatic melanoma.

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Squamous cell carcinoma of the skin shows distinct patterns of outcome-related proteins

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To date in squamous cell carcinoma (SCC) of the skin there is a lack of outcome-related markers to identify patients at high risk for multiple SCCs and metastases. Our aim was to assess expression of potential marker proteins incutaneous SCC by immunohistochemistry, proteins that had recently been shown to be associated with worse outcome in other tumors (Galektin-3, Moesin, Ezrin, hILP, EGLN, EBS, Rad17 and NY-ESO-1). As organ transplant recipients (OTRs) are known to be at a 100-fold higher risk to develop cutaneous SCC and to show a much more aggressive disease course than immunocompetent patients, protein expression was compared between these two patient groups. Furthermore, in-situ SCC (actinickeratosis, Bowen's disease) were compared to invasive SCC. Thus, a tissue micro array with specimens from 176 immunocompetent patients and 173 OTRs containing 46 in-situ SCC and 303 invasive SCC was designed. Eleven of these patients had SCC metastases. On the course from *in situ* to invasive SCC we found a significant increase in immunoreactivity for Moesin and hILP (P = 0.001, 0.045 respectively). OTRs had significantly higher levels of Ezrin expression (P = 0.039) than immunocompetent patients throughout all lesions. Patients with metastasizing SCC showed overexpression of Rad 17 (P = 0.025) in primary SCCs as compared to patients without metastases. In conclusion we found that also in cutaneous SCC distinct patterns of outcome-related proteins can be identified. In further studies an expanded range of markers has to be analyzed. Joint analysis of such markers may help to identify SCC with more aggressive potential.

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Modulation of MAP kinase signaling pathways in CD133-positive melanoma cells overcomes their resistance to apoptosis

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Background: Recent research shows that cancer stem cells (CSCs) are relatively resistant to apoptosis. For melanoma, subpopulations enriched in CD20 and CD133 (nest in) have been demonstrated to possess stem-like properties.
Therefore, we studied the effect of chemotherapeutic drugs on several melanoma cell lines including MV3 and BLM upon purification of the CD133-positive population. The aim of the present study was to determine the molecular mechanism(s), that are responsible for the resistance of CD133+ melanoma cells to selected anti-cancer agents.
Material and Methods: MTT assay, flow cytometry, MACS enrichment, Western blot, *in vitro* kinase assay, electrophoretic mobility shift assay (EMSA).
Results: CD133 (positive) MV3 cells were significantly more resistant to apoptosisthan their CD133 (negative) counterparts as evidenced by flow cytometry analysis using annexin V/PI, and by Western blot analysis through the detection of cytochrome c release and cleavage of caspase-9, caspase-3 and PARP. In addition, the activation of MAP kinase signaling pathways JNK, p38 and ERK together with their physiological substrates AP-1, ATF-2, and ELK-1 was noted in CD133-, but not in CD133+ cells. However, the combination of ERK inhibitors (PD98059) and the induction of both JNK and p38 pathways by H202 was found to overcome resistance of CD133+ cells to apoptosis.
Conclusion: These results suggest that the induction of MAP kinase pathways JNK and p38 in combination with ERK inhibitors may effectively target CD133+ melanoma cell that possess stem-like properties.

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Identification and functional analysis of a novel maker ofm2-differentiated tumor associated macrophages

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Macrophages are a major cellular subpopulation of tumors. Recent clinical and experimental studies have provided evidence that these phagocytic cells are causally involved in tumor promotion and progression by supporting angiogenesis, growth and invasion. In a tumor microenvironment tumor associated macrophages (TAMs) are polarized towards an M2-alternatively activated status or may represent a hybrid between M1/M2 differentiation.
By screening the tumor stroma of murine B16F1 melanoma and TSA breast carcinoma for the expression levels of typical markers for non-continuous endothelial cells and macrophages we identified a novel CD11b+ TAM subpopulation co-expressing the lymphatic endothelial cell marker LYVE-1 and the sinusoidal endothelial cell marker Stabilin-1. These LYVE-1+ macrophages could be generated in vitro by stimulating mBMMφ with the combinationtumor-supernatant/dexamethasone/IL-4 indicating an M2 phenotype.
In order to verify this hypothesis, we analysed the gene expression profile ofLYVE-1+ and LYVE-1- macrophage populations using Affymetrix 340 2.0 microarrays. Results showed an upregulation of many typical M2-markers like arginase, CD163 and Mgl-1/2 in addition to a novel surface molecule. Expression levels of selected genes where confirmed by RT-PCR, immunohistochemistry and western blotting.
After the generation of a custome made antibody against the novel surface protein, we confirmed its expression in subcutaneous TS/A mammary carcinoma and B16F1 melanoma. Further experiments characterizing tissue distribution and function of this protein in TAMs are in progress.

P255

Aberrations of the CDKN2A network components p16, p53 and RB1 in primary cutaneous B-cell lymphoma

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The cyclin-dependent kinase inhibitor 2A (CDKN2A) gene encodes the p16 (INK4A) inhibitor of the CDK4/retinoblastoma (RB1) cell proliferation pathway and, in addition, p14 (ARF) which controls p53 dependent pathways. CDKN2A thus codes for two cooperative tumour suppressor pathways. The inactivation of p16 has been implicated in the genesis of various nodal lymphomas of human and animal origin. Interestingly, in the group of primary cutaneous B-cell lymphomas (PCBCL), inactivation of p16 has previously been described to be associated with primary cutaneous diffuse large B-cell lymphoma, leg type. This entity has the most unfavourable prognosis of PCBCL. Aberrations of components of the CDKN2A tumour suppressor network other than p16, e.g. p53 und the RB1 gene have been made responsible for the pathogenesis of various other tumours, including some types of nodal lymphomas. However, so far the role of these latter genes has not been investigated in PCBCL. This prompted us to investigate the tumours of 22 patients (nine primary cutaneous follicle center lymphomas (PCFCL), seven primary cutaneous marginal zone lymphomas (PCMZL), six primary cutaneous diffuse large B-cell lymphomas, leg type (PCLBCL)) with respect to alterations of the p16, p53 and the RB1 gene. Fluorescence in situ hybridization (FISH) was performed by application of specific probes for p16 (9p21), p53 (17p13) and RB1 (13q14). None of the PCFCL or PCMZL showed alterations of p16. In 4/6 PCLBCL however FISH analysis revealed alterations of p16 (two biallelic deletions, one monoallelic deletion, one trisomy 9). Interestingly, this tumour type also most often showed deletions of p53 (3/6) and RB1 (3/6). On the other hand, only 1/7 PCMZL and none of the nine PCFCL showed deletions of p53 and only 1/7 PCMZL and 1/9 PCFCL revealed a deletion ofRB1. In conclusion, aberrations of the CDKN2A network components p16, p53 andRB1 seem to be mostly absent in PCMZL and PCFCL. In contrast, PCLBCL, which is characterized by an inferior clinical outcome, shows deletions of these genes in a significant percentage. Verification of their gene products and the products of their target genes on the protein level have to be investigated in the future.

P256 (V02)

Survival of cutaneous T cell lymphoma is dependent on regulation of Ferritin Heavy Chain by constitutively activated NF-κB

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Control of the intracellular redox balance is critical to protect cells from oxidative damage. Here, we report that inhibition of NF-κB causes a dysregulated redox balance and results in a caspase-independent cell death in malignant T cells from Sézary patients. Inhibition of NF-κB causes downregulation of ferritin heavy chain (FHC) expression which leads to a rapid increase of free intracellular iron and massive generation of reactive oxygen species (ROS). Finally, high concentrations of ROS induce cell death of malignant T cells. In contrast, T cells isolated from healthy donors do not display downregulation of FHC and, therefore, do not show an increase in iron and cell death upon NF-κB inhibition. Both, generation of ROS and induction of cell death were blocked by iron chelator desferioxamine (DFO). Moreover, in a murine T cell lymphoma model, we show that inhibition of NF-κB and subsequent downregulation of FHC significantly delays tumor growth *in vivo*. Thus, our findings identify FHC as a key mediator of cell death upon NF-κB inhibition and suggest FHC as a promising therapeutic target for lymphomas.

P257

Impact of ADAM10 mediated CD44 shedding on melanoma cellbiology

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The malignant melanoma is one of the most frequent and invasive human neoplasms. Because of ineffectiveness of the most conventional therapies the mortality rate caused by malignant melanoma is still very high. Increased plasma levels of the soluble form of CD44 may be associated with a poor prognosis for malignant melanoma patients.
CD44 belongs to a family of plasma membrane glycoproteins involved in adhesion processes and cell movement. It is the functional cellular receptor of hyaluronic acid (HA), a major component of the extracellular matrix. CD44-HA interactions promote cell proliferation of malignant melanoma cells. CD44 can be shed from the cell surface by proteolytic cleavage. The promoting effect of HA on cell proliferation could be abolished by secretion of soluble CD44 into the culture supernatant *in vitro* and *in vivo*.
We have recently identified the matrix metallo-protease ADAM10 to be critically involved in the constitutive shedding of native CD44 from human melanoma cell lines by performing inhibitor studies and siRNA experiments. ADAM10 specific inhibitors were able to block CD44 release from MM cells. ADAM10-expression could be specifically inhibited by siRNA techniques and this ADAM-10 blocking was able to reduce the constitutive CD44 shedding from MM cell lines. Functionally, ADAM10 silencing increased cell proliferation of MM cells suggesting that ADAM10 can influence MM cell proliferation by its implication in solCD44 shedding.
Here we analysed the impact of ADAM10 mediated CD44 shedding on tumor biology using a novel model of tumor spheroids from different melanoma cell lines. ADAM10 silencing could be demonstrated in the 3D spheroids with impact on CD44 shedding and cell proliferation as in monolayer cultures. Second, an inducible Tet-On system made of 1F6 melanoma cells that allows the inducible expression of soluble CD44 has been generated. The impact of soluble CD44 on tumor growth, cell proliferation and induction of apoptosis in a pre-existing tumor spheroid was investigated.

P258

A unique case of an erythrodermic TCR$\gamma\delta^+$ peripheral T cell lymphoma with TH2 phenpotype

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Peripheral T cell lymphomas expressing the $\gamma\delta$TCR represent rare cases of non-Hodgkin lymphomas and most often originate at extra nodal sites.
We report on a 78-year old patient with a peripheral TCR$\gamma\delta^+$ T cell lymphoma who initially presented with a sudden onset of erythrodermia, massive pruritus and eosinophilia. Within one month the patient developed generalized lymph adenopathy with the presence of tumor cells staining positive for CD3, CD4, CD5 and TCRVδ1and lacking expression of CD2, CD7, CD8, CD30 and of cytotoxic molecules (granzyme B, perforin). These cells were also present in the peripheral blood (CD4/CD8 ratio up to 12) whereas involvement of liver or spleen could not beobserved. Upon *in vitro* stimulation those cells readily produced IL4, IL5 and IL13, thus displaying a TH2 phenotype. This is in sharp contrast to the vast majority of TCR$\gamma\delta^+$ lymphomas showing a cytotoxic phenotype. The disease was not associated with HTLV1 or EBV infection.
Due to the known aggressive course of $\gamma\delta$T cell lymphomas we initiated systemic chemotherapy with CHOP, which was complicated by severe recurrent infections and stopped after five cycles because of disease progression. After short stabilization with extracorporeal photopheresis and systemic glucocorticoids the patient died 8months after initial diagnosis due to disease progression.
To the best of our knowledge this TCR$\gamma\delta^+$ peripheral T cell lymphoma is unique for the expression of a TH2 phenotype and its clinical presentation as erythrodermia.

P259

Mast cells : active participants in co-culture with skin derived tumorcells

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The coincidence of skin tumors and elevated mast cell (MC) numbers has been known for many years. Nowadays, the importance of the tumor-stroma and the here in located MC are roughly discussed. Several immunohistochemical studies indicated that MC might regulate tumor progression via secreted cytokines like IL-8. Modulation of angiogenesis, neovascularisation and tissue remodelling are discussed as well as immuno-modulating capacities of the MC. However, evidence for the functional role of Mc in tumorigenesis and progression is still missing and cannot be featured by this technique.
Addressing this problem, we established a mast cell tumour co-culture system, thus revealing possible supportive or suppressive effects of MC on tumour growth. Different melanoma and squamous cell carcinoma cell lines were co-cultivated with or without primary, dermal mast cells for 24 h and the gene expression of cytokine IL-6 and IL-8 in each individual cell line as well as in co-cultures was estimated in order to look for a potential modulation of this cytokine as a result of tumour-mast cell interaction. In addition to native MC, this panel was expanded by the use of anti-IgE activated MC. Co-culturing of MC led to an increase in IL-8 gene expression and IL-8 protein release from melanoma cells and IL-6 and IL-8 gene expression and protein release from squamous cell carcinoma cells, respectively. Moreover induction of IL-6and IL-8 was primarily regulated by Mc derived TNF-Ñ.
Our data suggest interplay between MC and tumour cells which results in altered cytokine release and may thus have an impact on tumor growth, invasion and neovascularisation.

P260

Specific Inhibition of Kallikrein 5 by the new epidermal Kazal-type InhibitorLEKTI-2

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Proteolytic degradation of extracellular proteins plays a crucial role in the physiological detachment of corneocytes from human stratum corneum. The presence of protease inhibitors regulates their proteolytic activity and contributes to the integrity and protective barrier function of the skin. Their principal importance in diseases has been revealed impressively in Netherton's disease, where the Kazal-type protease inhibitor LEKTI, encoded by its gene SPINK5, is absent. LEKTI domains are inhibitors of the epidermal serine proteases Kallikrein-related peptidase (KLK) 5 and 7. In order to identify KLK5 inhibitors is human epidermis, we analysed stratum corneum extracts for the presence of KLK5 inhibitors after high performance liquid chromatography. We identified a new KLK5-inhibiting peptide with high homology to LEKTI by electro spray mass spectrometry analyses. The encoding gene was identified as SPINK9 and the peptide was termed LEKTI-2. RecombinantLEKTI-2 exhibited protease inhibitory activity against KLK5 with a K(i) of approximately 200 nM but no protease inhibition against KLK7, 14 and other serine proteases like trypsin, thrombin, plasmin, chymotrypsin, elastase, matriptase and mast cell chymase. Fluorescence microscopy revealed LEKTI-2 expression in thestratum granulosum at palmar and plantar sites. No co-localization with KLK5 was observed. In conclusion, we report here the identification of LEKTI-2 as aKLK5-selective protease inhibitor in human epidermis.

P261 (V34)

Epigen, a ligand of the epidermal growth factor receptor, regulates sebaceousgland activity

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The epidermal growth factor receptor (EGFR), together with its seven ligands and the three related ERBB receptors, constitutes one of the most powerful and versatile signaling networks in higher vertebrates. Activation of the EGFR in the epidermis and hair follicle keratinocytes results is a wide range of effects depending on the ligand involved. For instance, heparin-binding EGF is important for keratinocyte migration during wound healing, excess of amphiregulin is associated with psoriasis-like lesions and the overexpression of transforming growth factor-alpha results in skin tumorigenesis. Epigen, the newest (and probably the last) EGFR ligand, has unique receptor binding properties, and is expressed in mouse and human skin. To study the biological functions of this growth factor, we generated transgenic mouse lines overexpressing epigen under the control of the ubiquitously active CMV/beta-actin promoter (Epigen-tg). Following pronuclear microinjection, five Epigen-tg mice carrying the transgene at different chromosomal sites were obtained. In all Epigen-tg mice hair growth was delayed as compared to control littermates, and, once hair became visible, it appeared greasy and grew in a patchy pattern. Although Epigen-tg mice were not sterile, they generated very small litters and consistently failed to transmit the transgene to their descendants (possibly because the transgene wasn't present in the germ line). They were therefore sacrificed, and expression of the transgene in the skin was confirmed by Northern blotting. Histological examination of skin sections and oil-red staining revealed depletion of hair follicles and extraordinarily enlarged sebaceous glands, suggesting that the greasy fur of the mice was a consequence of increased sebum production. The distribution of the differentiation markers keratin 6, keratin 14, loricrin and filaggrin was not altered in the skin of Epigen-tg animals. The prominent sebaceous gland phenotype observed in Epigen-tg mice differs from the changes elicited by other EGFR ligands in transgenic mouse models, supporting the concept that EGFR ligands are no tredundant, but contribute distinctively and specifically to the regulation of keratinocyte function. Tissue-specific overexpression of epigen will allow studying its role in hair follicle morphogenesis, cycle induction, and tumorigenesis.

P262

Acantholysis in pemphigus vulgaris is independent from apoptosis

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Pemphigus vulgaris (PV) is caused primarily by autoantibodies against the desmosomal cadherins desmoglein 1 and 3. Apoptosis has previously been detected in lesional skin of PV patients and after treatment of cultured human keratinocyteswith PV-IgG. However, the role of apoptosis in PV pathogenesis is unclear at present. In this study, we provide evidence that apoptosis is not required for acantholysis in PV. In skin lesions from two PV patients, TUNEL positivity but not cleaved caspase-3 was detected in single keratinocytes in some lesions but was completely absent in other lesions from the same patients. In cultures of human keratinocytes (HaCaT and normal human epidermal keratinocytes), PV-IgG from three different PV patients caused acantholysis, fragmentation of Dsg 3 staining and cytokeratin retraction in the absence of nuclear fragmentation, TUNEL positivity and cleaved caspase-3 and hence in the absence of detectable apoptosis. To further rule out the contribution of apoptotic mechanisms, we used two different approaches which are effective to block apoptosis induced by various stimuli. Inhibition of caspases by z-VAD-fmk as well as overexpression of FLIPL and FLIPS to inhibit receptor-mediated apoptosis did not block PV-IgG-induced effects indicating that apoptosis was not required. Taken together, we conclude that apoptosis is not a prerequisite for skin blistering in PV but may occur secondarily toacantholysis.

P263

Gene regulation in a keratinocytes cell line HaCat by all-trans-retinal and all-trans-retinol

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It is well-known that vitamin A is a critical modulator of growth and differentiation in skin cells. Several studies have shown that retinoic acid (RA) is the biologically active main form of vitamin A. RA serves as a ligand for two families of nuclear receptors. These include all-trans retinoic acid receptors (RARs) and 9-cis retinoic acid receptors (RXRx), which regulate the expression of different genes. Here, we present evidence that not only RA plays this important role, but also all-trans retinol (atROL) and all-trans retinal (atRAL) have potential to regulate genes. First, using the gene expression profiling analysis with whole genome BeadChip® Sentrix array, we found that (1) atROL down-regulates about 40 genes (fold-change from 2 to 10) and up-regulates about 50 genes (fold-change 2 to 11); (2) atRAL down-regulates about 60 genes (fold-change from 2 to 12) and up-regulates about 120 genes (fold-change 2 to 14) in a keratinocyte cell line HaCat. To avoid potential metabolism of atROL in keratinocytes this cell line was cultivated with a well-known alcohol / aldehyde dehydrogenase inhibitor citral, and the absence of atROL metabolites were checked by HPLC-chromatography. The following genes were selected for further investigations based on (1) the high fold-change parameter and (2) the connection to keratinocyte physiology and/or vitamin A metabolism: KRT1, KRT5, CYP26A, CYP26B, S100A8, ALDH3B, DHRS9, OAS1, KLK6, DEFB1 and ARG2.Next we verified our chip data with Real-Time PCR analysis of these genes. Using mRNA-decay analysis with actinomycin D we showed, that atROL and atRAL do not affect the mRNA stability of these genes in the keratinocyte cell line and gene regulation is due to elevated transcription. It is probable, that atROL and atRAL use the same mechanism of gene regulation as RA, namely through RARs and 9- RXRx. We are currently carrying out experiments to verify this hypothesis.

P264

CD44 knock-out results in an alteration of tight junction composition and function in keratinocytes

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Adult CD44 knock-out mice are characterized by alterations in epidermal structure and function, such as loss of apical polarization of lamellar body (LB) secretion and delayed barrier recovery after acute stratum corneum perturbation. Tight Junctions (TJ) play important roles in barrier function of epithelial cells and are thought to be involved in cell polarity. To elucidate whether TJ might be involved in the above mentioned alterations of CD44 knock-out mice we investigated TJ and TJ associated proteins, including the cell polarity complex aPKC/Par3/Par6 in developing and mature epidermis as well as primary keratinocytes of wild type (WT) and CD44 knock-out mice. During embryogenesis we observed a downregulation of Cldn-1,Par3 and cell signalling molecules important for TJ assembly, i.e. Rac1 and Tiam1, in knock-out compared to wild type mice at day 17.5. ZO-1 was mislocalized. Dye-penetration experiments using day 17.5 mice exhibited a disturbed permeability barrier of knock-out mice; electron microscopy investigations showed a loss of polarized LB secretion. In cultured cells after Ca-switch we observed a downregulation of Cldn-1 and Rac1 as well as alterations of localization of ZO-1 andPar3 in CD 44 k/o cells compared to WT cells. Trans epithelial resistance was decreased, permeability for FITC-dextrans was increased in k/o cells arguing for an impairment of TJ functionality. This study strongly suggests an influence of the transmembrane proteoglycan CD44 on TJ assembly and function via Rac1. This might be responsible for impaired barrier function and loss of cell polarity in CD44 knock-out mice.

P265

Connexin 43 mimetic peptide Gap27 accelerates normal wound healing but has no effect on keratinocytes from diabetic origin

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Connexins are transmembrane proteins that form Gap Junctions (GJ), communicating channels that allow the exchange of small molecules between adjacent cells. GJs are important for migration, differentiation and proliferation of cells. Connexin 43 (Cx43) has been shown to be ubiquitously expressed in human epidermis and to be down-regulated during early wound healing at the wound margins and in regenerating epidermis. The fact that Cx43 is still present at the margins of chronic wounds implicates that the down-regulation is important for effective wound closure. Phoshorylation of Cx43 on serine 368 (S368) has been shown to decrease gap junctional intercellular communication (GJIC).

We have shown previously that Gap27 accelerates wound closure in an *ex vivo* wound healing model and increases keratinocyte proliferation at the wound margins as well as in the regenerating epidermis of these models.

Here we show that the application of Gap27 into the *ex vivo* wound healing model as well as cultured cells resulted in a significantly decreased epidermal dye transfer by inhibiting the GJIC. Interestingly, the amount of S368 Cx43 was increased in the presence of Gap27 indicating phosphorylation to be involved in disruption of GJIC. Confluent keratinocyte and fibroblast cultures that were treated with Gap27 prior to a scratch wound assay showed significantly enhanced migration that resulted in a faster wound closure. In addition proliferation was increased. To further elucidate the effects of Gap27 as a possible approach for the treatment of diabetic ulcers, we investigated the effect of Gap27 on the migration and proliferation of human adult keratinocytes isolated from diabetic donors. We observed a significantly impaired migration of diabetic keratinocytes compared the cells from healthy origin. Surprisingly, Gap27 treatment did not affect migration or proliferation of human keratinocytes from diabetic origin whereas it significantly enhances migration and proliferation of non-diabetic cells.

These data show the importance of Cx43 in wound healing and suggest that the application of Gap27 might be beneficial for normal wound healing but not for diabetic wounds.

P266 (V28)

Alteration of tight junction proteins is an early event in psoriasis and might involve Interleukin-1beta

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Psoriasis is an inflammatory skin disease which affects about 2% of the western population. It is characterized by a hyperproliferation of keratinocytes, impaired barrier function and a pronounced infiltration of inflammatory cells into dermis and epidermis. Tight junctions (TJ) are cell-cell junctions which have been shown to form paracellular barriers for solutes but also for inflammatory cells. TJ proteins were demonstrated to be involved in various functions, including proliferation and differentiation. Altered localization of TJ proteins in the epidermis was described inplaques-type psoriasis. By using immunofluorescence microscopy, western blotting analysis and measurement of transepithelial resistance (TER) we investigated TJ and TJ proteins in psoriatic skin and in cultured keratinocytes as well as skin organ culture models. We observed that altered expression of TJ proteins is already found in early stage psoriasis. Occludin (Occl), ZO-1 and Claudin- (Cldn) 4 which are normally restricted to the upper layers of the epidermis are found in more layers, Cldn-1 and 7 which are normally found in all layers are negative in the basal cell layers and downregulated in the uppermost layers. JAM-A is slightly downregulated in the upper layers. In full-established psoriasis staining patterns of Occl, ZO-1 and JAM-A do not change while Claudins are further downregulated. Near transmigrating inflammatory cells, especially neutrophils, all TJ proteins are downregulated.Treatment of cultured keratinocytes with IL1B which is known to be present at elevated levels in psoriatic skin, results in an increase of TER at early points in time and a decrease of TER at later points in time. After injection of IL1B into an *ex vivo* skin organ model up- and downregulation of TJ proteins resembling TJ protein alteration in psoriasis, is observed in a dose dependent manner. Our results suggest that alteration of TJ proteins is an early event of psoriasis and not the consequence of long-time epidermal changes. IL1B might be involved in up- and downregulation of TJ proteins in a dose- and time dependent manner.

P267

Inter-alpha-trypsin inhibitor heavy chain 5 (ITIH5) expression is restricted to suprabasal keratinocytes in normal human skin, inflammatory skin diseases and differentiated 3D-skin models

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Inter-alpha-trypsin inhibitor heavy chain 5 (ITIH5) is a recently characterized novel member of the family of serum-derived hyaluronic acid associated protein (SHAP) which are now designated ITIH proteins. Since there is virtually no knowledge on the distribution and function of ITIH protein in this tissue, we have performed asystematic characterization of ITIH expression in healthy human skin and skin diseases. Using GeneChip® Human Exon 1.0 ST arrays we found that ITIH5 is the major inter-α-trypsin inhibitor heavy chain expressed in human skin. ITIH5 mRNA was detectable 13-fold more abundant in skin than in liver tissue which is the central expression site of ITIH1, ITIH2, ITIH3 and ITIH4. Since ITIH5 mRNA expression was absent in proliferating keratinocytes we analyzed ITIH5 expression in an *in vitro* keratinocyte differentiation model. Interestingly, expression of ITIH5 mRNA was significantly upregulated during the process of differentiation. Using an ITIH5 specific antibody we confirmed abundant expression of ITIH5 in differentiated keratinocytes of the human epidermis both by immunohistochemical and immunofluorescence staining. A similar ITIH5 protein expression pattern could be detected in the 3D-skin model where expression was restricted to the suprabasal layers of the epidermis-equivalent. Immunohistochemical analysis revealed a moderate staining for ITIH5 protein in normal skin and hair follicles, an abundant ITIH5 expression was detected in the suprabasal layers of patients with prurigo simplex subacuta and atopic dermatitis and expression was downregulated in samples of squamous cell carcinomas. ITIH5 may constitute a novel regulatory molecule of the human skin thathas an impact on extracellular matrix stability and viability of differentiating keratinocytes via its interaction with hyaluronic acid.

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Detailed domain mapping of the homodimer forming cytokeratin 16

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Recently, we have shown that the cytoskeleton is influenced in human skin aging. In this work we analysed the gene expression pattern of the complete family of cytokeratins in intrinsic cutaneous aging. We investigated more closely the function of one specific cytokeratin. K16, was identified to be strongly upregulated in aged human skin. Interestingly, K16 was found to form homodimers *in vitro*. In general cytokeratins form heterodimers, composed of an acidic and basic keratin protein. We were able to verify this homotypic interaction via *in vitro* pull down analysis and intracellularly in human keratinocytes, fibroblasts by FRET (fluorescence resonance energy transfer) experiments. These findings represented the base for detailed domain mapping of interacting regions in the K16 homodimer. We therefore designed fragments of the full length K16, corresponding to the known domains (1B, L12, 2A-L2, 2B) and also fragments shortened from the 5 and 3 prime end of the gene. The fragments were tested in Y2H experiments against the full length K16. As a biological positive control the K16/K6 heterodimer was used. According to the Y2H experiments and the following pull down analysis it becomes evident that the domains 1B and 2B, predicted to be key features in molecular assembly, may be important for the homotypic interaction. In future studies, we are going to further elucidate the general biological function of K16 homodimerization and the relation to the human skin aging process.

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Biocompatibility study on a biocellulose wound dressing

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Introduction: Biocompatibility is one of the main requirements for the safe use of medical devices. The assessment of the *in vitro* cytotoxicity is often a qualitative analysis based on the examination of cell damage and growth after direct or indirect contact with the material. According to the DIN EN ISO 10993-12 we prepared extracts of the biocellulose wound dressing Suprasorb X. Cell proliferation under the influence of the extracts was determined by measurement of the cell ATP content.

Methods: HaCaT-cells, primary fibroblasts and keratinocytes were cultured with extract from Suprasorb X (Lohmann & Rauscher, Germany) and increasing chlorhexidine concentrations as reference. Cell proliferation was investigated by means of the ATPLite (TM)-M kit (Perkin Elmer, USA). The luminometric ATP assay is based on the detection of light generated by the ATP dependent enzymatic conversion of D-luciferin by luciferase. Interleukin release was measured via ELISA specific for IL-6 and IL-8 (Milenia biotec, Germany).

Results: No significant influence of the extract from the biocellulose on the proliferation of human fibroblasts, keratinocytes and HaCaT-cells was found. The incubation of the cells with this extract did not change the release profile of IL-6 andIL-8 compared to the control. The tested chlorhexidine concentrations had a distinct negative effect on cell viability.

Discussion: In conclusion, the biocellulose extract does not exhibit a negative effect on the cells under the test conditions. The determination of ATP is expedient in cytotoxicity studies as it provides a stabile metabolic marker that enables direct monitoring of cell viability.

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Protective effect of polihexanide on HaCaT keratinocytes in co-culture withStaphylococcus aureus

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Introduction: Staphylococcus aureus is one of the most important pathogen of nosocomial infections and is common complication during the treatment of chronic wounds. It can exhibit a range of antibiotic resistency (MRSA). Therefore, wound dressings combined with antimicrobial agents are increasingly utilized in the treatment of critical colonized or infected chronic wounds. Polihexanide is regarded first choice for the treatment of chronic wounds because of its good skin tolerance beside its antimicrobial effects. Furthermore, a positive influence of polihexanide on wound closure was observed in individual clinical cases. So far, we investigated the influence of polihexanide human keratinocytes and fibroblast and found a positive influence on cell proliferation. Hence, we have used a co-culture system of HaCaT keratinocytes and Staphylococcus aureus to test the capacity of polihexanide to protect the cells from the bacterial damage.

Material & Methods: HaCaT keratinocytes were cultured with increasing concentrations of Staphylococcus aureus and with or without the addition of polihexanide in different concentrations and the extract of a polihexanide containing biocellulose wound dressing (Suprasorb X+PHMB, Lohmann & Rauscher). Viability and proliferation of HaCaT-cells was investigated by means of the ATPLite (TM)-M kit (Perkin Elmer). The luminometric ATP assay is based on the detection of light generated by the ATP dependent enzymatic conversion of D-luciferin by luciferase. Staphylococcus aureus was quantified via staining with SYTO-9 (Molecular Probes).

Results: Increasing Staphylococcus aureus concentrations had a distinct negative effect on HaCaT cell viability and proliferation. Polihexanide in increasing concentrations and the extract of the wound dressing were able to prevent cell damage and restore normal cell proliferation.

Conclusions: Polihexanide seems to be an ideal antimicrobial substance in wound dressings for treating chronic wounds because of its low cytotoxicity, good skin tolerance and positive influence on proliferation. Thus, the addition of polihexanide toa co-culture of HaCaT keratinocytes and Staphylococcues aureus protects the cells from the bacterial damage and allows normal cell growth.

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Comparison of different epidermal keratinocyte media for preservation of keratinocyte stem cells in serial cultures and epidermal transplants

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Hair follicle and epidermal basal layer human keratinocyte precursor cells play a central role in tissue homeostasis and wound repair and gain increasing interest as cell source for tissue engineered skin reconstructs. Whereas a high yield of stem cells would be favourable for integrity and long-term stability, they were lost during serial passages under generally used culture conditions.
In order to generate standardized cell culture protocols for preservation of keratinocyte precursor cells we focused our attention on different types of commercially available epidermal keratinocyte media.
Interfollicular keratinocytes (KC) isolated from the basal membrane (BM) of human skin biopsies as well as follicular KC isolated from the outer root sheath (ORS) of plucked anagen hair follicles were maintained in serial cultures using CNT-57- andCNT-07 medium, (CellnTec®), KG-medium (KGM, Clonetics®, Lonza®) andKG2-medium (KGM 2, Promocell®) under serum-free culture conditions.
In parallel cultures using the different cell sources and media, cell morphology was analyzed, viability and cell division rate were determined. By immunocytochemistry expression of markers of stem and transient amplifying cells (CK15, follistatin, CD71, p63,) as well as differentiation markers (involucrin) was determined. By clonality assays proliferative capacity, clonality and clonal conversion were compared. Multilayered epidermal transplants using both cell types under different culture conditions were reconstructed on feeder cells and characterized immunohistochemically.
For ORS-KC CNT-57 as well as KGM-2 media were superior to KGM with respect to cell viability, cell division - and passage rate. They support colony forming efficiency in serial cultures as well as preservation of stem cells in epidermal transplants. For epidermal BM-KC CNT 57/ 07 media were most favourable allowing a high cell viability and passage rate and a serial propagation of holoclones. In contrast, KGM medium supports differentiation and paraclone formation of isolated keratinocytes thereby leading to a lower passage rate.
Based on these findings, the selection of adequate progenitor targeted keratinocytemedia seems mandatory to protect epidermal stem cells and to prevent clonal conversion under serial propagation.

P272

Truncated Cockayne syndrome B protein represses elongation by RNA polymerase I

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Mutations in the CSB gene result in the human form of Cockayne syndrome (CS).CSB protein has been shown to be a component of RNA polymerase I (Pol I) transcription. In this study, we have analysed at which step of the transcription cycle CSB influences *in vitro* transcription by RNA polymerase I. We demonstrate that CSB stimulates elongation of RNA polymerase I in an ATP independent manner. Moreover, CSB can be crosslinked to the rDNA promoter and gene-internal sequences. Partial deletion mutants of CSB strongly repress Pol I *in vitro* transcription indicating an inhibitory function of truncated CSB. In addition, evidence is presented that mutant CSB inhibits the elongation step of Pol I transcription. Lack of CSB expression does not impair Pol I transcription showing that CSB is not essential for ribosomal transcription. Our results implicate that repressed Pol I transcription could be one factor contributing to the CS phenotype.

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The proteome and the impaired redox balance of ageing fibroblasts

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Comparative proteome analyses of the protein profiles of senescent and young fibroblasts are essential to understand the molecular mechanisms of cellular ageing. Using 2-DIGE and mass spectrometry a number of proteins revealed enhanced expression in old compared to young fibroblasts. Among these the manganese superoxide dismutase (SOD2), involved in antioxidative defence, showed the highest expression differences between young and old fibroblasts. To get insight into the effects of the imbalanced antioxidant defence in old fibroblasts, we here investigated whether the increased expression of SOD2 was accompanied by increased activity and whether the hydrogen peroxide (H202) detoxifying enzymes catalase and glutathione peroxidase are changed concomitantly. The activity of SOD2 was increased by 40% in old fibroblasts. As protein expression and activity of catalase are unchanged and expression and activity of glutathione peroxidase only slightly increased, we studied whether this would result in changed H2O2 levels in senescent fibroblasts. To determine the H2O2 levels in primary fibroblasts we established the HyPer-construct based on the bacterial H2O2 sensor OxyR. Unexpectedly, we couldnot detect any changes in the concentrations of H2O2 between young and old fibroblasts leading to the question why the increase in SOD2 did not result in enhanced levels of H2O2. Interestingly, in 2-DIGE and mass spectrometry analyses of young and old fibroblasts, the H2O2 detoxifying enzymes peroxiredoxin (Prdx) 2, 5 and 6 were found to be increasingly expressed in old fibroblasts. An increase in the protein expression of Prdx 5 was detected in old fibroblasts by immunoblotting. As superoxide anion radicals react with nitric oxide radicals to the oxidizing peroxynitrite, we analysed the general nitration levels in the fibroblasts. In old fibroblasts enhanced levels of nitrated proteins were detected by immunoblotting. Our data suggest that imbalances in the antioxidative defence in old fibroblasts might be partly compensated by additional antioxidative enzymes like the peroxiredoxins but are partly feeding in the generation of the damaging peroxynitrite.

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Epidermal stem cell markers on primary human keratinocytes

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Increased aldehyde-dehydrogenase (ALDH) activity is a well known marker for haematopoietic stem cells. The ALDH activity can be monitored using the artificial substrate ALDEFLUOR®. Degradation of ALDEFLUOR® by ALDH activity releases a fluorophore, which can be quantified in the cells by flow cytometry. To our knowledge, there is no information about ALDH expression in epidermal stem cells. We analysed the ALDH activity and surface expression of integrin alpha6, integrinbeta1 and CD200 of cells from epidermal cell preparations from human skin biopsies by flow cytometry.
Interestingly, in our experiments ALDH positive cell populations do show aco-staining with well known surface markers discussed as epidermal stem cell markers integrin beta1 and integrin alpha6. Moreover, a high proportion of ALDH/integrin double positive cells do show expression of CD200 a recently discovered marker of epidermal stem cells present in the human hair bulge (Ohyama, 2006). Further physiological analyses of the triple positive cells may allow us to analyse the stem cell activity of these cells.

P275

The EGFR in hair follicle development and cycle induction: studies in theWaved-5 mouse model

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The epidermal growth factor receptor (EGFR) fulfils essential functions in the homeostasis of the epidermis and hair follicle and its deregulation rapidly results indisorders as inflammatory responses, tumourgenesis, and impaired wound healing. Mice lacking EGFR die during embryonic development or during the first weeks of postnatal life, depending on the genetic background. Surviving EGFR-deficient mice develop a delayed and fuzzy coat, showing a severe phenotype of aberrant and premature hair follicle differentiation, epidermal atrophy, and low epidermal keratinocyte proliferation rates. However, the shortened life span and growth retardation of these animals precluded more detailed studies, forcing researchers to fall back on graft studies or on the overexpression of dominant-negative forms of the EGFR. Here, we employed the recently reported ENU-induced mutant mouse lineWaved-5 (Wa5) to analyze the effect of reduced EGFR activity in hair follicle morphogenesis and cycle induction during early postnatal life. Wa5 mice have a point mutation resulting in an antimorphic EGFR allele whose product acts as an dominant negative molecule, potently inhibiting the wild-type EGFR. Wa5 mice have wavy coats and partially formed eyelids at birth. Histological examination of defined regions of back skin revealed no differences in hair follicle morphogenesis between Wa5mice and control littermates at embryonic day 18.5, and postnatal days 0 and 8.However, the thickness of the subcutis of Wa5 transgenic mice is significantly decreased on day 8 as compared to control mice. In addition, while most hair follicles of control mice were in catagen stage VII or VIII at postnatal day 18, the majority of Wa5 hair follicles remained in catagen stage VI or VII. This resulted in a significant difference in hair cycle score on this day, indicating a retarded initiation of hair follicle cycling. The proliferation and survival of Wa5 keratinocytes is being currently evaluated. Wa5 mice differ from other mouse lines with altered EGFR activity in showing a rather mild skin phenotype. Our results suggest the presence of mechanisms ensuring nearly normal epidermal and hair follicle keratinocytes behavior in spite of very low levels of EGFR activity.

P276 (V17)

Wound healing defect of Vav3-/- mice due to impaired β2-integrin dependent macrophage phagocytosis of apoptotic neutrophils

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Vav proteins are guanine nucleotide exchange factors implicated in multiple leukocyte functions by relaying signals from a variety of receptors to Rho GTPases. While CD18 and Syk control neutrophil (PMN) recruitment and function in several models of inflammation and wound healing, evidence for the *in vivo* relevance of Vav in macrophages (MΦ) is scarce. Using Vav1-/-, Vav3-/- and Vav1-/-Vav3-/- mice, we here provide first evidence for a so far not reported role of Vav3 for MΦ functions during wound healing. Vav3-/-, as well as Vav1-/-Vav3-/- double knockout mice revealed a significantly delayed healing of full-thickness excisional wounds. Likewise, γ-irradiated Vav3 competent mice reconstituted with Vav3-/- bone marrow presented an impaired wound healing phenotype, confirming that Vav3 deficiency on leukocytes, but not on other cells, was causal for impaired healing. This was due to an impaired formation of the phagocytic synapse between apoptotic PMN and MΦ. In fact, confocal microscopy and immunoprecipitation revealed that activated Vav3 was recruited and co-localized with β2 integrins competent MΦ, but not in Vav3-/- MΦ upon adhesion to ICAM-1, the major ligand of β2 integrins in the phagocytic cup. β2 integrin-dependent phagocytosis of apoptotic PMN is the major stimulus for MΦ to release TGFβ1 which is responsible for the myofibroblast-driven wound contraction. In contrast to Vav3 competent mice, Vav3-/- MΦ revealed reduced adhesion to and phagocytosis of apoptotic PMN and significantly reduced release of active TGFβ1 *in vitro* upon co-culture of PMN with MΦ and *in vivo* as assessed in wound tissue lysates by TGFβ1 specific ELISA. Injection of either TGFβ1 or Vav3 competent MΦ, but not Vav3-/- MΦ into wound margins fully rescued impaired wound healing inVav3-/- mice. These data, in conjunction with an identical wound healing phenotype and impaired phagocytic synapse formation in β2 integrin (CD18)-/- mice, suggest that we have identified Vav3 as a critical downstream target in the β2integrin-dependent signalling pathway of phagocytotic synapse formation essentially required for cutaneous wound healing.

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The role of melanocortin receptor 1 polymorphisms in the regulation offibroblast function

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Melanocortin 1 receptor (MC1R) polymorphisms play a major role in the regulation of several signalling pathways in melanocytes. Alpha melanocyte stimulating hormone (α-MSH), which activates the MC1R initiating activation of the cAMP cascade has recently been shown to have impact not only on melanocyte but also on fibroblast function. In the present study we have investigated human native melanocyte and fibroblast cell lines with different MC1R polymorphisms. To evaluate the effect of MC1R polymorphisms on the receptor function we measured the intracellular cAMP concentration and the cellular proliferation upon stimulation with α-MSH. Our results indicate that the fibroblasts as well as the melanocytes show differences in the receptor function depending on the MC1R polymorphisms. In wild type fibroblasts the intracellular cAMP concentration and the cellular proliferation decrease upon α-MSH stimulation. In fibroblasts revealing MC1R polymorphisms, which correlate with a limited receptor function, both effects are significantly diminished. The same stimulation in wild type melanocytes results in an increase of cAMP concentration and cellular proliferation. Interestingly in cell lines with MC1R polymorphisms the increase of cAMP is less intense, while the cell proliferation is not significantly affected. We conclude that the MC1R polymorphisms are not only important for the regulation of the pigmentary system of the skin (i.e. melanocytes) but also have an impact on the function of the connective tissue (i.e. fibroblasts). Our findings can potentially be of importance in better understanding individual reactions within the scope of wound healing, development of hypertrophic scars, kelloids and fibrosis as well as aging.

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Ex vivo expanded hematopoietic progenitor cells increase angiogenesis and migration as well as proliferation of fibroblasts in murine dermal wound healing

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Ex vivo expanded hematopoietic progenitor cells improve dermal wound healing by paracrine mechanisms. In the present study we investigated the effects exerted by our immortalized hematopoietic progenitor cell line DKmix and their conditioned medium (CM) on angiogenesis, macrophages and migration/proliferation offibroblasts in a murine wound healing model. Endothelial cell-specific staining for CD31 showed increase capillary density in wounds treated with DKmix cells and their CM at day 6 compared to PBS treated wounds. Capillary density was significantly higher in DKmix cells treated wounds (5.60 ± 0.52% of total wound area; TWA) and in wounds treated with the CM of DKmix cells (6.92 ± 0.87% of TWA) than in PBS treated wounds (3.03 ± 0.38% of TWA). Immunohistological staining for CD68-positive macrophages revealed no differences among the three groups at day 6. *In vitro* we observed a significant dose-dependent increase in the number of tube-like structures of human endothelial cells with 10% CM (16.7 ± 3.3) which was even more pronounced with 20% (22.4 ± 3.3) and 50% (35.4 ± 2.3) compared to unstimulated control (3.2 ± 0.6). The migration of murine 3T3 fibroblasts significantly increased in adose-dependent manner when stimulated with 10% (1.53 ± 0.22-fold), 20%(1.75 ± 0.16-fold) and 50% CM (2.02 ± 0.22-fold) compared to unstimulated control. Likewise, we observed a dose-dependent effect of the CM on the cell proliferation of 3T3 fibroblasts. Proliferation was significantly enhanced with 10% (15 600 ± 2698 cells), 20% (18 975 ± 1195 cells) and 50% CM (34 300 ± 2479 cells) compared to unstimulated control (5125 ± 1105). DKmix cells improve skin-substitute wound healing by promoting angiogenesis as well as migration and proliferation of fibroblasts. These data indicate a participation of paracrine effects, inducing the formation of new capillaries, which is necessary to sustain the newly formed granulation tissue and the survival of keratinocytes.

P279

Everolimus, a rapamycin derivative suppresses fibroblast proliferation, collagen synthesis and skin fibrosis in a mouse model of scleroderma – future implications for the treatment of scleroderma and related disorders

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Treatment of scleroderma and related disorders of the skin remains a major therapeutic challenge. Here we assessed the *in vitro* and *in vivo* potency of everolimus, a rapamycin derivative, on proliferation, collagen synthesis and cutaneous fibrosis. Using the well known fibroblast mitogen basic fibroblast growth factor (bFGF), we first demonstrated that everolimus dose-dependently suppresses basal and bFGF-induced metabolic activity and proliferation of human dermal fibroblasts *in vitro*. Moreover, everolimus attenuated the inductive effect of the profibrotic cytokine transforming growth factor-beta1 (TGF-beta1) on intracellular collagen type I expression and secretion of procollagen type I C-terminal peptide in a dose-dependent fashion. This effect of everolimus was not due to suppression of collagen type I (alpha and beta chain) mRNA expression indicating that the drug acts posttranscriptionally. In fact, everolimus counteracted TGF-beta1-induced reduction in the relative amounts of various matrix metalloproteases (MMP1, 2 and 9) which degrade collagen and collagen metabolites. Moreover, in accordance with its role as an inducer of autophagy, ultrastructural analysis of everolimus-treated fibroblasts revealed signs of autophagy, i. e. autophagosomes consisting of degraded organelle content and double membrane structures, suggesting also increased intracellular procollagen turnover. In order to assess the *in vivo* significance of these data we finally utilized an established mouse model of bleomycin-induced scleroderma. Here, everolimus suppressed bleomycin-induced dermal collagen synthesis and fibrosis as shown by real-time RT-PCR analysis of collagen type I and III, collagen type I protein determination by pepsin digestion, and immunohistochemistry. Our data highlight the potency of everolimus as a novel suppressor of human dermal fibroblast activity. Based on our *in vivo* data everolimus may also become a promising therapeutic agent in future trials of fibrotic skin diseases including scleroderma.

P280 (V13)

Knock-down of (pro)-filaggrin in an organotypic skin model reproduces some of the features observed in atopic dermatitis and Ichthiosis vulgaris

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Loss-of-function mutations in the filaggrin gene have been shown to be associated with skin diseases displaying impaired terminal keratinocyte differentiation such as ichthyosis vulgaris and atopic dermatitis. We have thus investigated whether knock-down of filaggrin in an organotypic skin model would also lead to morphological phenotypes reminiscent of these diseases. Inaddition, we asked whether filaggrin knock-down in this system would have an impact on keratin-condensation/solubility and UV-sensitivity. Human primary keratinocytes were transfected with siRNAs specific for filaggrin, and seeded onto fibroblast–collagen suspensions to generate a multilayered epidermis. By H&E-staining we observed a complete loss of keratohyalin granules in filaggrin knock-down organotypic skin cultures, whereas the stratum corneum was formed regularly, displaying no signs of parakeratosis. However, ultra-structural investigations showed that keratohyalin granules, although still present, were strongly reduced in size. The stratum corneum appeared unaltered. We did not observe significant differences in the expression of other important epidermal differentiation associated genes, including loricrin, involucrin, matriptase-1, caspase-14, and several keratins by Western blot analysis and immunostaining. In addition the solubility of keratin-1, -10 and -2e was not affected by filaggrin knock-down, indicating a proper aggregation of keratin intermediate filaments. To investigate the impact of filaggrin deficiency on UV sensitivity, organotypic skin equivalents were cultured for 7 days and exposed to UVB (50–150 mJ/cm^2). In a first experiment a strong increase in apoptotic keratinocytes, as demonstrated by staining for cleaved/active caspase-3, was observed in the filaggrin knock-down samples compared to the controls. Our findings show that filaggrin knock-down in an organotypic skin model does not affect the expression and solubility of other differentiation associated proteins, and that morphological alterations of the epidermis are restricted to the granular layers, without influencing stratum corneum formation. The enhanced UV-sensitivity suggests an important role of filaggrin or its degradation products for the skin's UV-protection.

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ADAM10 and ADAM9 are the major collagen XVII sheddases in skin

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Collagen XVII / BP180 is an epithelial adhesion molecule that exists in a membrane-anchored form of 180 kD and a proteolytically released soluble ectodomain of 120 kDa. Previous studies identified ADAMs-9, 10, and 17 as candidate collagen XVII sheddases, and ADAM-17 deficient keratinocytes had 50% diminished collagen XVII shedding, suggesting that ADAM-17 is a major sheddase. However, here we show that ADAM17 only indirectly affects collagen XVII shedding, and that ADAMs 9 and 10 are the most prominent collagen XVII sheddases in primary keratinocytes. This conclusion is supported by the following results: a) collagen XVII shedding is not induced by short time stimulation with phorbol esters, known activators of ADAM17; b) constitutive and calcium influx-stimulated shedding is sensitive to the ADAM10 selective inhibitor GI254023X; c) calcium influx stimulated shedding of collagen XVII was lost in Adam10-/- cells, whereas wildtype or ADAM-9, -12, and -17-deficient cells responded normally; d) there was also a 55% decrease incollagen XVII ectodomain shedding from Adam9-/- keratinocytes; e) H2O2 enhancedADAM-9 expression in keratinocytes and in mouse skin and stimulated collagen XVII shedding, and this enhanced shedding was not seen in Adam9-/- keratinocytes or skin. We conclude that ADAM9 and ADAM10 can both contribute to collagen XVII shedding in skin, with an enhanced relative contribution of ADAM9 in the presence of reactive oxygen species. These results provide critical new insights into the identity and regulation of the major sheddases for collagen XVII in keratinocytes and mouse skin, and has implications for the treatment of Epidermolysis bullosa.

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Arginase-1 expression in human epidermal keratinocytes is regulated by differentiation status and all-trans-retinoic acid

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Arginase-1 contributes to the formation of the so-called natural moisturizing factor by converting arginine, a major breakdown product of filaggrin, into ornithine and urea. Here we investigated the regulation of arginase-1 in human epidermal keratinocytes (KC) *in vitro* and *in vivo*. cDNA microarray analysis showed that the expression of thearginase-1 gene was strongly increased in differentiated as compared to proliferating KC. Quantitative real-time PCR analysis confirmed a more than hundred-fold upregulation of arginase-1 mRNA during terminal KC differentiation induced by cultivating cells for several days at confluency. Treatment of KC under the same conditions with all-trans retinoic acid almost completely inhibited the increase of arginase-1 mRNA and protein expression. In contrast to a previous publication, which reported the presence of arginase-1 in the spinous layer of psoriatic epidermis and its absence in normal epidermis, we detected arginase-1 expression in the upper granular layer and the stratum corneum of both psoriatic lesions and normal epidermis. Finally, we developed an assay for the quantification of arginase-1 activity in the stratum corneum and found high inter-individual variations. In conclusion our data suggest that arginase-1 expression in the skin is confined to the last steps of KC differentiation and that its active form is present in the stratum corneum. Our finding that retinoic acid suppresses arginase-1 expression could be relevant for the 'dryskin' phenotype observed during retinoid therapy.

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Identification of conserved amino acid sequence motifs in keratin taildomains

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Type I and type II keratins contain a conserved central domain, that is critical for intermediate filament formation, as well as head and tail domains, which have more diverse amino acid sequences. The terminal domains of hard keratins are characterized by high frequencies of cysteine and proline residues whereas glycine and serine residues predominate in the terminal domains of soft keratins. The functions of the terminal domains of keratins are largely unknown. However, the clinical phenotypes of rare mutations in the tail domain of keratins 5 and 14 suggest essential roles of carboxy-terminal sequence elements. Here we compared the aminoacid sequences of human keratins with those of hair keratin-like proteins of the reptile, Anolis carolinensis, and identified highly conserved sequence motifs in keratin tail domains. The tail domains of some but not all type I keratins, including an Anolis hard keratin and human keratins 12, 14, 17, 18, 36, 39, and 42, as well as the tail domains of keratin 8 and of type III intermediate filament proteins contained a conserved sequence motif. Another previously unnoticed sequence motif was present in the tail domains of type II keratins such as a novel hard keratin of Anolis and human keratins 1, 5, 75, and 84. Our results suggest that carboxy-terminal sequence motifs of keratins have been conserved since the evolutionary divergence of the reptilian and mammalian lineages more than 300 million years ago. We propose that the functional characterization of keratin tail domains should be focused on the conserved motifs.

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Disturbed epidermal differentiation and increased skin thickness in Gly96/IEX-1 knock out mice

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The immediate early gene IEX-1, also known as IER-3/Dif2, is a growth and stress response gene that is involved as an regulater in signal transduction pathways. It consist of 156 amino acids and has no significant structure similarities to other known proteins. The murine ortholog is called Gly96/IEX-1. The IEX-1 expression is strongly dependent on the stimuli applied and on the cellular context. The complex regulation of the IEX-1 gene expression is regulated through a NFκB, a p53, 4 sp1, 2 radiation RE, 2 Ap1, a E-Box and a SOX binding sites in the promotor region. Transcription activation occurs after stimulation with cytokines, growth hormones, UVB irradiation or viral infection. IEX-1 overexpression can lead to cell proliferation but also to apoptotic effects depending on the cell type. For the way of action the celltype and the stimuli plays an important role. IEX-1 is describes to interact with the ERK1/2 and the AKT pathways in the cytosol. Additionaly it can change subcellular location and switch into the nucleus where it interacts neagtively with the p65 subunit of NFκB repressing p65 dependent gene transcription. In epidermis IEX-1 is constitutively expressed by most of the basal and some of suprabasal keratinocytes. Analyses of the skin of Gly96/IEX-1 knock out mice showed that in the untreated epidermis the thickness of the epidermis is significantly increase compared to wildtype littermates. Furthermore the expression of filaggrin, as one of the most important markers of epidermal differentiation, was upregulated and the immunreactivity offilaggrin is altered in the epidermis. It showed increased thickness of the stained band in the stratum granulosum. Caspase 3 staining revealed no difference in the amount of Caspase 3 positive keratinocytes between kockout and wild type mouse. Staining with the proliferation marker Mib showed slightly more positive cells in the epidermis of Gly96/IEX-1 knock out mice. These findings suggest that IEX-1 might play a role in regulation of skin homeostasis and might influence proliferation and differentiation but not of apoptosis.

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New insights into skin evolution: positive selection of the S100-fused-type-protein (SFTP) gene family

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Mammals have evolved various structural adaptations that allow them to survive better in changed environments. This is best characterized by the adaptation history of the epidermis, which is modulated to maintain the skin homeostatic barrier function. There is little knowledge, however, on the molecular mechanisms underlying epidermal evolution. Here we provide molecular evidences for supporting that epidermal adaptation is a process of natural selection. Firstly, we provide a complete identification of the S100-fused-type-protein (SFTP) gene family in humans. In addition to three known genes, filaggrin, trichohyalin and cornulin, we have identified four novel members of this family, including hornerin, filaggrin-2, repetin and trichohyalin-like 1. These genes are closely clustered within the epidermal differentiation complex at human 1q21 and share a conserved gene and protein structure. We found that they are expressed in the human and mouse skin but are selectively expressed in other less-complex epithelial tissues examined. In cultured primary keratinocytes, their expression were associated with Ca2+ stimuli and coordinated by mitogen-activated protein kinase (ERK, p38 and JNK) signalling pathways. Secondly, we determined all SFTP orthologs in other mammals (including the egg-laying platypus) and primates. We show that the SFTP family members follow a conserved one-to-one orthologous relationship in mammals while there is only one member (cornulin) in chickens, frogs and fishes, suggesting that the SFTP family may stem from gene duplication of a cornulin ancestor in a common mammalian ancestor after mammal-bird split (∼ 200–310 million years ago).These data are not compatible with the previous hypothesis of filaggrin present in non-mammals. Finally, examining the pattern of evolutionary change in this gene family, we uncovered the signature of positive selection in all mammalian SFTP members, which are confined to the repeat-containing domain, suggesting a process of natural selection. Together, our data indicate that the adaptation history of mammal epidermis is coincided with the origin and natural selection of the SFTP gene family and may help us understand the functional evolution of skin barrier.

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Topoproteomic approach to identify a ppERK 1/2-positive and -negative subpopulation of interfollicular epidermal stem cells by Multi Epitope LigandCartography (MELC) in psoriasis

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Interfollicular keratinocyte stem cells (IKSC) may be detected *in situ* utilizing complex combinations of markers. For this purpose Multi Epitope Ligand Cartography (MELC) may be especially advantageous being capable of reading out up to one hundred markers in one and the same tissue section. In the present study unaffected and affected skin of psoriasis patients was analyzed for the presence of IKSC and corresponding subpopulations. IKSC located in the basal epidermal layer were identified by MELC using the following topoproteomic signature: cytokeratin+/CD29+/CD49d+/CD49f+ and cytokeratin10-/Ki67-/CD71-. IKSC were quantified in skin biopsies from $n = 6$ psoriasis patients with normalization to 100 μm of horizontal skin width. We found all IKSC to be positive for the expression of Bcl-2 and c-KIT. The number of IKSC was significantly higher in unaffected psoriatic skin (2.81 cells/100 μm) as compared to affected psoriatic skin (1.22 cells/100 μm, $P < 0.0037$). IKSC were shown to be composed of a major ppERK1/2-negative and a minor ppERK1/2-positive subpopulation. In detail, there was a significantly higher amount ofppERK1/2-negative IKSC in unaffected psoriatic skin (2.54/100 μm) as compared to affected psoriatic skin (1.01/100 μm, $P < 0.001$). The ppERK1/2-positive IKSC subpopulation did not differ in both conditions (0.27 vs 0.21 cells/100 μm, respectively). The data allow the conclusion that IKSC are recruited and thereby reduced in number during the process of psoriatic epidermal proliferation. Moreover, the ppERK-positive IKSC subpopulation may represent an activated state for subsequent proliferation, as evidenced by its increased relative amount in affected psoriatic skin (17.1%) in comparison to unaffected psoriatic skin (9.6%).

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Role of ADAM-9 during wound repair

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ADAM-9 belongs to a family of transmembrane disintegrin-containing metallo-proteinases (ADAMs) involved in protein ectodomain shedding, cell–cell and cell–matrix interactions. Although the functions of many ADAM family members are known the specific biological function of ADAM-9 is still unclear. In this study, we have analysed ADAM-9 temporal and spatial distribution during wound healing. We demonstrated increased ADAM-9 transcripts expression during the first 7 days postwounding and, by immunolocalization, we detected ADAM-9 in all migrating and proliferating keratinocytes from day 3 to 7. In contrast, ADAM-9 expression in day 14 older wounds was mainly found in the suprabasal layers of the epidermis. In addition, in pathological conditions as in chronic ulcers, we detected a twofolds increased ADAM-9 transcripts expression as compared to normal healthy skin. These data together with our previous observations that ADAM-9 expression modulates migration of keratinocytes, suggested an important role for this protein during wound healing. To analyse how this protein would interfere with the healing process, we have produced excisional wounds on the back of animals with complete ablation of this protein. These experiments showed an accelerated wound repair in mice deficient for the Adam-9 as compared to control littermates being the excisional wounds closed earlier. No alterations in neutrophils, leukocytes as well as macrophages infiltrate were observed. However, epithelial migrating tongue was significantly longer inAdam-9-/- than control wounds. Since no differences in proliferation were observed *in vivo*, increased migration of keratinocytes was supposively responsible for this effect. *In vitro* investigations confirmed that migration but not proliferation of keratinocytes was increased. These results show for the first time that ADAM-9 can be a negative regulator of wound repair.

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Activation of the MEK5/Erk5 MAP kinase signalling pathway elicits a vasoprotective phenotype in primary human endothelial cells

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Mitogen-activated protein (MAP) kinases are important mediators of multiplebiological processes including proliferation, differentiation, stress responses and inflammation. One of the MAP kinase signalling cascades, the MEK5/Erk5 pathway, is activated by laminar shear stress in endothelial cells and is implicated in transducing the vasoprotective effect of flow on the vasculature. Here, we performed a microarray analysis of primary human endothelial cells (HUVECs) stably expressing a constitutively active form of MEK5 (MEK5D) to elucidate the downstream targets of Erk5 under static conditions. We provide evidence that ectopic activation of Erk5 can mimick various functions of laminar flow *in vitro* and induces a vasoprotective phenotype characterized by an antiapoptotic, antiangiogenic, antiinflammatory and antithrombotic gene expression pattern that strongly resembles that induced by laminar flow. Depletion of endogenous Erk5 by small hairpin RNA can revert the phenotype obtained with MEK5D expression confirming that the observed physiological consequences are not due to Erk5-independent signalling pathways. Our data suggest a role of the MEK5/Erk5 signal transduction cascade as major regulator of vascular integrity and implicate dysfunction of this pathway as a potential risk factor that affects the outcome of disorders involving the vascular system such as inflammatory skin diseases and disturbed wound healing.

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Melanoma cells induce an acute pro-coagulatory and pro-inflammatory response in endothelial cells

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Tumor cell spreading is accompanied by an intravascular pro-coagulatory activity and by inflammatory conditions that strongly support further tumor dissemination. These conditions are partially mediated by endothelial activation followed by release of von Willebrand factor (VWF), IL-8 or P-selectin. In particular, luminally exposed ultra-large VWF (ULVWF) seems to represent a central link between circulating cells including tumor cells and vascular endothelial cells (EC), due to its highly adhesive properties. In our study we have investigated the ability of invasive melanoma cell lines MV3 or WM9 or melanoma-derived soluble factors to trigger pro-coagulatory responses in EC. MV3 cells induce a massive release and immobilization of ULVWF at the luminal endothelial membrane via direct activation of the thrombin receptor PAR-1 on EC. As reported previously, melanoma-secreted MMP-1 is involved in PAR-1 cleavage which leads to adhesion of platelets and melanoma cells on ULVWF. WM9 cells, in contrast, activate the NFκB pathway in EC followed by an up-regulation of IL-6 and tissue factor (TF) expression. Exposition of TF into blood stream causes immediate thrombin generation, which leads to PAR-1 activation on EC and subsequent VWF release. Moreover, tumor cells can express components of coagulation pathway including TF and thrombomodulin (TM) on their surface. We could show that both melanoma cell lines express different amounts of TF and TM contributing to a different extends of thrombin generation. In conclusion, melanoma cells can directly (via secreted MMPs) or indirectly (via TF-mediated thrombin generation in blood plasma) activate PAR-1 on EC leading to instantaneous luminal ULVWF release. Binding of melanoma cells to ULVWF on EC supports adhesion and facilitates reciprocal communication, such as activation of the NFκB pathway in EC. Thus, ULVWF release and stabilisation may be enhanced by further up-regulation of TF or production of IL-6 which diminishes the activity of VWF-degrading protease ADAMTS13. We therefore hypothesize that the switch from an anti- to a pro-inflammatory and pro-coagulatory surface of EC plays a pivotal role in melanoma cell extravasation and tumor cell spreading.

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Mouse beta-defensin 3 and 14 expression are enhanced after chronic barrier disruption in EFAD-mouse skin: the defensins may prevent colonization with pathogenic *Staphylococcus aureus* but not with pathogenic *Proteus mirabilis*

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Mice fed an essential fatty acid deficient diet (EFAD) develop a red and scaly skin, impaired epidermal differentiation, and a disturbed skin barrier function, which resembles atopic dermatitis. Atopic dermatitis and psoriasis, diseases with an impaired skin barrier, are often colonizes by bacteria in particular *Staphylococcus aureus*. In contrast to atopic dermatitis, no infection occurs in psoriasis, and this was related to higher amount of antimicrobial proteins in psoriasis. Previously, we found increased expression of antimicrobial proteins after acute barrier disruption in mouse skin. Here we examined whether the chronic barrier disruption in EFAD-mice also influences defensin expression and whether this leads to changes in bacterial colonization of the skin. In EFAD-mice we found induction of the mouse beta-defensins mBD3 and hBD14, orthologues of the most important human defensins hBD2 and hBD3, as shown by real time PCR and by immunohistology. Remarkably, the skin of EFAD mice was not colonized by *Staphylococcus aureus*. The reason that we did not find this colonization in EFAD-mice may be that mBD3and mBD14 exert antimicrobial activity against *Staphylococcus aureus*. However, in EFAD mice only, but not in healthy control mice, we found colonization with the *Staph. sciuri*, *Staph. cohnii* ssp. urealyticum and *Proteus mirabilis* which may be smear infection from the intestine. *Staph. sciuri* and *Staph. cohnii* ssp. urealyticum resemble *Staph. epidermis* in human skin. Previously, it was described that hBD2and hBD3 are not active against *Proteus mirabilis*. Therefore, we expect that the orthologues mBD3 and mBD14 are also inactive against this bacterium. Together increased expression of mBD3 and mBD14 in EFAD-mice may prevent colonization with Staphylococcus aureus despite an impaired skin barrier. The defensins are not active against *Proteus mirabilis* colonization.

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Alternative proteolytic processing of hepatocyte growth factor controls skin repair

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Wound healing is a crucial regenerative process in humans. Recently, genetic deletion of c-Met in the murine epidermis demonstrated that the Hepatocyte growth factor (HGF)/c-Met pathway plays an essential role in skin repair. However, little is known on the function of this ligand-receptor complex during cutaneous repair in humans. We examined expression, integrity and function of the HGF/c-Met pathway in normal healing and non-healing human skin wounds. In normal healing wounds expression and phospohorylation of c-Met was most prominent in basal and suprabasal keratinocytes of the epithelial wound margin and several dermal cell types. In contrast, in non-healing wounds phosphorylation of c-Met was absent in the wounded epidermis and merely detected in the dermal compartment, suggesting limited c-Met activation. In wound exudates obtained from non-healing, but not healing wounds, HGF protein was a target of substantial proteolytic processing, which was different from the classical activation pathway by known serine proteases. Western blot analysis of rhHGF and comprehensive protease inhibitor analysis revealed that in non-healing wounds, HGF is a target of neutrophil elastase and plasma kallikrein. Proteolytic processing of HGF by each of these proteases significantly attenuated keratinocyte proliferation and wound closure capacity *in vitro*. Our findings reveal a novel pathway of HGF processing in the human system, in particular during skin repair. Furthermore, our results indicate that these unconventional proteolytic processing events of HGF have fundamental consequences on skin homeostasis. Under conditions in which inflammatory proteases are imbalanced and tipped towards an increased proteolytic activity, such as in chronic non-healing wounds, this event might compromise HGF activity due to the inactivation of the HGF molecule and/or the generation of HGF fragments that ultimately mediate a dominant negative effect. Overall, these studies provide new mechanistic insights in HGF/c-Met function and suggest a novel molecular pathomechanism underlying chronic wounds.

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The vitamin D receptor pathway is linked to the spliceosome

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The vitamin D receptor (VDR) is the most important linker between a signal from the outside of a cell, generated through the hormone 1a,25-dihydroxyvitamin D3 (calcitriol), and expression of vitamin D responsive genes. Beside calcium and phosphate homeostasis, the vitamin D pathway regulates proliferation and differentiation of keratinocytes. In this study we investigated the molecular environment of the vitamin D receptor in order to better understand mechanisms of keratinocyte differentiation and gene expression. Initially we accomplished a yeast2-hybrid (Y2H) analysis by screening the human vitamin D receptor against a cDNA gene library derived from human keratinocytes and found 46 potential VDR interactors. One of these proteins was the Ski-interacting protein SKIP, an already published interaction partner of VDR, which confirms the reasonable quality of our protein-interaction screen. A very interesting Y2H VDR-interactor turned out to be the RNA helicase DDX5 (DEAD box polypeptide 5). DDX5 is involved in alteration of RNA secondary structure, nuclear and mitochondrial splicing, ribosome and spliceosome assembly, transcription, cell growth and -division. According to the PRIMOS (http://primos.fh-hagenberg.at/) protein–protein interaction search database, this protein homologously interacts with the estrogen receptor (ER), a member of the nuclear hormone receptor family. As VDR and ER are members of the same protein family, DDX5 is thought to have similar effects on VDR. Y2H domain-analysis experiments showed that DDX5 interacts with the ligand-binding-domain (LBD) of the vitamin D receptor. Fluorescence-microscopy studies revealed a co-localization of both proteins in the nucleus of HaCat cells. Real-Time PCR data showed that mRNA expression of DDX5 is not regulated by the vitamin D signalling pathway, as it does not respond to calcitriol stimulation. The hypothesis that DDX5 is a co-activator of VDR mediated calcitriol activity has to be analysed in further functional studies.

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Prevention of skin hemorrhage during thrombocytopenia by blocking neutrophil β2-integrins

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Thrombocytes prevent bleeding by formation of a platelet plug. This classical haemostatic function is based on adhesion receptors that mediate the thrombus formation. Recently, we identified a novel haemostatic platelet function. During both inflammatory and angiogenic vascular remodelling, platelets were shown to regulate vascular integrity independent of their major adhesion receptors. This 'inflammatory hemorrhage' was discovered in thrombocytopenic mice subjected to models of dermatitis, melanoma, stroke and lung injury. We now demonstrate that granular contents of platelets regulate vascular integrity independent of their ability to form platelet plugs. By loss of anti-permeability factors, thrombocytopenia promotes organ hemorrhage. Thus far, the effector cells of inflammatory hemorrhage are unknown. Studying the role of neutrophils in models of dermatitis (Irritant contact dermatitis, Arthus reaction) we here show that blocking of β2-integrin function (CD18-/- mice) results in complete inhibition of skin hemorrhage. This demonstrates how anti-inflammatory treatment prevents skin hemorrhage.

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Electron microscopy in skin research

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Electron microscopy is a powerful tool to investigate ultrastructural details at the subcellular level. Also in dermatology, transmission- (TEM) as well as scanning electron microscopy (SEM) is often used. The methodological background of preparation and the following investigation in the electron microscope is sometimes difficult and complex. Especially if cryopreparation techniques like high-pressure freezing or cryosectioning should be applied. With three examples we show only a small application window of electron microscopy in the field of dermatological research. (i) For drug delivery, one of the used application pathways are the well established use of liposomes. We visualize the interaction of liposomes and adherentc ells by using freeze-fracture preparation and replica technique, in the TEM. Different pathways through the plasma membrane of the cells can be demonstrated. (ii)Conventionally prepared skin samples were compared with cyanacrylat biopsies. After fixation with osmium tetroxide or ruthenium tetroxide the lipid layers in the intercellular space as well as the lamellar bodies were visualized. (iii) Ultrastructural details, near to the life state of the sample, could only be visualized by using cryopreparation techniques like high-pressure freezing and freeze-substitution (HPF-FS). Differences in the ultrastructure of human skin are shown after the application of either, conventional fixation with aldehydes and osmium tetroxide followed by dehydration at room temperature, or the application of cryoprepared skin samples (HPF-FS). Furthermore, the well preserved antigenicity in such cryoprepared samples enables immunolocalisation of proteins and lipids. Microscopy Services, as a full service provider, offer a complete solution of your scientific questions, including specimen preparation (conventional as well as cryopreparation techniques), investigation in the electron microscope (SEM and TEM) and finally interpretation of the images.

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STRA6 a potent novel transporter for the active influx of retinol in human epidermal keratinocytes

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Retinoids play key roles in cell proliferation and differentiation. Retinyl esters and β-carotene are ingested and stored mainly in stellate cells of the liver. Demand for retinol results in the release of retinol–retinol binding protein complexes which are taken up by human skin. Recently the novel transport protein STRA6, which is a high-affinity cell surface receptor for retinol-RBP has been detected in bovine retinal epithelium cells. STRA6 removes retinol from RBP and transports it across the plasma membrane. To demonstrate whether similar transport processes take place inhuman skin cells, we analyzed expression of human STRA6 in normal human epidermal keratinocytes (NHEK), murine PAM212 cells and human dermal fibroblasts. qRT-PCR analysis detected a constitutive expression of STRA6 in NHEK, PAM212 cells and dermal fibroblast and expression could be significantly upregulated by ligands of the different nuclear retinoic receptors such as 9-cis-RA,13-cis-RA, all-trans-RA and targretin as well as retinol itself. In contrast we could not observe an increased STRA6 expression after stimulation with ligands of other class II nuclear receptors such as phenobarbital, dexamethasone and benzanthracene. To characterize the influx transport of retinol in NHEKs we established a functional uptake-transport assay and demonstrated that retinol uptake in keratinocytes dependends on expression level of STRA6 as well as time of incubation. Gene regulation studies revealed that RAR/RXR- as well as EGFR signalling pathways are involved in the upregulation of STRA6 in NHEK. In conclusion we were able to demonstrate that skin cells express STRA6, a novel transporter for active influx transport of retinol.

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Optimizing active RNA trans-splicing molecules for the treatment of cysticfibrosis

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Cystic fibrosis is one of the most common hereditary diseases. It is caused by a mutation in the cystic fibrosis conductance regulator (CFTR) gene resulting in a malfunctioning chloride channel. Usually a full-length gene is delivered into a 'diseased' cell in order to supplement the impaired protein expression, which raises the possibility of unregulated overexpression. Overexpression of the therapeutic gene might lead to tumor formation and/or loss of stemness of the corrected stemcells. Trans-splicing is a technology to 'reprogram' the sequence of endogenous mRNAs circumventing these problems. For this purpose we have established a FACS based high-throughput screen for the CFTR gene. With this screening system we have improved repair of the CFTR gene building reprogramming molecules (RTM) for correction of exons 5 to 24, making the molecule effective for over 85% of the CF patients. We have identified RTM's with a trans-splicing efficiency of up to 68%. The RTM's have been introduced into CFPAC-1 cells, a pancreatic duct cell line that is homozygous for the F508del mutation, to show endogenous repair. To verify the activity of our trans-splicing molecules the functionality of the protein was analysed by protein detection assays. Transfer into an adenoviral vector and evaluation in an animal model are the next planned steps.

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The transcriptional response to distinct growth factors is impaired in Wernersyndrome cells

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The Werner syndrome protein (WRN) is mutated in Werner Syndrome (WS) and plays a role in telomere maintenance, DNA repair and transcription. WS represents a premature ageing syndrome with severe growth retardation. Here we show that WRN is critically required to mediate the stimulatory effect of Vascular Endothelial Growth factor (VEGF), basic Fibroblast Growth Factor (FGF-b) and Epidermal Growth Factor (EGF) on the activity of RNA polymerase I (Pol I). Recombinant WRN specifically reconstitutes RNA polymerase I transcription in extracts from Werner syndrome fibroblasts *in vitro*. In addition, we identified a critical role for WRN during promoter clearance of Pol I transcription, but not in elongation. Notably, WRN was isolated in a complex with Pol I and was cross-linked to the unmethylated, active proportion of rDNA genes in quiescent cells suggesting a so far unknown role for WRN in epigenetic regulation. This together with alterations in Pol I transcription provide a novel mechanism possibly underlying at least in part the severe growth retardation and premature aging in Werner Syndrome patients.

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Psychosocial distress in psoriatic out-patients

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The aim of this study was to evaluate the psychosocial morbidity in psoriasis patients treated at the Göttingen University out-patient clinic. One hundred and thirty-five patients with chronic plaque psoriasis and 55 control patients treated for other skin diseases at the same center were evaluated for psoriasis and for psychiatric disease using the PASI, the Dermatology Life-Quality Index (DLQI) and the Hamilton Rating Scale for Depression (HAM-D). The majority of psoriasis patients suffered from moderate-to-severe disease and 28% of psoriasis patients had a definite diagnosis of psoriatic arthritis. DLQI was 3.2 ± 2.4 (mean $\pm$ SD) and, in a multivariate analysis, only PASI correlated with DLQI ($P < 0.01$). Twenty per cent of patients suffered from an at least mild depression (HAM-D > 8), compared to 5% of control patients. A multivariate analysis showed a correlation of HAM-D with age ($P < 0.02$) and gender ($P < 0.001$), but not with PASI. When asked whether they had psychosocial distress, 35.6% of psoriasis patients answered yes, which differed significantly from control patients (14.5%) ($P < 0.001$). Seventy-three per cent of psoriasis patients reported psychosocial distress to be related to psoriatic disease. When study participants were answered whether they suffered from depression, anxiety, nervousness or sleep disorder, patients with psoriasis reported a significantly higher prevalence of the disorders in all categories with 33% vs 9% for depression, 17% vs 2% for anxiety, 44% vs 22% for nervousness and 34% vs 16% for sleep disorder in psoriasis patients versus control patients. At the time of evaluation 3.7% of psoriatic patients regularly took anti-depressants and 2.2% took antipsychotic drugs, compared to 1.8% and 0% of control patients respectively. The majority of psoriasis patients with a HAM-D value indicative of an at least moderate depression (HAM-D > 14) did not receive antidepressants. In conclusion, psoriasis patients had an increased prevalence of depression compared to other dermatological outpatients, and most patients were not treatedad equately. In self-rating, a third of patients described themselves as suffering from depression, indicating a high prevalence of psychosocial distress not necessarily picked up in the presently used standardized instruments.

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Cytotoxic and inflammatory effects of PM10 in classrooms

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Outdoor particulate matter (PM10) is associated with a wide range of health effects and a European threshold limit of 50 $\mu g/m^3$ was established in 2005. However, most individuals spend at least 85% of their time indoors where particle concentrations are mostly higher than outdoors. Since children represent a vulnerable group, we investigated the health effects of indoor air PM10 collected in classrooms compared to outdoor air PM10. PM10 was collected in five schools in Munich during teaching hours. Cytotoxicity was assayed as a decline of cellular ATP concentration in human primary keratinocytes, human lung epithelial A549 cells and Chinese hamster V79 lung fibroblasts at concentrations up to 10 μg/ml. In addition, toxicity after metabolic activation was assayed in V79 cells expressing human cytochrome P450 1A1, 1A2, 1B1, 2A6, 2B6, 2C9, 2D6, 2E1, 3A4 or 3A5. For a genome wide expression analysis BEAS-2B bronchial epithelial cells were incubated with 10 μg/ml PM10. RNA was isolated and analyzed on Affymetrix HG U133A 2.0 expression arrays. While in A549 and V79 cells no toxicity was observed, in human primary keratinocytes PM10 at a concentration of 10 μg/ml caused a slight decrease in vitality. This cytotoxic effect was also found in V79 cells after metabolic activation by CYP1A1 or CYP2C9. Genome wide analysis of PM10 from outdoor and indoor air showed the overexpression of xenobiotic metabolizing genes (CYP1A1, CYP1B1) and of inflammatory cytokines (IL1A, IL1B, IL6, IL8). Indoor PM10 caused a lower induction of xenobiotic metabolizing genes but a up to six fold higher induction of inflammatory cytokines compared to outdoor PM10. Direct cytotoxicity and metabolic activation by cytochrome P450 isoforms 1A1 and 2C9 were statistically significant at a PM10 concentration of 10 μg/ml, which is about 10 000 times higher than exposure encountered in classrooms. We therefore expect no toxic effects of these particles in school children. The reduced induction of xenobiotic metabolizing genes but increased induction of inflammatory cytokines in indoor PM10 treated cells suggests that classroom PM10 is less toxic but has a higher inflammatory potential than outdoor PM10.

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Methods in hair research: How to distinguish anagen VI and early catagen in organ-cultured human hair follicles

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The organ culture of human scalp hair follicles (HFs) in the anagen VI stage of the hair cycle is the best currently available assay for hair research in the human system. In order to check whether a test agent promotes or inhibits hair growth in this assay, it is critically important to be able to assess with certainty whether the test agent prolongs anagen or prematurely induces catagen. However, objective qualitative as well as easily quantitative criteria for distinguishing early catagen from anagen VIHFs, which shows a very similar morphology, remain to be established. Here, we report the first classification system that allows to distinguish between anagen VI and early catagen in organ-cultured human HFs using both qualitative and quantitative parameters. Qualitative classification is based on assessing the morphology of the hair matrix (HM), the dermal papilla (DP), the distribution of melanin and the expression of the premelanosomal marker gp100 (Nki/beteb). These criteria are complemented by the following ten quantitative ones: 1) Quantification of DAPI+ cells in HM keratinocytes below Auber's line, 2) Quantification of Ki-67 + cellsin HM keratinocytes below Auber's line, 3) Quantification of TUNEL+ cells in HM keratinocytes below Auber's line, 4) Quantification of TUNEL+ cells in the central DP, 5) Quantification of the number of DP stalk fibroblasts, 6) Quantification of TUNEL+ cells among DP stalk fibroblasts, 7) Quantification of total number of nucleated gp100+ cells around the DP, 8) Quantitative immunohistomorphometry of tyrosinase activity-associated immunoreactivity, 9) Total melanin content in hair matrix on both sides of the DP and 10) Total intensity of gp100-associated immunoreactivity around the DP. Using this classification system, we tested several defined hair growth inhibitory agents to check the reliability of this classification. All test substances produced the expected premature catagen induction as identified by the novel classification criteria reported here. Therefore, this classification system offers an excellent sensitive, and reproducible tool to reliably distinguish between anagen VI and early catagen, and thus serves an important new tool for preclinical hair research in the human system.

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Comparison of the binding capacity of collagen from different origin for inflammatory cytokines and free radicals

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Introduction: Chronic wounds contain elevated levels of inflammatory cytokines (IL-1β, TNF-α) and reactive oxygen and nitrogen species (ROS/RNS). The overproduction leads to severe tissue damage and impairs wound-healing. Hence, the reduction of these mediators is a suitable way to promote normal healing. Collagen is known to be able to bind significant amounts of cytokines or inhibit the formation of free radicals. A variety of wound dressings containing collagen of different type and origin are used. Within the present study we investigated the influence of the collagen origin (bovine, porcine and equine) on the binding capacity for IL-1β, TNF-α and ROS/RNS.

Materials and methods: Following wound dressings have been used bovine – SuprasorbC (Lohmann & Rauscher), porcine – Nobakoll (MBP GmbH) and equine – Kollagenresorb (Resorba Clinicare GmbH). Samples were cut into equal pieces, taken in 1 ml of IL-1β or TNF-α solution (100 pg/ml), and incubated up to 24 h at 37°C. Concentrations of unbound cytokines in the supernatants were determined by specific ELISAs (Mabtech AB). Antioxidant potential was measured using the chemiluminescent ABEL® Antioxidant Test Kits containing Pholasin® specific for superoxide and peroxy nitrite (Knight Scientific Limited).

Results: Already after 1 h a highly significant decrease of the cytokine concentration was observed. Furthermore, the collagen wound dressings of different origin showed antioxidant capacity. Bovine collagen performed best on IL-1β and TNF-α binding, but porcine collagen was more effective against ROS/RNS formation. Equine collagen showed a similar antioxidant capacity as bovine collagen and a comparable influence on the cytokine reduction as porcine collagen.

Conclusions: Collagen possesses a high binding capacity for different inflammatory cytokines and is able to inhibit the formation of free radicals *in vitro*. Therefore, collagen containing dressings should be able to improve the healing outcome of chronic wounds by decreasing these excessive mediator concentrations. Nonetheless, the choice of the collagen origin does influence the wound dressing performance.

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Binding capacity of collagen from different origin for PDGF-BB

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Introduction: Chronic wounds contain elevated levels of neutrophil elastase which is responsible for the degradation of growth factors such as platelet-derived growth factor (PDGF). In order to support the normal wound healing process the protection of growth factors is essential. Previous studies have shown that a wound dressing composed of bovine collagen type I is able to bind significant amounts of PDGF-BB, protect it from proteolytic degradation and maintains its biological. The aim of this study was to investigate the influence of collagen origin on PDGF-BB concentration as well as biological activity *in vitro*.

Materials and methods: Following wound dressings have been used bovine - SuprasorbC (Lohmann & Rauscher), porcine – Nobakoll (MBP) and equine – Kollagen resorb(Resorba Clinicare). Samples were cut into equal pieces and incubated up to 2 h at37°C in PDGF-BB solution. Supernatants were collected and the wound dressing samples washed to recover bound PDGF-BB. Supernatants and washing solutions were incubated with normal human dermal fibroblasts (NHDF). PDGF-BB concentration was determined by specific ELISA (Quantikine Immunoassays for PDGF-BB, R&D Systems). Fibroblast proliferation was assayed by determination of dsDNA amount and ATP content.

Results: The bovine collagen wound dressing binds considerable amounts of PDGF-BB leading to a reduction of the effective concentration of the growth factor. The binding capacity of porcine and equine collagen for PDGF-BB was much. Bound PDGF-BB can be regained from bovine collagen by elution. The release from porcine and equine collagen was much less distinct due to the lower binding. The PDGF-BB elution from bovine collagen correlated with increased fibroblast proliferation compared to medium control.

Conclusions: Collagen is able to bind PDGF-BB at different rates depending on its origin. In particular, bovine collagen has a considerable binding capacity for the growth factor. During the binding, PDGF-BB is not only protected from proteolytic degradation but preserves its biological activity as well. Porcine and equine collagen showed no significant binding affinity for PDGF-BB.

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Influence of the collagen origin on the binding affinity for neutrophilelastase

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Introduction: Non-healing wounds contain elevated levels of neutrophil elastase which are responsible for the degradation of extracellular matrix and growth factors. These destructive processes prevent wound closure and lead to persisting wounds. It has been shown that the binding of the proteolytic enzymes by collagen wound dressings contributes to the treatment of chronic wounds. The aim of this study was to investigate the influence of the collagen origin on neutrophil elastase concentration *in vitro*.

Materials and methods: Wound dressings consisting of bovine, porcine and equine collagen have been used. Samples were cut into equal pieces (0.5 cm^2), taken in a final volume of 1 ml of neutrophil elastase solution, and incubated up to 24 h at37°C. Supernatants were collected and stored at -20°C. The concentrations of unbound protein in the supernatants were determined by specific ELISA (neutrophil elastase ELISA, milena biotec).

Results: All collagens tested exhibited binding capacity for neutrophil elastase. However, bovine collagen was most effective.

Conclusions: Collagen is able to bind neutrophil elastase at different rates depending on its origin. In particular, bovine collagen has a considerable binding affinity for neutrophil elastase. Although, collagen at large should be able to establish a physiological environment in chronic wounds and promote healing, the origin of the collagen does influence the wound dressing performance.

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Anti bacterial and antifungal effect of iodine containing gauze

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Introduction: *Staphylococcus aureus* is one of the most important pathogen of nosocomial infections. It can exhibit a range of antibiotic resistance (MRSA) thus complicating the patient's treatment. Facultative pathogenic enterobacteria, like *Klebsiella pneumoniae*, are normally innocuous, but they can lead to infection of wounds. Fungi such as *Candida albicans* are also facultative pathogenic but the colonization can result in mycosis. The spread of these pathogens can only be inhibited through consistent hygiene sanctions and preventive disinfectant actions. The antimicrobial activity of iodine has been discovered early for the use in medicine. The mechanism of action is the damaging of microbial proteins through oxidation of amino acids by elemental iodine. We have tested three different iodine containing gauzes according to the JIS L 1902 for antibacterial and antifungal activity.

Materials and methods: *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Candida albicans* were chosen to monitor the antimicrobial effect. According to the JIS L 1902norm samples of 400 mg of the iodine containing gauzes (Opraclean, 5% iodine; Lohman & Rauscher, 5% iodine; and Lohmann & Rauscher 'forte', 50% iodine) were used for testing. Gauze without iodine was used as reference material. The samples were incubated with the experimental pathogens (*Staphylococcus aureus*: 1.8×10^{-5} cfu/ml, *Klebsiella pneumoni*: 5.4×10^{-5} cfu/ml and *Candida albicans*: 1.6×10^{-4} cfu/ml) up to 24 h at 37°C under aerobic conditions.

Results: All three iodine containing gauzes showed a strong inhibitory effect on *Staphylococcus aureus* and *Klebsiella pneumoniae*. They were also able to inhibit the growth of *Candida albicans* significantly.

Conclusions: Iodine containing gauzes exhibit a distinct antibacterial and antifungal activity. Their use should help to prevent wound infections and treatment complications. Low concentrations of iodine seem necessary to achieve growth inhibition of *Klebsiella pneumonia*, and *Candida albicans*. Only *Staphylococcus aureus* showed an iodine concentration dependent reduction of viability.

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The *in vitro* formation of ROS/RNS is inhibited by polihexanide

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Introduction: Wound dressings combined with antimicrobial agents are increasingly utilized in the treatment of critical colonized or infected chronic wounds. Polihexanide is regarded first choice for chronic wounds because of its good skin tolerance beside its antimicrobial effects. An additional antioxidative effect would be a beneficial attribute of polihexanide as exudates of chronic wounds contain elevated levels of reactive oxygen and nitrogen species (ROS/RNS).

Materials and methods: Antioxidant potential of polihexanide (Cosmocil, ARCH Chemicals) and a polihexanide containing wound dressing (Suprasorb X+PHMB, Lohmann & Rauscher) was measured using the chemiluminescent ABEL® Antioxidant Test Kits containing Pholasin® specific for superoxide and peroxynitrite (Knight Scientific Limited, UK).

Results: Polihexanide exhibited a significant concentration dependent antioxidant potential. The wound dressing containing polihexanide was also able to inhibit the formation of ROS and RNS significantly.

Conclusions: It is believed, that the overproduction of reactive nitrogen and oxygen species in chronic wounds results in an elongated inflammatory phase and severe tissue damage. Hence, the reduction of these active species seems to be a suitable way to promote normal wound-healing. Polihexanide inhibits the formation of free radicals *in vitro*. Therefore, polihexanide as well as the wound dressing used should have an auxiliary influence on the healing of chronic wounds beside the antimicrobial effect.

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Polyacrylate-superabsorber binds inflammatory proteases *in vitro*

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Introduction: Non-healing wounds contain elevated levels of neutrophil elastase and matrix metalloproteinases (MMPs) which are responsible for the degradation of extracellular matrix and growth factors. These destructive processes prevent wound closure and lead to persisting wounds. It has been shown, that the binding of the proteolytic enzymes contributes to the treatment of chronic wounds. The aim of this study was to investigate the binding capacity of a polyacrylate-super absorber for elastase and MMP-2 *in vitro*. Polyacrylate-superabsorber containing wound dressings are able to take up large quantities of exudates while keeping the wound environment moist; an additional binding of matrix degrading proteases would be a beneficial attribute.

Materials and methods: Wound dressing samples (Vliwasorb, Lohmann & Rauscher) were cut into equal pieces (0.5 cm^2), taken in a final volume of 1 ml of protease solution (PMN elastase: 250 ng/ml and MMP-2: pg/ml), and incubated up to 24 h at37°C. Supernatants were collected and stored at -20°C. The concentrations of unbound protein in the supernatants were determined by specific ELISAs (neutrophil elastase ELISA, milena biotec; and Quantikine Immunoassays for total MMP-2, R & D Systems).

Results: The polyacrylate-superabsorber exhibited a high binding capacity for all proteases tested. Subsequently, only marginal amounts of elastase and MMP-2 could be eluted from the samples after incubation.

Conclusions: Polyacrylate-superabsorber is able to shortly bind large amounts of elastase and MMP-2 *in vitro*. Elution of the wound dressing revealed a strong, possibly irreversible binding of both proteases. The decrease of these matrix degrading proteases should aid the establishment of a physiological wound milieu *in vivo* and thus support the healing process.

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Screening for potential allergens with proteomic MALDI-TOF read-out

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Allergies have increased significantly in the US and worldwide (NHANES III Survey 2005). In an environment with millions of naturally (and synthetic) occurring antigens and allergens the human system has permanently to distinguish between an armada of different chemical substances and to decide to ignore/tolerate or react towards a specific substance. Thus, there is an increasing demand for the development of novel *in vitro* screening methods to identify potential sensitizers or non-sensitizers. One often characteristic of the majority of sensitizing chemicals is that they are electrophiles, which may interact with nucleophilic amino acids. Cysteine is one of the most dominant binding partners for electrophiles. These cysteine–electrophile interactions result in covalent protein or peptide modifications. The aim of the present investigation is to support the development of an easy but sensitive MALDI-TOF [Matrix Assisted Laser Desorption Ionization – Time Of Flight Mass Spectrometry (MS)] based screening method for electrophilic chemicals. In the present study, allergy inducing human contact sensitizers such as 2,4-dinitrochlorobenzene (extreme), cinnamaldehyde (moderate) and salicylic acid (none) have been examined for their reactivity with peptides. To explore especially the reactivity of cysteine, two different synthetic peptides were chosen: peptide-21(with 21 proteinogenic amino acids plus cysteine), and peptide-20 (as peptide-21 but lacking cysteine). The resulting peptide modification was determined via mass shift analyses using MALDI-TOF-technique. Both, the extreme sensitizer as well as the moderate sensitizer produced mass shifted peptide peaks of peptide-21. The non-sensitizer did not result in any mass shift related peptide-peaks.
Conclusion: The present study indicates that novel MALDI-TOF-technology-based *in vitro* assays may help to determine reactive electrophiles, and to distinguish between potential human sensitizers and non-sensitizers. (Work supported by EU-Project LSHB-CT-2005 - 018681, www.sens-it-iv.eu).

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In vivo investigation of hypoxia-induced angiogenesis in experimental autoimmune disease using [18F]FAZA and positron emission tomography (PET)

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Angiogenesis is critically involved in the pathogenesis of organ-specific autoimmune diseases. As the signals leading to angiogenesis remain poorly understood, we investigated glucose-6-phosphate-isomerase (GPI) auto-antibody induced arthritis in mice that closely resembles human rheumatoid or psoriasis arthritis (RA). Tissue hypoxia that is associated with inflammation can induce angiogenesis via stabilization of the transcription factors hypoxia inducible factor HIF-1α and HIF-2α in resident and infiltrating cells. To characterize the mechanisms underlying inflammation induced angiogenesis, we investigated hypoxia in GPI arthritis using PET and 18F-fluoroazomycin-arabinosid ([18F]FAZA) or 18F-fluoromisonidazole ([18F]FMISO) which are selective for hypoxic tissue. To induce arthritis, Balb/c mice received either GPI-serum or control-serum on days 0 and 2. After 5–7 days mice underwent *in vivo* PET investigation using [18F]FAZA and [18F]FMISO and, in addition, magnetic resonance imaging (7 Tesla MRI). Subsequently, we performed *ex vivo* H&E-staining, pimonidazole immunohistochemistry, real-time PCR and western blot of arthritic and healthy joints. *In vivo* PET images revealed a 2.8-fold increase of [18F]FAZA and a 3.6-fold increase of [18F]FMISO uptake specifically in arthritic joints directly visualizing hypoxia at the site of GPI arthritis. Western blot of arthritic ankles showed enhanced HIF-1α and HIF-2α protein expression and RT-PCR analysis demonstrated a 6–3000 fold enhanced expression of HIF1-/HIF2α, IL-1 β IL-6 and TNF on mRNA level. Pimonidazole immunohistochemistry confirmed the *in vivo* observed hypoxia inside the inflamed joint tissue. As a consequence of hypoxia, blood vessel, pannus formation and joint destruction were strongly enhanced. Thus, non-invasive *in vivo* examination of hypoxia-induced angiogenesis using [18F]FAZA or [18F]FMISO provides a new powerful tool to quantitatively evaluate hypoxia *in vivo* and the initial phases of angiogenesis in autoimmune diseases such as psoriasis or RA.

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Differential proteomic analysis and mass-spectrometric identification of allergen-regulated proteins in primary human keratinocytes

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Background: Over the past two decades, rates of allergies have increased in the US and worldwide (NHANES III Survey 2005). Nickel (Ni) represents the most common contact allergen. Aiming to characterize so far unknown molecular events underlying T-cell mediated human allergic contact dermatitis (ACD) a proteomic approach was chosen to identify Ni-regulated proteins specifically at the primary contact sites of the human body.
Methods: Primary human keratinocytes were stimulated with Ni and subsequent regulation of proteins was investigated using differential-in-gel-electrophoresis (DIGE) technology. Fluorescent-labelled 2D-protein-pattern was detected on a laser-scanner (FLA-5100, Fujifilm). Regulated proteins were analysed using Delta-2D-software (Decodon, Greifswald, Germany), selected spots were excised automatically (Proteineer-II, Bruker) and digested with trypsin. Mass-spectrometric identification was performed using MALDI-TOF. Verification of the results as well as analysis of phosphorylation levels was obtained by Western-blotting.
Results: Seventeen allergen-regulated epithelial proteins were identified including several heat-shock proteins. Differential distribution of phosphorylated isoformes of ap38-MAPK-pathway related protein was seen after stimulation with Ni. The differential distribution of its phosphorylated isoformes gives hints on a shift in function of this protein.
Conclusion: The proteomic identification and analysis of proteins, which are affected by the most common contact allergen Ni in primary human keratinocytes is an important step in increasing the understanding of molecular mechanisms involved in the development and the pathophysiology of human ACD. Furthermore, the differential regulation of p38-MAPK-pathway indicates its involvement in allergen-specific cellular signalling responses. (Work supported by EU-Project Novel Testing Strategies for *In-Vitro*-Assessment of Allergens, LSHB-CT-2005-018681, www.sens-it-iv.eu.).

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