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P001 (V04)

The role of cyclophosphamide-sensitive IL-10-producing CD4+CD25+ regulatory T cells in low zone tolerance to contact allergens

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The development and mechanisms of tolerance to allergens are poorly understood. Using the murine low zone tolerance (LZT) model, where contact hypersensitivity (CHS) is prevented by repeated topical low dose applications of contact allergens, we showed that suppressor CD8+ T cells of LZT inhibit the development of contact hypersensitivity (CHS). In this study, we analyzed the role and function of naturally occurring CD4+CD25+FOXP3+ regulatory T cells (nTregs) in LZT. FACS analysis of nTregs during LZT revealed a significant increase of CD4+CD25+FOXP3+ Tregs as compared to controls. Depletion of nTregs by the use of an anti-CD25 antibody (clone PC61) during the induction of LZT completely abolished the development of tolerance to allergens, demonstrated by the absence of suppression of a CHS reaction (significant ear swelling, hapten-specific T cell proliferation and Tc1 cytokinepattern). After pre-treatment of tolerized animals with low doses of cyclophosphamide (200 mg/kg), we observed a reduction in the number of nTregs and no development of suppressor CD8+ T cells resulting in an inhibition of LZT. As previously demonstrated, IL-10 is critical during the induction phase of LZT. ELISPOT analysis showed that CD4+CD25+ nTregs are the source of IL-10 in LZT. IL-10+CD4+CD25+ nTregs derived from WT mice, but not IL-10- nTregs allowed for the induction of LZTin adoptively transferred T cell-deficient mice, indicating that IL-10-producing nTregs are required during the induction phase of LZT. Our data show that CD4+CD25+ nTregs are regulators of immune responses to allergens by promoting the generation of allergen-specific suppressor CD8+ T cells and, consequently, the development of LZT.

P002

Attenuation of allergic contact dermatitis by the GABA B receptor agonist baclofen

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This study was designed to analyze the role of baclofen, a GABA B receptor agonist,in contact allergy. Proof of concept for a potential anti-inflammatory effect of baclofen*in vivo* was established using the ear swelling response in a mouse model with C57BL/6 mice for delayed-type hypersensitivity (DTH), using DNFB as a contactallergen. In this model, baclofen administered during the resensitization phase significantly reduced the ear swelling response in a dose-dependent manner ($P < 0.001$). Immunohistochemistry analysis revealed significantly lower infiltration of CD45+ cells within the ears of baclofen treated mice, indicating that leukocyte influxis reduced. This difference in leukocyte recruitment may be due to desensitization of chemokine receptors mediated by baclofen. Indeed, migration of leukocytes inresponse to several chemokines such as CTAK, MCP-1, Rantes, SDF 1 alpha,Fractalkine and IP-10 was significantly reduced in the presence of baclofen. We were able to rule out chemokine receptor internalization as a potential cause for reduced chemotaxis, as this did not occur in the presence of baclofen. Moreover, cytokinesecretion of activated PBMCs altered by baclofen, including a 40% reduction inTNF-a secretion. Finally, we treated two patients with known contact allergies. During the second week of baclofen treatment (20 mg/day), patch tests with their known contact allergens were repeated. This treatment did not cause adverse events. One patient with a previously week reaction to nickel (+) had a negative patch test response, whereas the other patient who previously showed numerous moderate to pronounced reactions (++ – +++) to several contact allergens still exhibited readily detectable positive patch test reactions at $t = 48$ h and $t = 72$ h. These results demonstrate protective role for a GABA B receptor agonist in contact allergy and points towards a possible therapeutic exploration of this approach.

P003

Proliferation of skin-homing lymphocytes induced by the α-nascent polypeptide-associated complex (NAC, Hom S2) in adult patients with atopic dermatitis

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In previous studies it has been shown that the serum of some patients suffering from atopic dermatitis (AD) contains IgE autoantibodies to antigens which may be present in inflamed skin. In order to investigate mechanisms of 'autoallergy' for AD we studied immunological responses to the auto allergen Hom S2, the humantranscriptional coactivator α-nascent polypeptide-associated complex (α-NAC) in detail. Sera of 165 patients with AD were screened for the presence of specific IgE antibodies to NAC. Specific proliferation of CFSE labelled blood lymphocytes from AD patients was investigated by flow cytometry. T cell clones (TCC) were generated from peripheral blood as well as from biopsies of lesional skin and the phenotype and cytokine patterns were determined. Furthermore, control experiments were performed in order to elucidate whether in the present study antigen presentation occurs dependently of monocytes. For this purpose proliferation of blood derived CD4+ T cells following stimulation with rNAC was assessed in the presence of either untreated or formaline fixed monocytes. Sensitization to rNAC was detected in >20% of adult AD patients. rNAC induced proliferation of circulating T cells was similar in patients and control individuals. rHom S2 induced a significantly higher proliferation of CCR4+ (compared to CCR4-) and of CLA+ (compared to CLA-) putative 'skin-homing' T cells from the circulation. rNAC-reactive TCC were preferentially CD8+ in the blood and CD4+ in the skin and produced high levels of IFN-γ. Limiting dilution assays revealed a high proliferation of skin-infiltrating lymphocytes in the presence of rNAC compared to control cultures. Marked proliferation of blood derived CD4+ T cells following stimulation with rNAC was observed in the presence of untreated but not of formaline fixed monocytes. NAC appears to be a potent auto antigen in adult patients with AD inducing both humoral and cellular responses which may be relevant for cutaneous inflammation in AD.

P004

Recombinant allergen-based micro array: detection of specific IgE to unexpected glove-derived allergens in a mouse model

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Most medical gloves are produced with a low content of natural rubber latex (NRL) protein. However, they may be substituted by proteins of foreign origin to maintain specific properties of the material. Aim of this study was to investigate the allergenicity and immunogenicity of unexpected proteins (i.e. soy and casein) compared to NRL proteins in a murine model in BALB/c mice. All respective allergen sources (extracts from three brands of NRL gloves, soy and casein) were able to induce significant allergen-specific IgE and IgG1-responses. In mean, highest IgE-induction occurred after immunization with NRL, succeeded by soy and casein. Certain individuals from each treatment group exhibited levels of specific IgE as high as due to NRL. To further analyse specific IgE-responses on a single allergen level we established a micro-array based on recombinant allergens for allergen-specific murine IgE detection. Besides specific IgE against rHev b 3, 6, 7, 8 and 11 (contained in the extracted NRL gloves), specific IgE against kappa casein (also contained in NRL gloves as unexpected protein) could be detected in mice immunized with NRL glove extract. These findings indicate a sensitization potential of contained foreign proteins which may lead to a shift and de novo increase in sensitization to the finished NRL products in man.

P005

Mast cells mediate pro-inflammatory effects of Vasointestinal Peptide (VIP)

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Vasoactive intestinal peptide (VIP) is a neuropeptide with a broad spectrum of biologic functions, including the modulation of innate and adaptive immunity. Recently, VIP has been shown to induce inflammation in murine and human lung. Because of this, VIP has been proposed to contribute to chronic inflammatory lung diseases including asthma and COPD. In contrast, little is known about the effects and functions of VIP on other organs including the skin. To assess skin responses to VIP we injected C57BL/6 mice intracutaneously with VIP and assessed its effects. Interestingly, we found that VIP induced strong and dose-dependent immediate hypersensitivity-like skin reactions in concentrations ranging from 100 nM to 100 μM as assessed by measuring skin thickness. Response maxima were detected 30 min after injection for all concentrations tested, and swelling responses and earthickness in all reactions returned to baseline levels at 6 h after injection. These findings suggested that VIP-induced inflammation involves the activation of skin mast cells. Therefore, we assessed the effects of VIP on mast cell-dependent skin inflammation by investigating passive cutaneous anaphylaxis in mice treated with the VIP receptor antagonist D-p-Cl-Phe6, Leu17 or vehicle. Notably, we found that mastcell-driven skin inflammatory responses to IgE and allergens are markedly down-regulated in VIP antagonist-treated as compared to vehicle-treated mice (−52.0%, $P < 0.05$). Taken together, our observations indicate that the pro-inflammatory effects of VIP in murine skin are, at least in part, mast cell-mediated, and that VIP contributes to type-I allergic skin inflammation by activating mast cells. These results characterise VIP as a potential target for novelanti-inflammatory strategies of treating allergic skin disorders.

P006

Proinflammatory slanDC (6-sulfoLacNAc+ dendritic cells) in atopic dermatitis

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Langerhans cells and inflammatory dendritic epidermal cells are suggested to play important roles in the pathogenesis of atopic dermatitis (AD). We previously described the population of slanDC which have a high T cell stimulatory capacity and which stand out by their high level production of IL-12 as well as TNFα. SlanDC were previously described in human blood as well as in the inflammatory infiltrate of psoriasis and rheumatoid arthritis. In this study we asked whether slanDC may contribute to the immunopathology of atopic dermatitis. With the help a slanDC-specific mAb biopsies obtained from patients with chronic AD lesions ($n = 15$) were studied. SlanDC were frequently found in the perivascular inflammatory infiltratein all tissues studied. The epidermis was always devoid of slanDC. When studying the phenotype of slanDC in blood ($n = 16$) of patients with AD we observed a significantly higher expression of CD86 (B7-1) compared to healthy controls. SlanDC cultured over night in the absence of any stimuli displayed a strong upregulation of HLA-DR, CD83 and CD86. Again, the expression of CD86 was significantly higher in slanDC from patients with AD. In accordance with their high proinflammatory capacity described previously by our group, LPS-stimulated slanDC of patients with AD displayed a much higher production of TNFz??as well as IL-12p40/70 compared to monocytes ($P = 0.007$ and 0.0001). Furthermore, slanDC of patients with AD when compared with that of healthy controls showed an unaltered capacity to produce high levels of IL-12, as studied on the single cells level. Taken together we demonstrate the presence of proinflammatory slanDC in lesional skin of atopic dermatitis and describe slanDC in blood expressing high levels of the costimulatory molecule CD86. Moreover, slanDC in blood of patients with AD can serve as high level producers of IL-12 as well as TNFα. We therefore conclude that slanDC may significantly contribute to the immunopathology seen in chronic AD.

P007

NiSO4 but not CoCl2 or CrCl3 induce Caspase-8 and -9 activation in lymphocytes and monocytes irrespective of the patch test reactivity of the blood donors

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Apoptosis is a normal phenomen to regulate the cellular homeostasis. There are two pathways for the apoptosis induction, the receptor mediated (activates Caspase-8) and the mitochondrial pathway (activates Caspase-9). We wondered, if nickel allergic and non allergic individuals would show different apoptosis of their PBMC upon stimulation with the respective antigen *in vitro*. Sixteen nickel allergic (patch test positive) and 20 non allergic individuals took part in the study. Lymphocytes and monocytes were isolated from the heparinized blood with magnetic beads after density centrifugation. One hundred four cells per well were incubated for 24 h with NiSO4, CoCl2 and CrCl3 (concentration 1×10^{-3} M–3.5×10^{-5} M). Active Caspase-8 and -9 was assessed by a luminometric assay. Nickel allergic and the non allergic individuals showed similar cellular response. Stimulation with nickel led to a strong activation of Caspase-8 and -9 in both, lymphocytes and monocytes of the nickel allergic and non allergic individuals (Maximum 20891 RLU). On the other hand, CoCl2 and CrCl3 did not induce apoptosis. We thus conclude, that nickel can induce apoptosis by both pathways – but without relevance for the nickel contact allergy. Further studies will elucidate potentially different metal-induced proinflammatory mechanisms.

P008

Cellular and humoral mechanisms of tolerance induction on specific immunotherapy (SIT) with birch pollen allergens

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The therapeutic benefit of SIT is well documented even though immunological alterations and pathophysiological cellular mechanisms affected by SIT and associated with the amelioration of allergic diseases are still subject to current investigations. In particular, the modulation of regulatory T cells (Treg) during SIT in correlation to successful allergy treatment is of major interest. In this study, we analysed the occurrence and frequencies of birch pollen allergen (Bet v 1)-specific Thelper (Th) 1, Th2 and type 1 Treg (Tr1) cells of fifteen birch pollen allergic patients as well as allergen-specific IgE and IgG4 antibodies in peripheral blood before SIT and 1, 3, 6, 12 months after initiation of SIT with birch pollen allergen. ELISPOT analysis of peripheral blood mononuclear cells of the birch pollen allergic patients showed a decrease of the initially Th2-dominated immune response to Bet v 1 in favour of a preferential Th1 response 1 month after beginning of SIT. Furthermore, the frequencies of Bet v 1-specific IL-10-secreting Tr1-like cells increased during SIT resulting in a decreased Th2/Tr1 ratio accompanied by an increase of Bet v 1-specific IgG4 whereas Bet v 1-specific IgE levels did not significantly change. These findings suggest that Bet v 1-specific Tr1 cells and Bet v 1-specific IgG4 may be directly implicated in the induction of tolerance against the major birch pollen allergen, Bet v 1. Our data thus support the current concept that induction of allergen-specific Treg may be a major event on SIT. Monitoring allergen-specific Tr1 cells in addition to IgG4 may represent useful prognostic markers on SIT in immediate type allergy.

P009

Lipoteichoic acid can attenuate mast-cell dependent allergicinflammation

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The activation of mast cells (MCs) is crucial for raising pathogen-related innate and adaptive immune responses and for inducing IgE-associated allergic inflammation. As of yet, very little is known about the combined effects of MC activators linked to both host defense [such as Toll-like receptor (TLR)-ligands] and allergy, i.e. IgE and allergen, even though both forms of stimuli often occur at the same time and skin site. Recently, the TLR2 ligand lipoteichoic acid (LTA) has been shown to lower human MC Fcepsilon RI expression and IgE-induced degranulation *in vitro*. Here, we speculated that LTA can also down-regulate MC activation and MC-dependent type I reaction *in vivo*, and we tested this hypothesis using a murine model of passive systemic anaphylaxis (PSA). To this end, we sensitized C57/BL6 mice intraperitoneally with DNP-specific IgE in the presence or absence of LTA and then monitored the anaphylactic response to DNP challenge by measuring rectal temperatures over a period of 90 min. Both experimental groups displayed a fast and virtually identical drop of core body temperature. In contrast, LTA-treated mice exhibited a very quick recovery (within 30 min) from the anaphylactic reaction as compared to the non-LTA treated mice, showing significantly lower body temperatures at the same time point after challenge. Interestingly, the application of LTA during the challenge phase did not result in a down-regulation of allergic responses. To identify the underlying mechanisms, we treated peritoneal MCs with DNP-specific IgE and with LTA followed by subsequent FACS analysis. Notably, LTA treatment significantly down-regulated MC-FcepsilonRI expression and binding of IgE (60%/30%; $P < 0.05$). LTA/IgE-complexes were not involved as the addition of LTA upon overnight incubation with DNP-specific IgE showed similar down-regulatory effects on IgE binding. Collectively, these data implicate that LTA functions as a negative regulator for MC-dependent type-I reaction through down-regulation of FcepsilonRI expression and IgE binding. Thus, LTA might be a promising and novel therapeutic tool to control MC-mediated type-I allergic responses.

P010

Establishment of a highly sensitive lymphocyte activation test (LAT) topara-phenylenediamine (PPD) to avoid severe skin test reactions in PPDallergic patients

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In patients sensitized to p-phenylenediamine (PPD), allergic reactions in response to black hair dye are increasingly eliciting severe skin reactions including massive cutaneous and subcutaneous edema and lymph node hyperplasia even necessitating hospitalization. In these patients, sensitization was mostly acquired by previous application of temporary black 'henna' tattoos. We examined nine patients with severe skin and lymph node reactions of the head and neck, temporary black tattoos in the history and recent use of permanent hair dyes. These patients responded vigorously to epicutaneous patch testing with PPD leading to bullous reactions with the risk of scar formation. Patch test titrations with 0.5%, 0.1% and 0.01% PPD revealed severe test reactions using 0.1% PPD but negative results with 0.01% PPD in most cases. We therefore intended to establish an *in vitro* test system to diagnose PPD allergy. To this end, PBMC from these patients as well as six patch test negative controls with or without history of using permanent black hair dye were activated with titrated concentrations of PPD with or without IL-2 supplementation. After 6 days, thymidine was added to the culture and T cell proliferation was determined 18 h later. In eight out of nine patients with severe allergic reactions to PPD, lymphocyte activation test (LAT) was positive with PPD-specific lymphocyte proliferation at least twofold above background. In only one patient, PPD-specific LAT was positive with IL-2 supplementation only, in all others, PPD specific LAT was reactive with and withoutIL-2. None of the control PBMC responded to PPD demonstrating lymphocyte proliferation not above unstimulated PBMC values. Identical results were obtained when the LAT with PPD was repeated several times over a 10-months-periodindicating a long-lasting presence of PPD specific memory T cells in peripheral blood of highly sensitized individuals. These data clearly show that LAT with PPD is a highly sensitive and specific *in vitro* test system to prove PPD sensitization in patients with severe reactions. With this sensitive *in vitro* test system we can avoid severe test reactions and booster effects following even titrated patch tests with PPD.

P011

Grass pollen extracts modify the mediator release from human primary keratinocytes

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Background: Keratinocytes play an integral part of the dermal immune system, e.g. by their ability to release mediators like cytokines and chemokines. Since keratinocytes are one of the first cells exposed to airborne allergen carriers such as pollen grains they are most likely involved in the outcome of the subsequent immune response. As recently shown, pollen grains function not only as allergen carriers but also contain oxidases and pollen associated lipid mediators (PALMs). Besides their capacity to activate granulocytes, PALMs also modulate dendritic cells function in a way that lead to aT-helper (Th2) responses *in vitro*.

Objective: The aim of this study was to investigate the effect of grass pollen extracts on human primary keratinocytes with respect to chemokine release in steady state or in an inflammatory *in vitro* skin model.

Methods: Primary human keratinocytes of non-atopic donors were stimulated with IFN-γ? and/or TNF-α?and subsequently incubated with aqueous grass pollen extracts (APE). Effects on chemokine and cytokine production were investigated on mRNA (Real-Time PCR) and protein level (ELISA).

Results: APE blocked the IFN-γ-induced production of Th1 chemokines CXCL10, CXCL11 and CCL5 as well as of the Th2 chemokines CCL17 and CCL22. A decreased release of these chemokines was also observed after co-stimulation with IFN-γ?and TNF-α?with APE. In contrast, the release of GM-CSF and IL-1β?was increased by APE in keratinocytes and in our inflammatory skin model.

Conclusion: Our data indicate that pollen derived factors provide a signal for human keratinocytes which leads to a modified mediator release. The induction of GM-CSF and IL-1β suggests that pollen itself harbour mediators which can induce a micro milieu promoting maturation and emigration of epidermal/dermal dendritic cells.

P012

Reduction of contact allergic ear swelling by topically applied cannabinoids: new treatment approaches for inflammatory skin diseases

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Cannabis preparations have been used in traditional medicine for the treatment ofinflammatory diseases. The major constituents of the plant Cannabis sativa are Δ9-THC, Cannabinol (CBN) and Cannabidiol (CBD). We recently demonstrated the anti-inflammatory potential of Δ9-THC in experimental contact hypersensitivity (CHS) in wild type mice. Because Δ9-THC can display psychoactive side-effects, we now examined the anti-inflammatory potential of topically applied CBN and CBD, which donot possess any psycho activity. CHS was induced in C57BL/6 mice by application of 0.2 % DNFB on the shaved abdomen and elicited by painting the ears with 0.3 % DNFB. Ear swelling was measured after 24 and 48 h. Δ9-THC, CBN and CBD or vehicle control were applied topically immediately before and 24 h after challenge. Contact allergic ear swelling was measured and histopathological analyses were performed on inflamed skin. To examine the effect of cannabinoids on lymphocyte activation *in vitro*, we measured the IL-2 and IFNγ??production of ConA-stimulated spleen cells in the absence or presence of Δ9-THC, CBN or CBD. Topical treatment with Δ9-THC, CBN or CBD significantly reduced contact allergic ear swelling even after repeated elicitation. CBD displayed the most effective anti-inflammatory action followed by Δ9-THC and CBN. Histological analysis demonstrated diminished numbers of infiltrating CD3+, Gr-1+- and MHC-II+-cells. When tested *in vitro* CBN and CBD significantly reduced IL-2 and IFNγ secretion of activated lymphocytes. Taken together, our results demonstrate that the non-psychoactive cannabinoids CBN and CBD have an anti-inflammatory effect in the sk*in vivo* which may be mediated in part by reduction of cytokine secretion and recruitment of inflammatory cells to the skin. Therefore, plant derived cannabinoids such as CBD, which was the most effective compound in our studies, may be useful for the treatment of inflammatory skin diseases.

P013

Differentiation between cross-reactive carbohydrate determinant- and peptide-specific IgE-binding: comparison of three automated detection systems

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Cross-reactive carbohydrate determinants (CCD) are probably the most widely occurring IgE epitopes, their clinical relevance, however, still being discussed. They seem to decrease specificity of *in vitro* allergy diagnostics. This study investigated whether the CCD-phenomenon is probably dependent on the detection method. Sera of 34 insect sting allergic patients with IgE to the venom of both, honey bee (HB) and yellow jacket (YJ), were investigated with CCD-allergens: bromelain (BRO),horse radish peroxidase (HRP) in CAP FEIA (CAP) (Phadia), allergens coupled to a solid ImmunoCAP matrix, Immulite (IML) E 2000 (DPC Biermann), based on liquid phase technology, and with HB and YJ extracts as well as the non-glycosylated recombinant allergens Api m 1 (HB) and Ves v 5 (YJ)(ALK-Abello) in the ADVIAC entaur Allergy Screen Assay (Bayer HealthCare Diagnostics Division), based on a reverse sandwich architecture with monoclonal mouse anti-human IgE covalently bound to paramagnetic particles in the solid phase and capturing the sample IgE. All sera had IgE to both insect venoms in CAP, whereas it was 88% in IML, 33% in ADVIA. In IML 3/4 HB-IgE-negatives were highly positive for YJ-IgE, 1/4 was only positive for HRP- and BRO-IgE, and another one was negative for YJ-IgE. In CAP, 24/34 (71%) had IgE to BRO, 27/34 (79.4%) to HRP. In IML, 20/34 (59%) had IgE to BRO, 25/34 (74%) to HRP. In ADVIA, 29/33 had IgE to YJ-, 14/31 to HB extract, 11 to both. 12/34 bound only to Api m 1, 26/34 to Ves v 5, 8/34 to both. IML-results not corresponding with CAP showed accordance with ADVIA in 4/4 HB-IgE negatives, 3/4 being positive for YJ-IgE in both systems. The serum only HRP- and BRO-IgE-positive in IML had YJ-IgE in ADVIA. CCD-IgE were detected in both, CAP and IML, HRP being the dominant CCD-allergen. As in CAP the CCD-IgE-detectionvia BRO and HRP was comparable, these epitopes seem to be equally presented on the different ImmunoCAPs. The differences between BRO and HRP in IML might be due to the CCD-presentation in these allergens in liquid phase. Increase in test specificity may be achieved by parallel testing of CCD- and relevant recombinant allergens.

P014

TRAIL-R2 expression and function in murine mast cells

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Tumour necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) belongs to the TNF family. Another member of the TNF family, CD95 ligand, has been shown to induce apoptosis in murine mast cells. To explore whether TRAIL is also capable of controlling survival of murine mast cells, we investigated the expression and function of mDR5 (TRAIL-R2, KILLER), the only murine TRAIL receptor with an active death domain, in the murine mast cell line C57 and in bone marrow-derived mast cells (BMMC). Using FACS analysis, both cell types were found to constitutively expressmDR5. The immature C57 cells showed more pronounced expression than BMMC. Comparing BMMC cultures incubated with IL-3 alone or IL-3 and stem cell factor (SCF), SCF was observed to reduce mDR5 expression, suggesting that maturation of murine mast cells is associated with downregulation of mDR5. In C57 cells, aggregation of mDR5 with TRAIL resulted in decreased viablity and increased apoptosis measured by FACS analysis of PI uptake and binding of annexin V. C57 cells were especially sensitive to apoptosis induced by human TRAIL, which incontrast to its murine counterpart fails to bind to the decoy receptor mDcR1 (TRAIL-R3). BMMC were resistant to TRAIL alone, but after the addition of the RNA synthesis inhibitor actinomycin D also exhibited apoptosis in response to mDR5 stimulation. Our results indicate that TRAIL participates in the regulation of mast cell apoptosis and may help to develop more specific tools for the treatment of mast cell disorders.

P015

The perforin-positive portion of CLA-positive cytotoxic T lymphocytes correlates with total serum IgE-levels in patients with extrinsic atopic dermatitis

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Allergens penetrating the skin elicit a strong IgE response in mice. Evidence for a similar pathophysiological pathway in humans is lacking, but might be found within the CLA+ lymphocytic population. Since we recently demonstrated a role for the perforin-granule system in the regulation of IgE, expression of perforin was investigated by flow cytometry in CLA+ lymphocytes and other lymphocytic subpopulations of 22 patients with exacerbated extrinisc atopic dermatitis (f:m = 14:9; 23 ± 8 years of age). Total serum IgE-level was determined using the PharmaciaCap-system (5868 ± 7200 kU/l). The SPSS software was employed for statistical analysis. The perforin+ portion within the CLA+ CD8+ and the CLA+ CD8hi+population (32 ± 18% and 32 ± 20%, respectively) was significantly higher as compared to perforin+ portion within the total of CD8+ cells and CD8hi+ CTLs (26 ± 0% and 19 ± 8%, respectively). Perforin expression was not found in CLA+ CD4+lymphocytes. A significant positive correlation existed between total serum IgE-levels and the perforin+ portion of CLA+ CD8hi+ CTLs. This was most significant in patients with IgE-levels higher than 1500 kU/l ($n = 12$, Pearson coeff. = 0.67, $P = 0.01$). In these patients, IgE-levels also correlated significantly with the perforin+ portion of all CLA+ cells and of CLA+ CD8+ cells, but not with other T- and NK-cell subpopulations investigated nor with SCORAD. The perforin+ portion in the total of CD8hi+lymphocytes decreased in patients with IgE-levels higher than 8000 kU/l. The raise of the perforin+ portion of CLA+ T lymphocytes in correlation with raising IgE-levels may be interpreted as an attempt of the perforin system, as a known IgE-downregulator, to counteract elevation of IgE. This may serve as evidence that the skin is a major site for the clash between IgE-inhibiting and IgE-driving forces in humans as well.

P016

Proteomic identification of allergen-protein interactions at the human epidermal immune barrier

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Over the past years occurrence of allergies to small molecular weight chemicals in the western civilisation has significantly increased. The most widespread contact sensitizer is nickel (Ni) causing T cell mediated type IV allergy. To better understand molecular mechanisms underlaying this type of allergy we identified allergen/Ni-interacting proteins in primary human keratinocytes, as the first sensitizing event in contact hypersensitivity (CHS).

To investigate Ni-interacting proteins in primary keratinocytes we used immobilized metal ion affinity chromatography (IMAC) on human cell lysates. After 2-dimensionalgel electrophoresis (2-DE), gel laser scanning (Fujifilm, FLA 5100) followed by spot picking (Bruker Daltonics, Proteineer II), trypsin digestion and mass spectrometric analysis (Bruker Daltonics, Reflex II) more than 15 Ni-interacting epithelial proteins were identified in isolated human keratinocytes. Comparative analysis from previous studies revealed differential as well as similar Ni-interacting molecules in human B-cells, *in-vitro* generated dendritic cells (DC) and primary human keratinocytes. Among others, involucrin (a marker for keratinocyte differentiation) and several heat shock proteins were detected, which may be involved in initial cellular stress responses (danger signals) towards heavy metal Ni.

Conclusion: Immunoproteomic identification of Ni-interacting proteins in primary keratinocytes is an important step in increasing the understanding of molecular mechanisms involved in the development and the pathophysiology of human nickel allergy. Results indicate a pivotal role for heat shock proteins and are discussed in light of Polly Matzinger's danger theory. (This work was supported in part by the European Union, as part of the project Novel Testing Strategies for *In vitro* Assessment of Allergens (Sens-it-iv), LSHB-CT-2005 -018681, www.sens-it-iv.eu).

P017

Regulation of specific IgE-production *in vitro* by CD8-positive T cells in patients with extrinisic atopic dermatitis

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CD8+ cytotoxic T lymphocytes are known to regulate IgE in mice and rats. Their function in the human IgE-system is less clear. We recently reported about a role of these cells in the regulation of total IgE-levels *in vitro* in patients with extrinsic atopic dermatitis (AD). These studies were extended, experiments were focused on specific IgE-production.

Total serum IgE-levels ($n = 12$) as well as specific IgE-levels ($n = 10$) of patients with exacerbated extrinsic AD were determined using the Pharmacia Cap-system. The peripheral CD8+ T cell compartment was characterized in parallel by flow cytometry. 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+ T cells by Milteny beads and, as an additional control, (iii) CD8-depleted PBMC reconstituted with CD8+ T cells. After depletion of >95% of CD8+ T cells, remaining PBMC produced more total as well as specific IgE as compared to controls. This was significant at day 8-10. Total IgE-levels correlated with the portion of perforin+ CD8+ T cells of the donor (e.g. day 9, Pearson coeff. = 0.7, $P = 0.04$). Specific IgE-levels correlated withCD28-expression on lymphocytes and with the percentage of peripheral CD4+ Tcells.

Our data suggest that depleting the CD8+ T cell compartment removes an IgE-regulating population. This leads to elevated specific and total IgE-levels. Since perforin is known to regulate immunoglobulin production in general, one may speculate that CD8+ cytotoxic T lymphocytes use their perforin-granule system to participate in IgE-regulation in humans.

P018 (V02)

The lymphatic marker podoplanin effects wound healing

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The 38 kDa mucin-type glycoprotein podoplanin promotes migration and adhesion of endothelial cells by reorganization of the cytoskeleton. Our previous studies showed that podoplanin deficiency results in congenital lymphedema. However, its biological function has remained mainly unknown. In the present study we investigated the effect of podoplanin on wound healing, in particular the blood- and lymphatic-vessel formation and the potency of podoplanin as a marker for lymphatic vessels ingranulation tissue. Full thickness wounds were applied to the back skin of wild type and podoplaninheterozygous (±) mice. The wounds were measured over a certain time course. Skin samples including the wound edges were used for immunohistochemistry and immunofluorescence stainings. Western blot analysis of isolated cutaneous fibroblasts and keratinocytes of wild type and podoplanin ± mice confirmed podoplanin expression *in vitro*. Subsequently adhesion assays of these primary isolated cells expressing different levels of podoplanin were performed.

Immunohistochemical stainings for podoplanin showed an increase of podoplanin expression in keratinocytes of the wound edges within 7 days of wound healing. Lymphatic vessels invading the granulation tissue revealed a reduced diameter inpodoplanin ± mice as compared to wild type mice. Surprisingly, circular full thickness wounds of podoplanin ± mice showed a significantly reduced wound size 24 h after wounding. Podoplanin expression levels did not alter the duration of the wound healing process. When we applied angeld full thickness wounds to reduce wound contraction we did not see any differences of the wound sizes of wild type orpodoplanin ± mice. Adhesion assays of primary isolated keratinocytes or fibroblasts of wild type or podoplanin ± mice demonstrated significantly reduced adhesion capacities of podoplanin± cells as compared to wild type cells.

In conclusion, we found podoplanin expression increased in the wound healing process influencing wound contraction *in vivo*. Further experiments will be performed to elucidate the impact of podoplanin on specific components of the process of wound contraction.

P019

Guanylate-binding protein-1: a novel marker in skin lesions of human lupuserythematosus

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The large GTPase human guanylate binding protein-1 (GBP-1) is a key mediator ofangiostatic effects of inflammation in vascular endothelial cells (EC). GBP-1 regulates the inhibition of EC proliferation and invasiveness induced by proinflammatory cytokines. It has further been shown to be highly associated with EC and monocytes/macrophages *in vivo* and has been detected in vascular skin tumours, e.g.Kaposi's sarcoma; however, it is not expressed in normal healthy skin. The aim of this study was to investigate whether GBP-1 may be a potential marker in skin lesions of cutaneous lupus erythematosus (CLE), an inflammatory autoimmune disease with primarily skin manifestations. Protein concentrations of GBP-1 were determined in primary keratinocytes from normal healthy donors (NHD) with and without stimulation by interferon (IFN)-α?and -γ?using western blot analysis. The expression of GBP-1 was also analyzed in 30 paraffin-embedded skin biopsyspecimens from patients with various subtypes of CLE using a specific ratmonoclonal antibody against GBP-1. In addition to genuine and UV-induced skin lesions as well as non-lesional skin from patients with CLE, skin biopsy specimens from NHD and patients with CLE were investigated by immuno histochemistry several hours after UV-light exposure. Western blot analysis revealed that GBP-1 was induced by IFN-α?and -γ?in primary keratinocytes from NHD. Moreover, we found that this protein was expressed by keratinocytes and vascular endothelial cells in genuine and UV-induced skin lesions from patients with different subtypes of CLE in contrast to non-lesional skin of the same patients. No expression was seen in skin biopsyspecimens 24 and 72 h after UV irradiation prior to lesion formation in patients with CLE and in skin biopsy specimens from NHD with and without UV irradiation. In conclusion, we demonstrate for the first time that GBP-1 expression is up-regulated by IFN-α?and -γ?in primary keratinocytes. Furthermore, the expression of GBP-1 seems to be highly associated with skin lesions of CLE suggesting that this protein plays a role in the pathogenesis of this disease.

P020

Distribution of mast cells in tissues of patients with different skintumours

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Mast cells are likely to play a role in tumour cell-immune system interactions. As elevated numbers of tryptase-positive mast cells have previously been reported for bone marrow infiltrates of different lymphomas, the present study was designed to assess mast cell numbers and distribution, vasculature and the influence of the nerve system in different skin tumours.

Skin biopsies of patients with cutaneous B-cell- (BL), pleomorphic T-cell lymphomas(PTL), mycosis fungoides (MF), dysplastic nevi (DN) and malignant melanoma (MM) were subjected to histochemical staining with toluidine blue and immunohistochemistry for mast cell tryptase and chymase, c-Kit, CD34, CD31 andvasoactive intestinal polypeptide receptor 2 (VIPR2/VPAC2) and compared to normalcontrol (C) tissues. In sections of all three lymphoma types, elevated numbers of mast cells were foundin the marginal zones of the tumour infiltrates when compared to control tissues (C: 53 ± 15/mm^2; BCL: 85 ± 48/mm^2; MF: 108 ± 22/mm^2; PTL: 135 ± 81/mm^2). In the BL (44 ± 23/mm^2) and PTL (48 ± 28/mm^2) infiltrates, decreased numbers of tryptase-positive cells were present whereas in MF infiltrates (85 ± 46/mm^2), the numbers were increased to control specimen. Analysis of staining pattern revealed an activated mast cell status in the infiltrates if compared to the marginal zone. In sections of DN (98 ± 26/mm^2) and MM (96 ± 39/mm^2) the number of cells stained with mast cell markers were elevated compared to normal skin (58 ± 22/mm^2), especially closed to the vasculature. In all investigated pathological tissues higher numbers ofCD31-positive blood vessels were detected. VPAC2 expression, mainly expressed atmast cells, is upregulated in tissues of patients with MF, DN and MM.

In summary, the present studies demonstrate an altered mast cell tissue distribution and staining pattern in different types of skin tumours. These changes may reflect a role of mast cells in tumour-immune system interactions.

P021

In vivo evidence for persistent activation of macrophages, oxidative damage and accelerated aging of ulcer-adjacent fibroblasts in chronic venous leg ulcers

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So far, *in vivo* evidence for oxidative stress-induced impaired wound healing is scarce. Here we analyzed the oxidative damage in inflammatory cells and its pathogenic impact on the potential acceleration of irreversible aging of dermal fibroblasts in chronic venous leg ulcers of six patients with non-healing ulcerspersisting for >6 month despite of consequent compression therapy. Immunostaining of skin biopsies from ulcer margins revealed an abundant and persistent inflammatory infiltrate mainly consisting of CD68+ macrophages. These were highly activated with substantial up-regulation of the classical activation markers] 2-integrins and iNOS as compared to moderate and transient induction in acute wounds. In chronic, but not in acute wounds, macrophages were identified to be the major source of ROS at toxic concentrations with strong expression of 8OHdG, indicating oxidative stress-induced DNA damage.

Protein oxidation assessed by the formation of carbonyl derivatives in oxyblotanalyses was significantly increased in the ulcer margin, but not in clinically unaffected skin from the same leg of the same patient. Interestingly, a 50 kDa protein was highly oxidized in all ulcers but not in unaffected skin suggesting it to be a preferred target for oxidation in non-healing ulcers. Importantly, resident fibroblasts highly expressed the DNA-damage associated? H2AX, a marker indicative of cellular growth arrest and senescence. In addition, immunostaining for p16, which was recently identified as a robust *in vivo* marker for irreversible skin aging, showed a substantially increased number of p16-positive fibroblasts in the dermis adjacent to chronic venous leg ulcers but not in unaffected skin from the same patients. These data suggest that the continuous activation of macrophages results in a persistent pro-oxidant milieu with subsequent acceleration of localized and irreversible senescence of fibroblasts eventually resulting in functional incapacity to promote wound healing. Hence, rigorous debridement or even shaving of the ulcer margins with irreversibly senescent fibroblasts may be the therapy of choice in recalcitrant leg ulcers.

P022

Transcriptional profiling identifies an interferon-associated host immune response in invasive squamous cell carcinoma of the skin

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Squamous cell carcinoma (SCC) and basal cell carcinoma (BCC) represent the two most common types of non-melanoma skin cancer. Both derive from epidermal keratinocytes but show a distinct biological behaviour.

Here we present transcriptional profiling data of a large cohort of tumour patients (SCC, $n = 42$; BCC, $n = 114$). Differentially expressed genes reflect the known morphological features of SCC and BCC including cytokeratin pattern, cell proliferation and tumour-matrix interaction. Importantly, SCC showed upregulation of interferon (IFN)-associated genes (Mx1, IRF1, IFI30, CXCL9, IDO) and of MHC-molecules which correlated closely with the expression of matrixmetalloproteinases (MMP3, MMP12). In situ hybridisation and immunohistological examinations confirmed the lesional expression of IFN-inducible genes (MxA,CXCL9) which was accompanied by a CXCR3+ cytotoxic inflammatory infiltrate. Upregulation of indoleamine 2, 3-dioxygenase (IDO) was observed at the edges ofinvasive SCC.

These analyses revealed a close correlation between a specific MMP-expression pattern, indicating invasive growth, and the induction of an IFN-associated host immune-response, suggesting a role for endogeneous induction of the IFN-system which may participate in tumour immuno surveillance and immuno editing. Our results support earlier mouse data, showing the importance of the IFN-system in tumourimmune-control. Of note, the IFN-signature was almost absent in organ transplant recipients under long term immunosuppression, what might be one explanation for the significant higher incidence of SCC in these patients.

P023

The collagen VII hypomorph develops pseudosyndactyly, a key symptom of recessive epidermolyis bullosa, as a result of aberrant contractile fibrosis-implications for therapy

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Dystrophic epidermolysis bullosa (DEB) is a severe skin fragility disorder caused bymutations in the gene encoding collagen VII, COL7A1. The key symptoms include blistering of skin and oral/esophageal mucosa, nail dystrophy and development of mutilating deformities of hands and feet. Analysis of disease pathogenesis is aprerequisite for development of biologically valid therapies. To this end, we generated a collagen VII hypomorph by intronic insertion of a PGK-Neo-cassette into Col7a1.Homozygous mice exhibit a phenotype closely resembling severe human recessive DEB: blistering of skin and oral mucosa, nail dystrophy, pseudosyndactyly and growth retardation. The phenotypic abnormalities were caused by an aberrant splicing of the collagen VII mRNA due to interference of the PGK-Neo cassette and, consequently, reduction of collagen VII levels in the skin to about 10% of the wildtype levels. This led to frequent dermal-epidermal separation below the laminadensa. In the extremities of homozygous animals, blister formation was followed by an aberrant wound healing with persistent inflammation and increased expression ofTGF-beta 1 and connective tissue growth factor, CTGF. This caused excessive fibroblast-to-myofibroblast differentiation and strong deposition of tenascin C in the dermal matrix. The fibrotic tissue changes and contractile forces established by themyofibroblasts led to development of pseudosyndactyly, loss of digits and, in somecases, complete synecchia of the paw. Bony structures remained intact, except forosteopenia, as shown by X-ray and MR imaging. The severe dermal fibrosis and abnormal microenvironment at sites of repeated mechanical injury may also underlie the development of epithelial skin cancer predominantly in those areas. Our observations explain the mechanisms and progression of the mitten deformities in human recessive DEB and disclose potential novel targets for pharmacological intervention. It is conceivable that anti-inflammatory agents or inhibitors of TGF-beta1 signalling may prove valuable in the prevention of the aberrant contractile fibrosis. Our hypomorphic mouse model is ideally suited for testing such treatment regimensfor DEB.

P024

Caspase-14 expression is increased in lesional skin of atopic dermatitis

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Caspase-14 is an aspartate specific proteinase, whose expression is restricted to the suprabasal epidermal layers. Activation of caspase-14 is associated with terminal differentiation. A significantly reduced expression of caspase-14 was reported forpsoriasis, which is characterized by a disturbed epidermal differentiation. Caspase-14-deficient mice showed scaly skin with reduced stratum corneum hydration and increased transepidermal water loss, a sign of disturbed skin barrier function. These effects in the deficient mice were related to a reduced proteolytic cleaving of profilaggrin to filaggrin. Filaggrin is important for keratin assembly during epidermal differentiation and skin barrier formation and filaggrin break down products are important for stratum corneum water binding. Recently, it was found that loss-of-function mutations in the filaggrin gene are present in about 20% of atopic dermatitis (AD) patients. It was subsequently proposed that these filaggrin mutations are responsible for reduced skin barrier function and dry skin. In turn, this implies that the cause of disturbed skin barrier function in the remaining 80% of AD patients remains to be determined. We showed previously that filaggrin protein expression is impaired in non-lesional and lesional skin of our patients with AD in general. Therefore, we asked whether the expression of caspase-14 is reduced in AD. We performed immunohistochemical staining of normal skin (healthy controls), psoriatic and AD lesional and non-lesional skin using a polyclonal anti-caspase-14 antibody. We found that expression of caspase-14 was significantly reduced in lesional, but not in non-lesional skin of psoriasis compared to normal skin, confirming previous observations. In contrast, AD lesional skin showed a significantly increased expression of caspase-14 with strong staining of the entire suprabasal epidermal layers. In non-lesional AD skin an unchanged or a slightly increased expression of caspase-14 was found. These surprising findings of increased caspase-14 expression in lesional skin were confirmed by Western blot analysis of protein extracts from the epidermis. Treatment of AD with betamethasone or pimecrolimus slightly reduced caspase-14 expression. The present results argue against a reduced expression of caspase-14 as a cause for the reduced filaggrin expression and the disturbed skin barrier function in AD.

P025

Does the human cytokeratin 8 represent a novel target protein to *S. aureusadhesion*?

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Staphylococcus aureus is a major human pathogen which causes a variety of infections and toxinoses ranking from superficial skin infections to deep seated infections. Furthermore *S. aureus* is also the major cause of hospital acquired infections of surgical wounds. For its pathogenicity *S. aureus* expresses a multiplicity of virulence factors. Prerequisite for colonization and the resulting infection is the adherence of *S. aureus* to host proteins. Adherence to human extracellular matrixproteins is mediated by adhesins, so called MSCRAMM (microbial surface components recognizing adhesive matrix molecules). An adhesion assay, using a Cytokeratin 8 (CK 8) protein, expressed recombinantly in *E. coli*, has shown a strong binding of *S. aureus* to CK 8. These binding affinity is comparable with the adherence of *S. aureus* to already known host target proteins, e.g. fibrinogen and aortic elastin. Control experiments exclude unspecific binding (e.g. stickiness, off target binding etc.). Moreover, we could not show any affinity of CK 8 to *E. coli*, which underlines the specificity for CK 8- *S. aureus* interaction.

CK 8 is a specific protein of the simple epithelia and is not expressed in the skin epidermis, thus the biological relevance for the pathogenesis of this interaction has to be addressed. It is known that CK 8 and its heterodimer Cytokeratin 18 (CK 18) are expressed in the secretory cells of the glandular globule of eccrine sweat glands (Langbein L, Rogers MA, Praetzel S, Cribier B, Peltre B, Gassler N, Schweizer J. J Invest Dermatol 2005; **125**: 428–444). So we presume, that this may be a target structure, which could play a role for the adherence and the ensuing infection.

P026

FGFR3 mutations in solar lentigo suggest a common genetic basis with seborrheic keratosis

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Solar lentigo (SL) is a benign skin lesion appearing on sun-exposed areas especially in elderly people and therefore represents a hallmark of (photo) aged skin. It has been proposed that SL may subsequently evolve into adenoid seborrheic keratosis (SK). However, little is known about the genetic basis of SL. In human SK, FGFR3 mutations have been recently identified. We therefore screened 30 SL for FGFR3 mutations using a SNaPshot multiplex assay. Because PUVA lentigines show theV600E B-RAF hotspot mutation, we additionally investigated this mutation in SL byallele-specific PCR. Five of 30 (17 %) SL displayed activating FGFR3 mutations. Four of these mutations have already been described in human SK. None of 28 SL available for B-RAF analysis revealed the V600E mutation. Our results suggest thatFGFR3 mutations are involved in the pathogenesis of SL. The occurrence of these mutations both in SL and SK support the previously proposed relationship between these lesions. Our findings furthermore substantiate previous speculations that UV exposure may be a causative factor for FGFR3 mutations in human skin.

P027

Evidence for founder effect of filaggrin mutations p.R501X, c.2282del4 and p.R2447X in ichthyosis vulgaris

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The molecular basis of ichthyosis vulgaris (IV), the most common hereditary disorder of cornification in humans, has recently been ascribed to loss-of-function mutations in the gene encoding the protein filaggrin (FLG), which is important for the formation of the stratum corneum during epidermal differentiation. Here we genotyped 38 IV patients originating from Austria, Germany, the Netherlands, Belgium and Scotland, who were homozygous, compound heterozygous or heterozygous for the most common European FLG mutations p.R501X, c.2282del4 and p.R2447X, as well as12 normal controls for eight different single nucleotide polymorphisms (SNPs). Lying within a 54 kb region, three SNPs mapped within exon 3 of FLG, while two were flanking the gene up to 16 kb in centromeric and three up to 15 kb in telomeric direction. We assessed significant differences between the genotypes of IV patients and controls with nearly complete association of the SNP alleles with the mutations carried on conserved haplotypes and therefore conclude that the high frequency of these FLG mutations is the result of a founder effect rather than mutational hot spots. Our results support the idea, that the high prevalence of FLG mutations in European populations could be the result of an advantage for heterozygous carriers, since increased trans epidermal antigen transfer due to skin barrier impairment may lead to better stimulation of the immune system, however this hypothesis requires further investigation.

P028

Identification of a recurrent mutation in variegate porphyria patients from Sweden and Chile: evidence for an independent novel founder effect

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Due to a founder effect, variegate porphyria (VP) (OMIM 176200) represents the most frequent type of acute porphyria in both South Africa and Chile. This neurocutaneous porphyria is inherited in an autosomal dominant manner within complete penetrance and associated with a partial deficiency of protoporphyrinogen oxidase (PPOX), the seventh enzyme in the pathway of hemebiosynthesis. Clinically, affected individuals can present both skin lesions on the sun-exposed areas of the body and life-threatening acute neurovisceral attacks, which may be precipitated by various porphyrinogenic factors. Although PPOX genealterations usually represent private mutations, we interestingly detected a novel-recurrent mutation, designated 1330delCT, in both Chilean and Swedish families with VP. This raised the question whether these patients and families from distinctgeographic areas are distantly related and share a common ancestral genetic background. Haplotype analyses using micro satellite markers flanking the PPOX gene on chromosome 1q22-23 revealed that 1330delCT represents a novel founder mutation in both countries although the mutation arose on different genetic backgrounds in the Chilean and Swedish population, respectively. Our data for the first time indicate that an identical single gene defect in a monogenetic variant of the porphyrias can arise on two distinct genetic backgrounds thereby giving rise to an independent founder effect in two populations affected with VP.

P029

The end of a paradigm: a homozygous mutation in the UROD gene gives rise to hepatoerythropoietic porphyria but is not associated with fecal isocoporporphyrin excretion or decreased enzymatic UROD activity

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Hepatoerythropoietic porphyria (HEP), the homozygous form of hereditary porphyriacutanea tarda (PCT), results from a profound deficiency of uroporphyrinogendecarboxylase (UROD). Usually, the disease already manifests in early infancy and is associated with severe photosensitivity, blistering, scarring, and mutilation. Biochemically, fecal excretion of isocoproporphyrin and a marked decrease of erythrocyte UROD activity are the hallmarks of HEP. Here, we report on two unrelated German patients with HEP who were initially diagnosed with congenital erythropoietic porphyria (CEP), a recessively inherited type of porphyria due to marked uroporphyrinogen III synthase (UROS) deficiency that is also characterized by severe photosensitivity and disease onset in the first months of life. After excluding UROS gene mutations, however, we surprisingly detected a recurrent homozygous missense mutation in the UROD gene, designated F55I, that has not been detected yet in either clinically overt PCT or HEP. Haplotype analysis indicated that F55I arose on a common ancestral background in both patients. The functional significance of the mutation in disease pathogenesis was demonstrated by prokaryotic expression studies and computational analysis of the mutant allele in a three-dimensional UROD model. Our results for the first time provide evidence that hereditary PCT and HEP are not always associated with fecal isocoproporphyrin excretion and reduced erythrocyte UROD activity, thereby eliminating one of the oldest paradigms in porphyria research.

P030

Increased hydrolytic activity in Netherton syndrome keratinocytes can bereduced by transfection with rAAV2-LEKTI vectors

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Background: Netherton Syndrome (NS, MIM 256500) is a severe autosomal-recessive disorder characterized by variable erythrodermis, hairshaft anomalies, and atopic diathesis. NS is caused by mutations in SPINK5 (serine protease inhibitor Kazal-type 5) onhuman chromosome 5q31-32, which encodes the 1064 aa serine-protease inhibitor LEKTI (lympho-epithelial-Kazal-type related inhibitor). Insufficient levels ornon-functional truncation of LEKTI seems to be the reason for the defectivepermeability barrier observed in NS. The aim of this study was to reconstitute the Spink5-gene defect in keratinocytes of Netherton syndrome patients by gene transfer of functional Spink5-cDNA.

Methods: A recombinant adeno-associated virus vector type 2 was constructed containing the 3.3 kb human cDNA encoding LEKTI under control of a CMV-promoter (rAAV2-LEKTI). Keratinocytes of four Netherton patients were isolated from biopsies and expanded on a feeder layer of 3T3 fibroblasts. rAAV2-LEKTI transfected Netherton cells were compared with Mock-transfected cells (rAAV2-GFP) and normal human keratinocytes (NHK) for differences in LEKTI expression by RT-PCR and FACS analysis and for changes in hydrolytic activity by a fluorometric proteaseassay.

Results: In homogenates of untransfected or rAAV2-GFP transfected NS keratinocytes(without functional LEKTI protein), the hydrolytic activity is significant higher than inrAAV2-LEKTI transfected NS keratinocytes and healthy keratinocytes (with functional LEKTI protein), respectively. This difference in hydrolytic activity is observable only after incubation of the keratinocytes in high-calcium medium for 3 days beforehomogenization. Thus calcium seems to be the important factor for LEKTI function .

Conclusion: In view of these results rAAV2-LEKTI gene transfer could be a promising future toolfor reconstituting the defect in NS.

P031

The R620W polymorphism in PTPN22 confers general susceptibility for the development of alopecia areata

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Alopecia areata (AA) is a common form of hair loss affecting approximately 1–2% of the general population. The progression of AA can be extremely variable as well for females as for males. A classification in three subtypes is established referring to the amount of hair loss. The etiopathogenesis of AA is not completely understood. However, AA is thought to be a tissue-specific autoimmune disease directed against the hair follicle, and may be associated with other autoimmune diseases. To date, it has been postulated that various genes related to immune response are associated with AA, but only the involvement of the major histocompatibility complex (HLA) has been confirmed through independent replication. Genetic association for a number of autoimmune diseases has been repeatedly reported for the R620W (c.1858C > T rs2476601) variant of the protein tyrosinephosphatase non-receptor type 22. A recent study including 196 English patients with AA has suggested that this variant of the PTPN22 gene also contributes to susceptibility to AA. We attempted to replicate this finding by genotyping a case-control sample of Belgian-German origin that includes 435 patients with AA and 628 healthy controls. Significant results were obtained for the overall collective of patients with AA ($P = 0.007$). Subdividing the sample according to severity of AA, family history and age atonset, we detected lowest P-values for patients with the severe form of AA (Pcorr = 0.036), with a positive family history (Pcorr = 0.042) and with an age at onset ≤?20 years (Pcorr = 0.048). Our results suggest the R620W variant of PTPN22 as a general risk factor in AA with the strongest effect observed among patients with a severe type of AA, a positive family history or an early onset of disease.

P032

EXO1, a hormone-depending and age-related gene, is expressed in human sebocytes

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Introduction: EXO1 (exonuclease 1) is a member of the RAD2 nuclease family and has 5′ to 3′ exonuclease as well as RNase H activity. It is involved in mismatch repair, DNA replication and recombination processes, which play a significant role in the generation of ageing. In a recent study, EXO1 has been shown to be differentially expressed in human SZ95 sebocytes treated with a hormone mixture consisting of growth factors and sex steroids at concentrations corresponding to those circulatingin 20- (f20) and 60- (f60) year old females.

Materials and methods: The expression of EXO1 was tested in SZ95 sebocytes maintained under hormone-substituted conditions at mRNA and protein levels via real-time PCR and western blotting, respectively. Furthermore, SZ95 sebocytes were transiently transfected with EXO1 siRNA in the presence of cationic lipids (3–4.5 μl) and synthetic ribooligonucleotides (nM area, both from QIAGEN). The confirmation of the gene regulation was also performed by means of real-time PCR and western blotting.

Results: EXO1 protein was found to be expressed in human SZ95 sebocytes and was significantly upregulated in the cells receiving the hormone treatment at f60 levels. In addition, the expression of EXO1 was successfully inhibited (−65%) via RNA interference at protein level.

Conclusion: The evaluation of age-associated genes via RNA interference could facilitate our understanding on the molecular mechanisms involved in ageing. Moreover, our data imply that EXO1 may be a hormone-dependent gene and may be implicated in the impairment of the DNA repair accompanying the ageing process.

P033 (V32)

Variants of genes coding for IL-12β?and for IL-23R are associated with psoriasis vulgaris and psoriatic arthritis in large German study groups

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Only recently, overlap of variants of the IL-23R pathway in genetic contribution to Crohn's disease and to skin manifestation of psoriasis vulgaris (PsV) was recognized. In an effort to identify concordance and deviation of genetic factors for PsV and psoriatic arthritis (PsA), we analyzed four genetic variants in IL12B and IL23R in a case-control study of 1.114 PsV patients, 748 PsA patients and 937 healthy controls before and after stratification for the major psoriasis risk allele (PSORS1).

For both, PsV and PsA, we observed the strongest association with 2 SNPs and the corresponding haplotype of IL12B: OR = 1.50 [1.28–1.75] (rs6887695) in PsA and OR = 1.50 [1.27–1.76] (rs3212227) in PsV, respectively, with P-values in the range of 10–7. Similar results were obtained at haplotype level. After stratification for the PSORS1 risk allele, the association to both IL12B SNPs and the risk haplotype was considerably stronger in PsA patients carrying the PSORS1 risk allele (OR = 1.70[1.34–2.16], P-value $<1.35*10^{-5}$, rs6887695) as compared to non-carriers. In contrast, differences in patients with PsV were less significant. After correction for multiple testing with performing permutation analyses, results remained significant.

Our results confirm recent reports that variants of the IL23R pathway as susceptibility factors for PsV and extend these findings also to PsA. Our data indicate that these variants, though, do confer susceptibility to psoriasis in general and are not specific risk factors for joint affection. Nevertheless, our findings indicate a stronger susceptibility for PsA, but not for PsV in patients carrying risk alleles at both PSORS1and IL23R related loci, despite the lower prevalence of the PSORS1 risk allele in PsA patients. This could indicate that both loci interact to confer susceptibility especially to PsA.

P034

Shprintzen-Goldberg syndrome associated with a novel missense mutation in TGFBR2

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Shprintzen-Goldberg syndrome (SGS) is a rare disorder characterized by a Marfan-like habitus, mental retardation and craniosynostosis. Cardiac abnormalities such as aortic root dilation have also been noted as well as several skeletal abnormalities. Its nosological status is unclear as it is hard to delineate SGS from similar disorders such as Furlong, Marfan type II, Camurati-Engelmann and Loeys-Dietz syndromes. It has been suggested that these conditions represent a phenotypical spectrum associated with aberrant TGF-ß signalling. In support of this notion, we found a novel TGFBR2 missense mutation in a patient with features of SGS.

P035

A case of Lelis syndrome

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Lelis syndrome is a very rare ectodermal dysplasia that was originally described in Lithuania and later in Brazil. Classically, core symptoms are described as acanthosisnigricans, dysmorphic facial features, anodontia and anhidrosis. Inheritance is uncertain. We describe a patient of Pakistani descent born from consanguineous parents. We show that the acanthosis nigricans is, in fact, an epidermolytic hyperkeratosis. Our patients' descent suggests that the disorder may be autosomal recessive.

P036

Granulomatous rosacea and Crohn's disease in a patient homozygous for the Crohn-associated NOD2/CARD15 polymorphism R702W

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NOD2/CARD15 belongs to the N-terminal caspase recruitment domain family of proteins that are involved in regulating NF-kB activation in response toinflammatory stimuli transduced through Toll-like receptors. Mutations in theNOD2/CARD15 gene are associated with Blau syndrome, Crohn's disease and early-onset sarcoidosis. Specifically, the polymorphism R702W (arginine totryptophan) is strongly associated susceptibility to Crohn's disease in Caucasian populations. Skin abnormalities (other than cutaneous manifestations of Crohn's disease) have not been previously associated with this particular mutation. We report on a female patient homozygous for R702W who developed granulomatous rosacea at the age of 12 years old.

We hypothesize that granulomatous rosacea may belong to the spectrum of cutaneous findings in Crohn's disease. Alternatively, it may be a disease that is specifically associated with NOD2/CARD15 polymorphisms or mutations.

It would be of considerable interest to examine the NOD2/CARD15 gene in patients with granulomatous rosacea.

P037

A novel missense mutation in the second extracellular domain of GJB2, S183F, causes a new syndrome of focal palmoplantar keratoderma with deafness

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Gap junctions are intercellular channels that mediate rapid intercellular communication. They consist of connexins, small transmembrane proteins that belong to a large family found throughout the animal kingdom. In the skin, several connexins are expressed and are involved in the regulation of epidermal growth and differentiation. One of the most important skin expressed gap junction genes is GJB2, which codes for connexin 26 and is associated with a wide variety of disorders. Most mutations cause a unique phenotype. Despite extensive research, this phenomenon is poorly understood. The identification of novel diseases caused by mutations in GJB2 may help to shed light on the genotype-phenotype correlation and help elucidate the function of different parts of the protein. Here, we report on a family with the first GJB2 mutation in the second extracellular domain (S183F) causing a syndrome of subtle focal palmoplantar keratoderma with sensorineural deafness. Using fluorescent fusion proteins, we show that the mutation induces apartial protein transport defect. S183F affects the second extracellular loop. Like the first one, it is important for connexon-connexon docking. Because most mutations in E1 also lead to focal palmoplantar keratoderma and likewise perurbs proteintransport, we hypothesize that this symptom may be specifically associated with trafficking defects.

P038

Severe early onset photosensitivity and Fanconi renotubular syndrome in a girl with homozygous hereditary coproporphyria and concomitanturo porphyrinogen decarboxylase deficiency

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Hereditary coproporphyria (HCP) is an acute cutaneous porphyria that results from anautosomal dominantly inherited partial deficiency of coproporphyrinogen oxidase (CPOX), the sixth enzyme in heme biosynthesis. A 3-year-old American girl showed severe photosensitivity, blistering, erosions, and miliae on the hands and face since the age of 12 months. Additionally, she suffered from an early-onset proximal renal tubular dysfunction, diagnosed as Fanconi renotubular syndrome. Biochemical analysis revealed highly increased urine and stool levels of coproporphyrin, with a markedly elevated fecal coproporphyrin III/coproporphyrin I ratio. Interestingly, additional enzymatic analyses also showed a decrease of uroporphyrinogende carboxylase activity in red blood cells by 72% of the normal value. Under the clinical and biochemical suspicion of homozygous HCP with possibly accompanying porphyria cutanea tarda we initiated molecular genetic studies in the index patient and her family. Automated sequencing analyses of the CPOX gene revealed compound heterozygosity for two missense mutations, designated R328C and G333D, respectively, indicative of homozygous HCP. The causality of these mutations in disease etiopathogenesis was confirmed by eukaryotic *in vitro* expression of the mutant alleles. Subsequent sequencing analyses of the coding regions of both the uroporphyrinogen decarboxylase and the protoporphyrinogenoxidase gene largely excluded further mutations and, thus, the possibility of either associated hereditary porphyria cutanea tarda or variegate porphyria. This is the first report on a patient with homozygous HCP associated with acquired uroporphyrinogen decarboxylase deficiency and severe renotubular dysfunction, resulting in a complex genetic syndrome that has not been described to date.

P039

Molecular genetic studies in diffuse and segmental variants of familial cutaneous leiomyomatosis

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Multiple cutaneous and uterine leiomyomatosis (MCUL; OMIM 150800) is anautosomal dominant disease characterized by leiomyomas of the skin and uterineleiomyomas. MCUL has been reported in association with different forms of renal cancer, referred to as hereditary leiomyomatosis and renal cell cancer (HLRCC; OMIM 605839). Both disorders result from heterozygous germline mutations in the fumarate hydratase (FH) gene that acts as a tumour suppressor. Interestingly, variations in the clinical presentation of cutaneous leiomyomas have been observed. Besides a diffuse and symmetric distribution these tumours might also manifest a rather segmental and band-like pattern manifestation pattern that might be due tomosaicism. In nine unrelated families with diffuse and segmental skin phenotypes of MCUL we performed DNA mutation analysis using a PCR based approach. We identified nine mutations in the FH gene, eight of which were novel ones. These eight sequence deviations consisted of two frame shift, two splice site, one nonsense and three mis sensemutations, which were absent in ethnically matched control individuals. Our mutation analysis strategy led to the identification of disease causing mutations in all patients with cutaneous leiomyomas, supporting the notion that this disease is genetically heterogeneous The simultaneous occurrence of both diffuse and segmentally distributed tumours in patients from the same family most likely reflects a type 2 segmental manifestation. This form of cutaneous mosaicism might be explained bythe combination of a heterozygous germline mutation with postzygotic loss of heterozygosity (LOH).

P040

Association between HLA class I phenotypes and clinical manifestations inAdamantiades-Behçet disease patients in Germany

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Diagnosis of Adamantiades-Behçet disease (ABD) is based on a defined set of clinical criteria since no single pathognomonic sign, biological or genetic test exists. Aetiology and pathogenesis still remain obscure. Hypotheses discuss an infectiousorigin, autoimmune/autoinflammatory mechanisms and a genetic predisposition. Aconsistent link is the one with the MHC (HLA), specifically with the HLA-B51 allelle. HLA-B51 is associated with ABD worldwide and present in 48.3% of ABD patients in Germany. Aim of this study was to investigate frequencies of HLA class I haplotypes, their associations with clinical signs and to determine possible prognostic HLA markers. The study population included 267 ABD patients of Turkish (T), 227 of German (G)and 96 ABD pat. from 29 other countries of origin who are permanent residents in Germany. G ($n = 1426$) and T ($n = 268$) healthy blood donors served as controls.Typing of HLA A, B and C was executed by a lymphocytotoxic test. Chi-square test, correction after Yates and Fisher exact test were used to carry out statistical analyses. Most frequent HLA haplotypes in G and T ABD pat. compared to controls were A2 with 66.5% ($P < 0.01$) and B51 with 58.8 % ($P < 0.00001$). Certain HLA antigens (A9, A32, B7, B14, B35, Cw6) were more frequently found in T ABD pat. than in T controls($0.00001 > P < 0.0035$). Among ABD pat., B51 was associated with ocular lesions ($P < 0.001$), incl. hypopyon, retinitis and loss of vision, folliculitis ($P = 0.002$) and erythema nodosum ($P = 0.010$), Bw4 with eye, lung, CNS involvement, arthropathy and a positive pathergy reaction ($0.002 > P < 0.047$). More than 30 HLA haplotypes were either positively (A2, B5, Bw4, Bw6) or negatively (A1, A3, A9, A10 etc) correlating with certain manifestations ($0.001 > P < 0.05$). ABD patients develop clinical signs associated with HLA phenotypes, ethnic origin and gender, however, a pathogenetic or prognostic dependence is still unproven. B51 has been firmed as a prognostic indicator for a severe eye involvement. HLA allelles negatively correlating with complications may even disclose beneficial properties.

P041 (V21)

Structural and functional consequences of CYLD mutations giving rise to familial skin appendage tumours

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Mutations in the CYLD gene on chromosome 16q12-13 are associated with autosomal dominant tumour disorders of skin appendages, including Brooke-Spiegler syndrome, familial cylindromatosis, and multiple familial trichoepithelioma (MFT). These diseases are characterized by the development of distinct skin neoplasms, including cylindromas, trichoepitheliomas, and spiradenomas. To date, however, the structural and functional consequences of CYLD splice site mutations have not been assessed and genotype-phenotype correlations have not been reported yet. We performed molecular studies in five families with cutaneous appendage tumours and detected five novel CYLD sequence deviations consisting of one missense, one nonsense, and three splice-site mutations localized in exons 15, 16, and 17, respectively. Functional analyses revealed that all mutations exert their pathogenic effects through variable degrees of mRNA degradation. Interestingly, however, complete mRNA decay wasnot observed although this could have been expected as a consequence of the nonsense and splice-site mutations. Pedigree and haplo type analyses confirmed that in one of the affected individuals the splice site mutation occurred de novo. Based on RT-PCR experiments we show that CYLD is expressed in peripheral blood mononuclear cells and demonstrate the consequences of the three splicing defects, which all lead to exon skipping events, on the cDNA level. In a patient who clinically and histopathologically only showed trichoepitheliomas we detected a T-to-Gtransversion, resulting in a missense mutation and causing MFT. Only three of the 32 distinct CYLD mutations reported so far are missense mutations and in all instances these were exclusively associated with the manifestation of trichoepitheliomas. Thus, we hypothesize that missense mutations in the CYLD gene, as in our case, might be associated with a milder disease phenotype consisting of trichoepithelioma only. For the first time, we show the structural and functional consequences of splicing defect sin CYLD, expression of CYLD in peripheral blood cells, and suggest agenotype-phenotype correlation between CYLD missense mutations and MFT.

P042

Compound heterozygous mutations in the coproporphyrinogen oxidase andprotoporphyrinogen oxidase gene in a novel type of dual porphyria

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The heme biosynthesis diseases variegate porphyria (VP) (OMIM 176200) andhereditary coproporphyria (HCP) (OMIM 121300) belong to the group of neurocutaneous porphyrias and result from a partial enzymatic deficiency of protoporphyrinogen oxidase (PPOX) and coproporphyrinogen oxidase (CPOX), respectively. Clinically, affected individuals can present both skin lesions on the sun-exposed areas of the body and life-threatening acute neurovisceral attacks that can be precipitated by various porphyrinogenic factors. The simultaneous manifestation of two different forms of porphyria in the same patient, a phenomenon referred to as dual porphyria, is extremely rare and a combined PPOX and CPOX deficiency has not been reported to date. A patient with blistering, erosions, and photosensitivity revealed a biochemical profile indicative of both VP and HCP. We performed DNA mutation analysis and detected two novel mutations in the PPOX and CPOX gene, respectively. For the first time we provide molecular evidence that compound heterozygosity for two types of acute porphyrias can give rise to a uniquevariant of dual porphyria that has not been described yet.

P043

Novel and recurrent mutations in K1 in palmoplantar keratoderma with tonotubules

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Palmoplantar keratodermas (PPK) are a large group of disorders characterized byhyperkeratosis on palms and soles. PKK of Vörner is the most frequently inheritedPPK. It is transmitted in an autosomal dominant mode and starts within the first yearof life. It is characterized by a diffuse, yellowish or erythematous hyperkeratosis, sometimes covering bullous lesions, that is sharply demarcated with an erythematous margin. The majority of cases of PKK of Vörner are caused by mutations in keratin 9 which is specifically expressed in the suprabasal layers of the epidermis in palmoplantar skin. However, keratin one mutations have been identified in a small number of cases. A peculiar subset which is clinically identical to PKK of Vörner but shows characteristic ultrastructural findings of 'tonotubular' keratin has been found tobe caused by mutations in the 1B domain of keratin 1. These tubular structures have not been described in other keratin disorders and imply a distinct role of the 1B domain in filament formation. We report three unrelated families with PPK with 'tonotubular' keratin and have identified novel and recurrent mutations in the 1Bdomain of K1 suggesting a genetic hot spot in this geno dermatosis.

P044 (V12)

Cell therapy restores skin function in an immunocompetent mouse model for dystrophic epidermolysis bullosa

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Recessive dystrophic epidermolysis bullosa (RDEB) is a skin fragility disorder caused by mutations in the collagen VII gene, COL7A1. So far no therapy is available, and in the most severe forms scarring and mitten deformities of the extremities have an important impact on quality of life of the affected individuals. We recently generated a viable transgenic mouse model, the Col7a1flNeo/flNeo collagen VII hypomorph, which displays the key symptoms of severe RDEB, skin blistering, growth retardation, soft tissue scarring, and pseudosyndactyly. As there are continuing concerns about the safety and feasibility of gene therapy approaches for hereditary diseases, we investigated the efficacy of fibroblast therapy in our model. Five week old hypomorphic mice were injected intradermally, into a defined dorsal skin area (2×3 cm), with wild type or Col7a1flNeo/flNeo fibroblasts ($10–20 \times 106$ cells, non-gene corrected, in 0.9% NaCl). Prior to dissection, treated and untreated skin areas were exposed to a functional test employing mechanical rubber-stress. The animals weresacrificed 1, 3, 5, 7, 14 and 21 days post-injection, and skin specimens from treated and untreated areas were subjected to histopathological and indirect immunofluorescence analysis. Seven days post injection, the deposition of collagenVII at the DEJZ was clearly increased, and this persisted for at least 21 days (maximum observation period). Deposition of collagen VII was only slightly increased after Col7a1flNeo/flNeo fibroblast injection. Occasionally, a transient inflammatory cell infiltrate was found in the skin. Intriguingly, microblisters, regularly present in untreated skin of the hypomorph, were absent in treated skin, even after strong mechanical stress. These observations clearly demonstrate that the supplemented collagen VII is able to stabilize the skin. This study, the first therapy approach in animmune competent small animal model, delivers evidence that treatment of this severe genodermatosis with allogeneic fibroblasts may be of clinical use in preventing the detrimental sequence of dermal-epidermal separation, scarring, fibrosis, mutilating deformities and finally skin cancer.

P045

Thy-1 induces secretion of matrix metalloproteinase-9 and CXCL8 from neutrophils

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Neutrophils are the first cells at sites of inflammation. On their way from blood to the site of infection neutrophils have to adhere to endothelial cells (EC), to transverse the basement membrane and subsequently to travel through the interstitial matrix. Recently, we have shown that Thy-1 is an adhesion molecule on activated dermal EC and fibroblasts mediating the binding of neutrophils via Mac-1. Thus, human Thy-1 is an alternate EC receptor for the leukocyte integrin Mac-1 that contributes toleukocyte recruitment to sites of inflammation thus providing a new pathway for adhesion and transmigration of neutrophils. Here, we studied whether Thy-1 mediated adhesion of neutrophils mediates only the physical contact or further influences neutrophil functions. Since MMP-9 plays an important role for the migration of neutrophils through the basement membrane we analyzed secreted MMP-9 after interaction of neutrophils with Thy-1. Indeed, binding of neutrophils to recombinant Thy-1 stimulated secretion and activation of MMP-9 from neutrophils resulting in an enhanced migration through a collagen-IV barrier. Accordingly, blocking Thy-1 on activated dermal EC or fibroblasts decreased theMMP-9 secretion from neutrophils in co-cultures. Next, we investigate whether the interaction of neutrophils with Thy-1 regulates the secretion of CXCL8 and thus might support the attraction of additional neutrophils atsites of inflammation. Binding of neutrophils to Thy-1 induced the release of CXCL8.Blocking of Thy-1 on activated dermal EC or fibroblasts in co-cultures with neutrophils decreased the CXCL8 secretion confirming the role of Thy-1 in regulation of CXCL8 release. In summary, Thy-1 mediates not only the adhesion of neutrophils to activated dermal EC and fibroblasts but also regulates neutrophil function. These results support the general concept that the function of 'adhesion molecules' may not only be to provide mechanical support but might also be to regulate functions such as motility or release of chemotactic factors.

P046

CD8+CD11c+ dendritic cells are required for tolerance to contact allergens

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The induction of tolerance may represent a strategy aiming at preventing harmful immune responses. Low zone tolerance (LZT), induced by epicutaneous application of low doses of contact allergens, requires the generation of CD8+ suppressor T cells that inhibit the development of Tc1-mediated contact hypersensitivity (CHS). In this study, we analyzed the role of dendritic cells (DC) for tolerance induction to allergens. CD11c-DTR (diphteria toxin receptor) mice were used in which systemic administration of diphteria toxin leads to a transient and rapid decline of CD11c+ DC in lymph nodes (LN) and spleen. Notably, depletion of CD11c+ DC by injection of diphteria toxin during the induction of LZT abolished the characteristic features of LZT, including reduced ear swelling, generation of CD8+ suppressor T cells of LZT and skewing to a Tc2 cytokine pattern. As compared to PBS-treated CD11c-DTR animals, an unrestricted CHS reaction was observed in these mice resulting in a marked ear swelling mediated by the development of Tc1-effector T cells of CHS. To evaluate the role of CD8+CD11c+ DC, we performed adoptive transfer experiments of CD8+CD11c+ LN-derived DC obtained from tolerized WT and CD11c+ DC from tolerized ICSBP−/− mice (interferon consensus sequence-binding protein) lacking relevant numbers of functional CD8+CD11c+ DC. Transfer of CD8+CD11c+ DC induced a tolerance reaction in mice shown by an inhibited inflammatory skin reaction and generation of regulatory T cells, whereas recipients of DC generated from ISCBP−/− mice developed a CHS reaction, indicating that CD8+CD11c+ DC are involved in the development of LZT. In addition, reconstitution of transgenic DC-depleted CD11c-DTR mice with CD8+CD11c+ DC before tolerance induction restored the tolerance development. Our findings identify CD8+CD11c+ DC as key APC for LZT to allergens that are necessary and sufficient for priming of regulatory T cells during induction of LZT and validate DC as a novel target for directed therapy of allergic diseases.

P047

Epigenetic control of the expression of cathelicidin and CD14 by 1,25-dihydroxy vitamin D3 in keratinocytes

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Hormonally active vitamin D3–1,25-dihydroxyvitamin D3 (1,25D3) – acts as a signalling molecule in cutaneous immunity by increasing pattern recognition through Toll-like receptor-2 (TLR2), and increasing the expression and function of antimicrobial peptides. Here we show that the actions of 1,25D3 on keratinocyteinnate immune responses are influenced by histone acetylation and require the steroid receptor coactivator 3 (SRC3), which mediates inherent histone acetyl transferase (HAT) activity. SRC3 was detected in the suprabasal and granularlayer of the skin, similar to cathelicidin expression. HAT activity was important tokeratinocyte cathelicidin expression as the combination of histone deacetylaseinhibitors (HDACi) (butyrate or trichostatin A) and 1,25D3 increased cathelicidin andCD14 expression and enhanced the antimicrobial function of keratinocytes against *Staphylococcus aureus*. This treatment, or activation of TLR2, also directly increasedacetylation of histone 4. Small interfering RNA silencing of the vitamin D receptor orSRC3 blocked the induction of cathelicidin and CD14 by 1,25D3. HDACi could not reverse this effect or influence cathelicidin in the absence of 1,25D3, suggesting that both are necessary for function. These studies demonstrate that the epigenetic control of gene transcription by histone acetylation is important for 1,25D3-regulated antimicrobial and TLR function of keratinocytes, essential elements of the innate immune response of the skin.

P048 (V05)

CD137 ligand induces dendritic cell maturation and migration via TNF-α?and CCR7

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T cell activation via dendritic cells is an important step in the adaptive immune response, which requires dendritic cell maturation, migration to lymph nodes and presentation of antigen to T cells. CD137 receptor expressed on activated T cells is apotent costimulatory molecule. CD137 ligand is expressed by humanmonocyte-derived dendritic cells. Here we show that CD137 ligand activation ofdendritic cells leads to their maturation in an autocrine fashion, mostly via the releaseof TNF-α?as shown by the use of neutralizing anti-TNF-α?antibodies that clearly inhibited the expression of cell maturation markers such as CD83. During maturation, chemokine receptors such as CXCR4 and CCR7 are upregulated in DCs. We were able to show that activation of CD137 ligand leads to upregulation of the chemokinereceptor CCR7. Moreover, CD137 ligand activation mediates migration of dendritic cells via upregulation of the CCR7 chemokine receptor, as we could demonstrate *in vivo* by an MIP-3β-dependent SCID mouse migration model. These findings present direct evidence for the role of CD37 ligand in DC chemotaxis, and deliver new insights into the multiple effects of CD137 ligand activation in human dendritic cells during the initiation of an adaptive immune response.

P049

UV-induced regulatory T cells stimulate antigen presenting cells to further induce regulatory T cells

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UV-induced regulatory T cells (UV-Treg) inhibit sensitization in an antigen-specific fashion. There seems to be a tight cross-talk between UV-Treg and antigen-presenting cells (APC) since the migratory behaviour of UV-Treg can be reprogrammed by tissue-specific APC in an antigen-specific manner. In turn, UV-Treg inhibit APC to present antigens. Subcutaenous injection of antigen-loaded APC, which have been coincubated with UV-Treg activated in an antigen-specific fashion, did not result in sensitization of the recipient mice. This inhibition appears to be antigen-independent since APC coupled with TNBS, the water soluble analogue of trinitrochlorobencene (TNCB), were not able to sensitize *in vivo* against TNCBupon coincubation with activated dinitrofluorobencene (DNFB)-specific UV-Treg. Upon antigen-specific activation, UV-Treg released high amounts of interleukin (IL)-10 which might be responsible for the inhibition of APC. Accordingly, addition of a neutralizing anti-IL-10 antibody prevented the inhibition of APC by UV-Treg. To determine whether APC pretreated with UV-Treg in turn are able to induce Treg, APC were coincubated with activated DNFB-specific UV-Treg. APC were isolated 48 h later, coupled with TNBS and injected into naïve recipients. Five days after injection splenocytes were obtained from the recipients and injected into naïve mice which were sensitized against TNCB 24 h later. Five days later ear challenge with TNCB was performed. Mice which had received T cells from donors treated with Treg-exposed APC were significantly suppressed in their contact hypersensitivity response against TNCB. This indicates that activated UV-Treg can alter APC in such a way that they further induce Treg when injected into naïve recipients.

P050 (V33)

Interaction between dendritic cells and CD4+CD25+ T cells is required to suppress the sensitization phase of CHS

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Contact hypersensitivity (CHS) is induced by sensitization of individuals againsthaptens. This sensitization phase, in contrast to the elicitation phase, occurs in thedraining lymph nodes (dLN) where the antigen-specific priming of CD8+ T cells by dendritic cells (DCs) takes place. Our initial studies showed that CHS reactions can be significantly reduced by injection of CD4+CD25+ regulatory T cells (Treg) before sensitization. Thus we aimed to investigate the means by which the injection of Tregs leads to the reduction of the immune reaction in a murine CHS model. To prove that Treg, which are not able to migrate into LN, fail to suppress sensitization against haptens, we separated the Treg population in CD62L+ (LN homing) and CD62L- ('non-LN-homing') cells before injection and subsequent sensitization. After challenging the mice at the ears, we could demonstrate that administration of 'non-LN-homing' CD4+CD25+CD62L- T cells did not affect the ear swelling reaction, although both subpopulations (i.e. CD62L+ and CD62L- Treg) showed the same suppressive capacity *in vitro*. Therefore we further analyzed the influence of CD4+CD25+CD62L+ T cells on the most important cells involved in the sensitization phase, namely DCs and CD8+ T cells. In *in vitro* co-cultures of Treg and bone marrow derived DCs (BMDC) we could detect a close contact between these two cell types causing a reduction of the antigen-presenting capacity of the BMDCs. Likewise *in vivo*, injected Treg migrated to the LN and showed a high affinity interaction with the DCs after sensitization, which was demonstrated by fluorescence microscopy of cryosections of the dLN stained with fluorescence markers for Treg and DCs or CD8+ T cells, respectively. Functional changes of the DCs after contact with Treg *in vivo* became apparent when DCs were isolated from Treg injected mice and cultured together with CD8+ T cells *in vitro*. Here, proliferation of CD8+ T cells was significantly lower when *in vivo* Treg-exposed DCs were used as stimulators as compared to control DCs. On the whole, our data indicate that the T cell stimulatory capacity of hapten-presenting DCs is suppressed by Treg and thus provides a means by whichCD25+ regulatory T cells suppress the priming of CD8+ effector cells during the sensitization phase of CHS.

P051

Antimicrobial defence of human hair follicle epithelium: inducible expression of RNase 7 and psoriasin and constitutive expression of hornerin

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Human skin is under permanent stress of microbial invasion. However, it manages to combat and contain infection by maintaining a physical barrier including desquamation and mucous secretion and a chemical barrier by producing antimicrobial peptides (AMPs). As the hair follicle ostium represents a potential port of microbial entry into the skin, but only very rarely shows clinical signs of infection, this especially here suggests the presence of effective AMPs. To test this hypothesis we stained cryosections from normal human scalp skin and cultured biopsies treated with and without lipopolysaccharides (LPS) for RNase 7, psoriasin and hornerin immunoreactivity (IR), three of the lately discovered AMPs in the skin. Indeed, using different immunohistochemical methods like ABC and tyramide signalamplification (TSA) all tested AMPs could be detected in the hair follicle epithelium. RNase 7 IR is found throughout the entire outer root sheath (ORS), with a gradual decrease in intensity processing from distal to proximal, and with a lower IR than inthe epidermis. The basal layers of the ORS show often stronger staining than thesuprabasal and differentiated ones. This is consistent with the RNase 7 IR in the epidermis. Psoriasin is only observed in the uppermost layers of the perifollicular epidermis, in the ORS mostly distal of the orifice of the sebaceous duct and weakly in the hairshaft keratin. Hornerin IR is detected in the entire ORS. It is very strong in the distal ORS including all layers except the basal one, whereas in the more proximal parts of ORS it is much weaker and homogeniously distributed. Stimulation of normal human scalp skin fragments in serum-free organ culture with lipopolysaccharides from different gram negative microbes significantly up-regulates RNase 7 and psoriasin IR in the distal part of the ORS, whereas hornerin remains at about the same level. There is no difference between the LPS of distinct species (validation by quantitative immuno histomorphometry). To summarize, this pilot study confirms that the hair follicle epithelium has an antimicrobial defence system... [abstract was shortened, because it exceeded space limitations]..

P052

Characterization of regulatory T cells in skin lesions and peripheral blood of patients with scleroderma

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A lack or suppressive defect of CD4+CD25+ regulatory T cells (Treg) has been shown to be involved in a variety of human autoimmune diseases. Recently, our group demonstrated that the number of Foxp3+ Treg in skin lesions of lupuserythematosus was significantly reduced compared with other chronic inflammatory skin diseases. Furthermore, a significant reduction in the number of peripheral CD4+CD25+ Treg was found in patients with an active flare of systemic organ manifestation of the disease. In this project, we were interested to analyze the frequency and phenotype of Treg in scleroderma, a heterogeneous autoimmune disease with a large variability in the extent of cutaneous and organ involvement. The number of Treg in skin biopsy specimens from patients with systemic scleroderma (systemic sclerosis) and localized scleroderma (morphea) was investigated usinganti-Foxp3 and anti-CD4 monoclonal antibodies for immunohistochemistry. In addition, characterization of peripheral blood CD4+CD25+ Treg from patients with the different forms of the disease was carried out by flow cytometry analyzing the expression of Foxp3 and Treg subpopulations. The quantitative analysis of CD4+ Tcells in skin lesions of patients with scleroderma revealed a significantly lower number when compared with other chronic inflammatory skin diseases, such a slupus erythematosus, psoriasis, and atopic eczema. Moreover, the frequency of Tregin the skin of scleroderma expressed as percentages of Foxp3+ cells from CD4+cells in the inflammatory infiltrate was similar to lupus erythematosus but significantly reduced compared with other control diseases, irrespective of the type of scleroderma. In peripheral blood, our preliminary data show a higher number of total Foxp3+ Treg in patients with scleroderma compared with healthy controls while the number of CD4+CD25 high Foxp3+ Treg was normal suggesting a higher number of activated T cells in this disease. In conclusion, our results demonstrate a significant reduction of Treg in the skin of patients with different forms of scleroderma. Further investigations will evaluate if there is also a global peripheral dysfunction of CD4+CD25+ Treg in this autoimmune disease.

P053

A novel single chain fragment variable against DEC205 increases tumour antigen targeting capabilities leading to improved tumour therapy

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Antigen presentation by dendritic cells (DCs) is due to the expression of membrane bound receptors that guide antigens to the major histocapatibility class I and II (MHCI/II) compartments for processing and presentation. A prototype receptor for antigenuptake is DEC-205. DEC-205 is expressed specifically by DCs and greatly increases antigen presentation. Our first aim was to create a novel single chain fragment variable (ScFv) specific for DEC-205 in order to target DCs *in vivo*. The novel ScFv was created through RT-PCR using degenerative primers on total RNA from a hybridoma cell line (HB290) producing a monoclonal antibody for murine DEC-205.The isolated variable heavy and variable light regions were subcloned into an expression vector fused to both a 6X His tag and c-myc tag then expressed as anative protein in the *E. coli* stain TG1 using IPTG induction. Our second aim was to combine the ScFv for mDEC-205 with various melanoma tumour antigens. We created fusion proteins using monopeptide mGP100, polypeptide mGP100 or a combination of polypeptides from mTRP2 and mGP100. In C57/Bl6 mice, initial immunohistochemical staining of cytospins from CD11c+ isolated cells displayed a positive staining of ScFv comparable to the monoclonal antibody. FACs of six day bone marrow derived dendritic cells indicated a binding affinity of the ScFv to be 53%of the monoclonal antibody. In further experiments, C57/Bl6 mice were injected with the ScFv-mGP100 fusion protein in the footpad or left untreated. Using immunohistochemistry the mice injected with ScFv, show a higher concentration ofc-myc positively stained cells in comparison to the untreated littermates. We then examined the tumour suppressive effects of the ScFv-mGP100 in the B16 transplantable tumour model. When the vaccination regime is implemented after a visible tumour has form at the site of s.c tumour cell injection, we observed a concentration dependent suppression of tumour growth from the ScFv-mGP100 incomparison to mGP100 peptide injected or WT mice. Furthermore the induction ofGP100 specific CD8+ T cells examined via ELISPOT IFN-γ?assays indicated the superior antigen targeting and presentation of the ScFv-mGP100 fusion protein. Together our data indicate the novel ScFv is a viable method of targeting various tumour antigens to dendritic cells in order to elicit an improved immune response.

P054

CD4+CD25+ regulatory T cells reduce the transendothelial migration ofCD4+CD25- T cells

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In vivo recruitment of T cells from the blood stream to the site of inflammation is highly dependent upon the transmigration of these cells through the vascular endothelium during inflammation. We have previously shown that CD4+CD25+regulatory T cells suppress contact hypersensitivity reactions by inhibiting rolling and adhesion of effector T cells to the endothelium of the inflamed tissue (Ring S et al. Eur J Immunol 2006 Nov; **36** (11): 2981–92). Therefore, regulatory T cells might alter the capacity of endothelial cells (ECs) to mediate transmigration of effector cells into the tissue. In the present study, we wished to examine the potential role ofCD4+CD25+ Treg cells in modulating the migratory behaviour of conventional-CD4+CD25- T cells through the endothelium *in vitro*. To this end, we generated primary endothelial cell (ECs) cultures derived from Balb/c lungs and used these ECsto analyze the capacity of CD4+CD25+ T cells to modulate the chemokine-mediated migration of CD4+CD25- T cells through EC monolayers *in vitro*. Migration ofCD4+CD25- as well CD4+CD25+ T cells through an unstimulated EC monolayer was poorly induced upon a CCL19 gradient. In contrast, pre-treatment of the ECs with proinflammatory cytokines enabled CD4+CD25- and CD4+CD25+ T cells to migrate through the monolayer. The most pronouced effect was observed after stimulation of the ECs with TNF-alpha together with IFN-gamma. Cocultures of CD4+CD25- and CD4+CD25+ T cells during transwell assays resulted in a reduced T cell migration through a TNF-alpha/IFN-gamma treated EC monolayer by about 40%. Themigration of both subpopulations was comparably inhibited. On the whole, these data indicate that CD4+CD25+ T cells possess the potential to reduce the chemokine-mediated migration of CD4+ CD25- T cells through the endothelium, providing a possible means by which Treg suppress inflammatory skin reactions *in vivo*.

P055

Steuerung der Syndecan-4 vermittelten Motilität Dendritischer Zellen überPKC-delta abhängige PCK-alpha Assoziation

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Die Aktivierung und Migration von Dendritischen Zellen (DZ) ist eng an eineInteraktion mit der extrazellulären Matrix (EZM) verknüpft. Die EZM, in der eineinterzelluläre Kommunikation und Migration stattfindet, setzt sich größtenteils auszellgebundenen und freien Glykosaminoglykanen und Proteoglykanen zusammen. Syndecane SDC (SDC) sind transmembranäre Heparansulfatproteoglykane. SDCfungieren als Korezeptoren, die extrazelluläre Signale, wie z.B. Zytokine, binden undsequestrieren und darüber die Zellmigration beeinflussen. Die Regulation der SDCExpression während der LPS induzierten DZ Maturation mit Aufregulation von SDC4und Abregulation von SDC-1 beeinflusst deren Motilität. Hier wollten wir klären, wieSDC-4 in DZ eine Verbindung zum Zytoskelett eingeht und über welcheMechanismen diese reguliert wird. Anhand von Westernblot und Koimmunpräzipitationsstudien zeigen wir einePKC-delta abhängige Assoziation von PKC-alpha mit SDC-4 in maturierten DZ. EineVerbindung zum Zytoskelett erfolgt über aufreguliertes Vinculin. Die Aktivierung vonPKC-delta mittels Lysophosphatidylcholin (LysoPC) führt zu einer verstärkten SDC-4Phosphorylierung, einer verminderten SDC-4/PKC-alpha Assoziation und schließlichzur Abnahme der DZ Zellmotilität in videomikroskopischen Untersuchungen. Die PKC-delta abhängige SDC-4 Phosphorylierung verhindert somit eine Assoziationvon SDC-4 mit PKC-alpha und nachfolgend mit Vinculin und darüber zumZytoskelett. Die PKC-alpha Assoziation mit SDC-4 scheint wichtig für die MotilitätDendritischer Zellen. Über die Aktivierung/Hemmung von PKC-delta ließen sichmöglicherweise neue Ansätze zur gezielten Immunmodulation über DendritischeZellen finden.

P056

Effect of toll-like receptor 2 ligand lipoteichoic acid on development of atopic dermatitis

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Atopic Dermatitis (AD) is a chronic inflammatory skin disease induced by infiltrating Th cells. While Th2 cells dominate the acute flares of AD as seen in atopy patch tests, chronic AD lesions are indistinguishable from other forms of eczema and are dominated by Th1 cells. However, the mechanism underlying this change of inflammatory pattern in AD remained elusive. It is well known that >90% of our AD patients show colonization with *Staphylococcus aureus*, its role for this well documented change of inflammation pattern has not been investigated so far. To this end, we established a model for the initial phase of AD by adoptively transferring OVA-specific Th2 cells with or without OVA into the skin of naive mice. The increase of ear thickness after adoptive transfer correlated with antigen-specific inflammation. While injection of Th2 or OVA alone only lead to minor alterations the transfer of Th2plus OVA provoked an inflammatory reaction and resulted in a strong ear swelling after 24 h. One important pathogen associated molecular pattern (PAMP) of *Staphylococcus aureus* is the cell wall component and TLR2-ligand Lipoteichoic acid (LTA). Interestingly, the presence of LTA during the AD-like inflammation resulted inprolonged and increased dermatitis compared to Th2 plus OVA alone, aninflammation pattern identical to OVA-specific dermatitis observed after transfer ofTh1 cells. Investigations using TLR2-deficient mice as recipients showed complete abrogation of this effect demonstrating that LTA binding of TLR2 is operative in this change of inflammation pattern. These data indicate that *S. aureus* derived LTA shifts transient cutaneous inflammation mediated by Th2 cells towards a chronic and persistent form of dermatitis implicating an important role of LTA in the process of AD development and chronification.

P057

Naturally occurring T-cell responses against the tumour antigens MageA3, MelanA and Survivin in stage IV melanoma patients

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Background: Malignant melanoma is a highly immunogenic cancer. T-cell responses against defined epitopes restricted by certain HLA types such as A2 can be detectedin most of stage IV patients usually after *in vitro* restimulation. However, very little is known about the breadth and frequency of anti melanoma immune responses restricted by other HLA types.

Objective: In this study we investigate the naturally occurring T-cell responses in stage IV melanoma patients regardless of their HLA type against the tumour antigens Survivin (Su), MelanA (Mel) and MageA3 (MA3) using overlapping peptides covering the whole amino acid (aa) sequence of these antigens.

Methods: PBMC are being stimulated for 7 days with peptide pools consisting of peptides derived from the published aa sequences for Su, Mel and MA3. Every peptide pool consists of five to six peptides, which are 13 to 16 aa long and overlap by11 aa. T-cell responses against peptide pools were analysed in an ELISPOT assay on day 7. Cultures that showed reactivity were tested against single peptides contained in the individual peptide pools to define the fine specificity of the immune response. Individual peptide-specific responses were then checked by flow-cytometry and intracellular cytokine staining (ICS).

Results: Fifteen patients were analysed. One patient showed no T-cell responses, the other patients showed reactivity against 2 to 15 (median 5) peptide pools. The most frequently recognized aa sequences were Su 1-32, Su 88-120, Mel 22-51, Mel 41-73,MA3 120-153, MA3 143-178 and MA3 266-293. For 10 patients these T-cell responses could be further defined. Peptides with the aa sequence Su 18-32, Mel59-73 and MA3 265- 283 were recognized most frequently. Mel 59-73 and MA3265-283 contain previously defined peptide sequences for HLA DR4 and HLA A2 respectively. Su 18-32 however contains a previously unpublished T-cell epitope. ByICS it could be shown that Su 18-32 was recognized by CD4-cells only.

Conclusion: The Elispot assay together with overlapping peptides is an efficient method to determine pre existing T-cell responses in stage IV melanoma patients. Here we show that Survivin 18-32 contains a previously unpublished helper cell epitope, which needs to be further defined for optimal aa sequence and HLA restriction.

P058

Broad T cell reactivity against a tumour-associated self antigen in melanoma patients and healthy individuals without obvious signs of autoimmunity

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To avoid immune escape by down regulation or loss of antigen by the tumour cellstarget antigens are needed which are important for the malignant phenotype and survival of the tumour. We could identify a CD4+ T cell epitope derived from the human melanoma-associated chondroitin sulfate proteoglycan (MCSP) (also known as high molecular weight-melanoma-associated antige n = HMW-MAA), which is strongly expressed on >90% of human melanoma lesions and is important for the motility and invasion of melanoma cells. However, MCSP is not strictly tumour-specific as it is also expressed in a variety of normal tissues. Therefore, self tolerance should prevent the induction of strong T cell responses against these antigens by vaccination strategies. On the other hand, breaking self tolerance to this antigen by effectively manipulating the immune system, might mediate anti-tumour responses although it would bear the risk of autoimmunity. We could sucessfully isolate CD4+ T helper cells from the blood of a healthy donor recognizing peptide MCSP693-709 on HLA-DR11 expressing melanoma cells. Broad T cell reactivity against this antigen could be detected in the peripheral blood of both, healthy donors and melanoma patients, without any apparent signs of autoimmune disease. In some patients, a decline of MCSP-specific T cell reactivity was observed upon tumour progression. Our data indicate that CD4+ T cells are capable of recognizing a membrane glycoprotein that is important in melanoma cell function, and it may be possible that the sizable reactivity to this antigen in most normal individual scontributes to immune surveillance against cancer.

P059

Skin mast cells enhance resistance to animal venoms

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Animal envenomation can cause significant morbidity and mortality both in animals and humans. The typical route of envenomation is via the skin and the subsequent symptoms can range from annoying itch to severe ulcerations or even death, depending on the type of venom and the amount delivered into the skin. In the skin, mast cells (MCs) are ideally located and equipped to sense and react to these exogenous toxins. It has been proposed that the activation of MCs by snake or insect venoms contributes to the detrimental effects of the venoms. We show, in contrast, that skin MCs significantly reduce snake, spider, and honeybee venom-induced pathology in mice. Subcutaneous (s.c.) injection of the venom from a rattle snake (Crotalus atrox) in MC-deficient KitW-sh/KitW-sh (Wsh) mice results in the development of significantly larger hemorrhagic lesions as compared to wildtypeKit+/+ mice (27.3 ± 1 mm vs 18.7 ± 1 mm diameter, $P < 0.001$). Engraftment of MCsinto the skin of Wsh mice 6 weeks prior to venom injection largely restores the enhanced resistance to the venom (22.7 ± 0.4 mm diameter, $P < 0.05$ versus Wsh mice). Furthermore, a single intradermal injection of venom from the brown recluse spider (Loxosceles reclusa) or five s.c. injections of honeybee (Apis mellifera) venom, in concentrations reflecting the actual amount of venom usually administered by the respective animals, leads to hypothermia, hematuria and death in MC-deficient, but only to mild hypothermia in Kit+/+ mice or MC engrafted MC-deficient mice. To test whether similar protective mechanisms of MCs may exist in humans, we assessed the ability of bee venom to induce degranulation of human MCs (LAD-2 and human cord blood-derived MCs; hCBMCs). Bee venom potently induced the release of β-hexosaminidase from both cell types (i.e. >80% degranulation). This activation of MCs may lead to the release of proteases which can subsequently detoxify venom components, as we show by MALDI-TOF mass spectrometry that bee venom stimulation of hCBMCs, but not LAD-2, almost completely degrades the toxic components of the venom. These findings identify skin MCs as important effector cells in controlling the toxicity of a wide range of animal venoms and indicate that MC-derived proteases may be apromising treatment option for spider bites, bee stings or other animal envenomationsin the skin.

P060

Loss of IFN-g inducibility of the MHC class II antigen processing pathway in head and neck cancer: evidence for posttranscriptional as well as epigenetic regulation

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Abnormalities of the major histocompatibility complex (MHC) antigens by tumour cells impair the cellular immune response and promote tumour evasion from immune surveillance. So far, studies analyzing the MHC class II expression levels in HNC are limited. Therefore, we investigated the constitutive and interferon (IFN)-γ-regulated expression profiles of MHC class II APM in various HNC cell lines and also analyzed the MHC class II expression in HNC lesions. HNC cell lines analyzed *in vitro* lacked constitutive MHC class II surface expression. Despite of the IFN-γ-mediated induction at the mRNA level, six out of ten cell lines did not show any relevant MHC class II surface expression that might be attributed to a post transcriptional dysregulation of specific MHC class II APM components. One cell line displayed a loss of IFN-γ?induced CIITA-expression that corresponded to impaired MHC class II surface expression, which could be linked to hypermethylation of the IFN-γ-responsive CIITA-promoter IV. *In vivo*, immunohistochemistry analyses of 35 patients revealed that about 86% of HNC tissues exhibit a negative or only marginally positive staining, whereas 14% displayed a heterogenous or highly positive MHC class II surface expression. There was no statistical correlation between tumour differentiation and the MHC class II expression in HNC lesions. Taken together this study suggests a high frequency of MHC class II abnormalities in HNC *in vitro* and *in vivo*, which could occur at different steps of the antigen processing pathway. This information may have a significant impact on practical and clinical aspects of tumour vaccination strategies.

P061 (V10)

T cells are required for initiation of autoimmunity in experimental epidermolysis bullosa acquisita

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Epidermolysis bullosa acquisita (EBA) is a prototypical organ-specific autoimmune disease caused by autoantibodies against type VII collagen, the major constituent ofanchoring fibrils of the dermal-epidermal junction. While mechanisms of autoantibody-induced blister formation have been extensively studied, the initiation of autoimmunity and autoantibody production in autoimmune blistering diseases are still poorly defined. In the present study, we addressed the role of T cells for the production of blister-inducing autoantibodies in mice immunized with type VII collagen. To detect auto reactive type VII collagen-specific T cells, lymph node cells from immunized SJL mice ($n = 6$) were stimulated *in vitro* with recombinant antigen and their proliferation was measured by both radioactive thymidine incorporation and flow cytometry analysis of carboxy-fluorescein diacetate succinimidyl ester labelled cells. Interestingly, using synthetic peptides of the immunogen, different patterns of T and B cell epitopes in mice immunized with type VII collagen were demonstrated. In contrast to wild type mice ($n = 6$), immunization of SJL athymic nude mice ($n = 6$) lacking T cells with type VII collagen did not induce an autoimmune response and blistering phenotype. Importantly, lymphocytes from immunized wild type mice ($n = 6$) passively transferred into SJL nude mice ($n = 6$) produced auto antibodies over a period of 3 months resulting in subepidermal blistering in these animals. These results demonstrate that T cells are required for the initiation of autoimmunity against type VII collagen in experimental EBA and provide a basis for developing T cell-directed immunomodulatory strategies for this and related autoimmune diseases.

P062

Efalizumab induces T cell hyporesponsivness

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The lymphocyte function associated-molecule-1 (LFA-1) α-chain (CD11a)-binding monoclonal antibody (mAb) Efalizumab (E) is a new treatment option in moderate to severe psoriasis. Although LFA-1 is ubiquitously expressed on peripheral blood leukocytes, general opinion holds 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 this 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-bound anti-CD3, PHA), does effectively block the allogeneic mixed leukocyte reaction and anti-CD3-driven T cell proliferation induced under suboptimal conditions. The further observations (i) that the expression-level of LFA-1 on antigen-presenting cells (APC) compares favorably with that on Tcells but (ii) that E exerts its inhibitory effect solely on the T cell side, argues against physical hindrance being the only reason for the E-induced downregulation of the Tcell response. Our findings suggest that binding of E to its ligand induces events which increase the threshold of T cells to a proliferative stimulus via the T cell receptor. They also provide an explanation as to why E is highly effective in patients with stable psoriasis, but often fails to control disease flares.

P063

Accumulation of epidermis-derived CCL27 in skin-draining lymph nodes following topical application of a contact sensitizer induces accumulation of CCR10-positive cells

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The chemokine receptor, CCR10, is expressed by skin-homing T cells. CCL27 is aCCR10 ligand that is constitutively produced by epidermal keratinocytes. CCR10 and CCL27 appear to be important components of T cell-mediated cutaneous immunity, but whether they influence LN homing by T cells is unknown. Herein, we analyzed LN and skin expression of CCL27 by real-time RT-PCR, Western blot, and ELISA inmice before and after administration of a potent contact-sensitizing agent. CCL27protein was detected in skin-draining LN by Western blot and ELISA although CCL27mRNA transcripts were low. CCL27 protein was present at higher levels in skin-draining LN compared to gut-draining LN and spleen. A single topical treatment of mouse skin with the contact sensitizer 2,4-dinitro-1-fluorobenzene (DNFB) resultedin a 13-fold increase in CCL27 protein accumulation in skin-draining LN and a five-fold elevation in CCR10 mRNA (normalized to the T cell marker, CD2). DNFB treatment also resulted in rapid depletion of CCL27 from the epidermis within 30 min of application. CCL27 was also detected in lymph from healthy human volunteers and was elevated fivefold in patients with chronic lymphedema. In summary, we describe a novel mechanism for the recruitment of CCR10-positive T cells to skin-draining LN following the rapid release of preformed CCL27 from epidermis andlymphatic-mediated accumulation in skin-draining LN.

P064

Development of testing strategies for the *in vitro* differentiation between contact allergens and irritant chemicals

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Due to the 7th amendment to the cosmetic directive (76/768/EEC) the selling of cosmetics with substances tested *in vivo* on animals is prohibited as of 2009. Exceptions can be only made as of 2013 if there are no alternative testing methods available. Furthermore, since June 1, 2007, commercially available chemicals (about 30.000) have to be re-evaluated concerning their allergenic potential due to the EU-regulation REACH. At the moment there are only three accepted and validated *in vivo* tests for the assessment of the sensitising potential of a chemical but no *in vitro* test. Thus, for ethical and financial reasons there is a strong need for reliable *in vitro* test systems, not least because of the world wide increase of allergic diseases of the skin and airways. For the development of such tests there are many different approaches available. Taken together, these tests have to be based on the mechanistic coherencies of the sensitisation phase of contact allergy to gain reliable information about the sensitising potential as well as to provide a differentiation from irritant effects of chemicals. One of these approaches is the evaluation of the maturation of dendritic cells by analysing cytokine production and activation marker expression. Our recent data reveals a critical impact of the solvent type and concentration, e.g. acetone versus DMSO, on the cytokine detection, but not the expression of activation markers. Furthermore, the widely used detection of surface marker expression is hampered by the fact that a number of contact sensitizers induced upregulation of e.g. CD86 at concentrations correlated with higher levels of cell death. This correlation was also observed for irritant chemicals that show no increased activation marker induction at non-toxic concentrations. This makes the choice of both the solvent and the chemical concentrations one of the vital points in the development of standardized *in vitro* assays for the identification of contact and respiratory sensitizers and their differentiation from irritants.

P065

Evidence for a major role of IL-18 in cutaneous lupus erythematosus

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Cutaneous manifestations belong to the most common clinical features of lupuserythematosus (LE). It was the aim of this study to analyse differences in the inflammatory response of keratinocytes from patients suffering from cutaneous LE compared to healthy controls. Main findings include that keratinocytes from LE patients express higher IL-18R on their cell surface in response to TNFα?or IFNγ?stimulation. Furthermore, in response to IL-18 these cells produced large amounts of TNFα, which was not related to a higher carrier frequency of the -308A TNFα?promoter polymorphism. Of note, in the presence of IL-18 LE keratinocytes failed to express IL-12p40. IL-12 has previously been shown to protect keratinocytes from UV induced apoptosis. Keratinocytes from LE patients were more prone to die upon exposure to IL-18 and this increased apoptosis could be abrogated by blockade of endogenously produced TNFα?as well as by addition of exogenous IL-12. In biopsies from lesional LE skin IL-18 was highly expressed. Our results demonstrate anintrinsic difference in the inflammatory response of keratinocytes and point to anautocrine feedback loop involving TNFα, IL-18 and IL-12 family members. Our results suggest that IL-18 is on top of the cytokine hierarchy in cutaneous LE and therefore point to a potential benefit of a local therapy which blocks IL-18 activity for cutaneous LE manifestations.

P066 (V36)

Cutaneous interleukin (IL)-15 is important for anti-tumoural immunity in mice

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In K14-IL-15 transgenic (tg) mice cutaneous IL-15 overexpression results in increased antigen-presentation, NK cell- and CD8+ effector T cell activation. Since NK cells and CD8+ effector T cells are involved in anti-tumoural immune responses, we were interested to determine the effects of cutaneous IL-15 production on the regulation of protective immunity. Therefore, wildtype (wt) and K14-IL-15 tg mice were subcutaneously injected with TS/A mammary carcinoma cells and interestingly, K14-IL-15 tg mice showed a significantly reduced tumour growth. Immunofluorescence stainings of tumour tissue revealed markedly increased numbers of granzyme A expressing CD8+ effector T cells and NK cells infiltrating the TS/A tumours ofK14-IL-15 tg mice whereas we did not observe alterations in tumour infiltrating macrophages or granulocytes compared to wt mice. To analyze the specific role of both cell populations for anti-tumoural immune responses in K14-IL-15 tg mice we isolated NK cells and CD8+ effector T cells from tumour bearing tg mice, adoptively transfered them into naive wt recipients and inoculated recipient mice with TS/A cells. The transfer of NK cells from tumour-bearing K14-IL-15 tg mice did not influence progressive growth of injected TS/A cells in recipient mice. However, inoculated TS/Acells were rejected in recipients that were treated with CD8+ T cells from tumour-bearing K14-IL-15 tg mice suggesting strong MHC class I-restricted anti-tumoural immunity. To examine whether CD8+ T cells are indeed essential for the reduced tumour growth in K14-IL-15 tg mice we depleted either CD8+ T cells or NK cells by using specific antibodies prior to injection of TS/A tumour cells. NK cell depleted K14-IL-15 tg mice still showed reduced tumour growth whereas tumours grew progressively in CD8-depleted tg mice. Interestingly, in tumour draining lymph nodes from K14-IL-15 tg mice we observed an increased number of IFN-g+ and CD43+CD8+ T cells whereas the overall number of CD8+ T cells was not changed compared to wt controls indicating that reduced tumour growth in K14-IL-15 tg mice is associated with tumour infiltrating cytotoxic CD8+ T cells. These results indicate that cutaneous IL-15 over expression leads to strong protective anti-tumoural immunity via stimulation of MHC class I-restricted cytotoxicity.

P067 (V13)

TGFβ-differentiated Foxp3+ T regulatory (Treg) cells protect scurfy mice

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Scurfy mice have a deletion in the forkhead domain of Foxp3 and are deficient inthymic-derived Foxp3+ Treg. They develop a lympho proliferative syndrome withCD4+ (and CD8+) cell mediated multi-organ inflammation, and die between 3–4 weeks of age. Transfer of thymic-derived FoxP3+ Treg into neonatal Scurfy mice prevents the development of disease. Recently, it has been shown that TCR stimulation of naïve CD4+CD25- T cells *in vitro* in the presence of TGFβ?and IL-2 induces the expression of FoxP3 and an anergic/suppressive phenotype *in vitro* and *in vivo*. To determine whether the mechanism of action of the TGFβ-induced Treg was similar to thymic-derived Treg, we reconstituted newborn Scurfy mice with TGFβ-differentiated polyclonal Treg. 21 days after transfer of TGFβ-induced Treg, Scurfy mice show drastically reduced cell numbers in peripheral lymph nodes and spleen in comparison to scurfy controls. Furthermore, on histopathologic analysis, transfer of TGF-induced Treg prevented the development of inflammation in skin, liver and lung. The TGFβ-induced Treg retained the expression of FoxP3 *in vivo* for 21 days and migrated to the site of inflammation in the skin. Thus, TGFβ-differentiated Foxp3+ Treg appear to possess all of the functional properties of thymic-derived Treg. As both antigen-specific and polyclonal TGFβ-differentiated Treg can be easily generated in large numbers *in vitro*, they represent a potent population for the cellular immunotherapy of autoimmune and inflammatory diseases.

P068

Neutralization of TNF-alpha augments the suppression of Th2 cells byIL-10-treated DC-and TGF-ß-induced regulatory T cells (iTreg)

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Recently we have demonstrated that regulatory T cells (Treg) that also suppress Th2cytokine production can be induced in the presence of IL-10-DC and TGF-ß. Asanti-TNF-alpha therapy has been shown to enhance the suppressive function of Tregin autoimmune diseases, the aim of this study was to analyze whether neutralization of TNF-alpha would also increase the suppression of Th2 cells. Therefore, freshly isolated CD4+CD25- T cells from grass pollen allergic donors were stimulated with autologous, mature monocyte-derived allergen-pulsed dendritic cells (DC) alone ortogether with induced Treg (iTreg: CD4+CD25- T cells of the same donor previously cultured with IL-10-DC and/or TGF-ß with or without addition of anti-TNF-alpha). Only the combination of IL-10-DC and TGF-ß leads to the induction of iTreg that can suppress proliferation and cytokine production (Th1 as well as Th2 cytokines) of peripheral T cells while proliferation and Th2 cytokine production were only slightly reduced by iTreg induced with IL-10-DC or TGF-ß alone. In all conditions, the addition of anti-TNF-alpha further increased the inhibitory potential of iTreg especially concerning the inhibition of Th2 cells. These data demonstrate that TNF-alpha might be exploited as novel therapeutic target in allergic diseases.

P069

Enhanced *in vivo* secretion of antimicrobial peptides in psoriasis vulgaris andatopic dermatitis

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Inducible antimicrobial peptides (AMP) have been isolated from psoriatic scale extracts and are discussed to be responsible for the rare infection rate observed in psoriasis patients. In contrast, reduced expression of AMP was recently described in patients with atopic dermatitis to explain the high rate of microbial infections. Aim of this study was to perform a comparative analysis of the *in vivo* protein expression and secretion of human beta defens in (hBD)-2 and -3, psoriasin (S100A7) and RNase 7 in patients with psoriasis vulgaris (PV) and atopic dermatitis (AD). Untreated patients with PV and extrinsic AD (n = 22, 14 f/8 m, 2–66 years) as well as gender and age-related healthy volunteers were included in this study. Punch biopsies were taken from lesional and non-lesional skin of the patients and corresponding localisations of the controls to investigate the protein expression of AMP by immunohistochemistry. *In vivo* secretion of hBD-2 and -3, psoriasin and RNase 7 was determined by ELISA performed with skin-derived washing fluids. Enhanced immunoreactivity of all AMP under investigation was observed in lesional and partly in non-lesional skin of PV and AD patients when compared to the healthy controls. PV patients showed the strongest staining. However, AD patients clearly showed an increased staining when compared to healthy controls suggesting that AMP expression is induced in AD. Analyses of skin washing fluids revealed secretion of psoriasin, RNase 7 and hBD-2 in high amounts from lesional skin of PV patients. Although we detected lower amounts of AMP on the surface of AD skin as compared to PV (psoriasin: 145.5 ng/ml vs 2.7 μg/ml, RNase 7: 0.7 vs 3.86 ng/ml), our results revealed that the AMP levels secreted on AD skin were upregulated when compared to healthy skin. These results indicate that the skin of patients with AD has the capacity to express and to induce the expression of AMP and counter the hypothesis of a general induction defect of AMP in AD. However, because the expression levels of AMP in AD skin are significantly below the levels found in PV, the role of AMP expression for the infection rate of AD patients has still to be determined.

P070

Defects in dendritic cell (DC) function in IL-1 receptor antagonist (RA) deficient mice are responsible for progressive disease in L. major infection

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Previously, we have shown that DC-derived IL-1α/β?facilitates Th1 induction ininflammatory disease models (infection with Leishmania major, allergic asthma ofmice). DC from Leishmania-susceptible BALB/c mice produced less IL-1 as compared to resistant C57BL/6 mice; substitution of IL-1α?during T cell priming *in vivo* protected from progressive disease and abrogated aberrant Th2 development. With the aim of determining the role of IL-1 for resulting immune responses in more detail, we utilized IL-1RA−/− BALB/c mice. IL-1RA−/− mice exhibit excessive IL-1α/β?signalling due to a lack of its antagonist. In contrast to our expectation, IL-1RA−/−BALB/c mice infected with standard high dose (HD; 2 × 10E5 L. major) or physiologic allow dose (LD; 1,000 parasites) inocula displayed enhanced lesion progression as compared to wild type mice and had to be euthanized in wk6 and wk10, whereas controls progressed more slowly. In parallel, we found significantly higher lesional parasite burdens and decreased IFN-gamma production by draining lymph node (LN) cells, whereas the levels of IL-4 and IL-10 were unaltered in IL-1RA−/− mice. Interestingly, both *in vivo* in LN cultures and using bone marrow-derived cells orCD11c+ DC isolated from infected LN, a markedly impaired production of IL-12 was observed with IL-1RA−/− cells. As compared to DC from wild types, IL-1RA−/− DC displayed a more mature phenotype with significantly increased surface levels of MHC class I, II and cost imulatory molecules and a reduced phagocytotic capacity.Incubation with IL-1RA during culture of DC *in vitro* resulted in the generation of regular immature DC capable of normal L. major uptake and restoration of their ability to produce inflammatory mediators (e.g. IL-12p40) upon infection. In summary, apart from effects of IL-1α/β?on T cell priming towards Th1, excessive IL-1 signalling *in vivo* due to absence of IL-1RA led to alterations in DC function even under steady state conditions resulting in pathological activation of DC. This raises this possibility that blockade of IL-1RA *in vivo* may alter the function of antigen presenting cells as well as T cells in chronically treated patients.

P071

Desmoglein 3-specific antibodies generated in an HLA-class II-transgenic mouse model of pemphigus vulgaris are pathogenic

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Pemphigus vulgaris (PV) represents a severe blistering autoimmune disorder characterised by IgG autoantibodies (autoab) predominantly against desmoglein 3(dsg3), a transmembranous component of the desmosomes. Recent studies suggest that autoab directed against epitopes of the NH2-terminal adhesive region of dsg3 are pathogenic whereas autoab recognizing epitopes of the COOH-terminus are non-pathogenic. The aim of our study is to characterise the antibody response to human dsg3 protein in HLA-DR0402-DQ8-/huCD4-transgenic mice. Following repetitive immunizations with recombinant human dsg3-protein, serum samples of HLA-transgenic mice are investigated for dsg3-reactivity and their ability to induce acantholysis. Applying protein constructs spanning single extracellular domains of thedsg3-protein in an ELISA system, enables us to monitor the development of the IgG response to human dsg3-protein. IgG from dsg3-immunized mice demonstratesdsg3-specificity as shown by ELISA and recognizes native dsg3 using indirect immunofluorescence on human skin samples. Initially, serum antibodies bind to epitopes of the extracellular domain 5 (EC5) located at the COOH-terminus of the dsg3-ectodomain. Repetitive immunizations with dsg3-protein lead to an intramolecular epitope-spreading demonstrating IgG-reactivity to epitopes of theNH2-terminal EC1 and EC2 domains. Dsg3-reactive IgG is capable of inducing acantholysis in both cultured normal human and HaCaT keratinocytes *in vitro*. Incubating human skin samples ex vivo with serum samples of dsg3-immunized mice induces suprabasilar acantholysis resembling PV, accompanied by positive direct immunofluorescence. Noteworthy, mouse sera showing exclusive EC1-reactivity are pathogenic *in vitro* and induce acantholysis in human skin biopsies, whereas COOH-reactive serum samples do not demonstrate pathogenic capability. These findings are in line with previous studies on autoantibody reactivity in PV patients. Thus, HLA-class II-transgenic mice provide an excellent *in vivo* system to characterise the auto antibody response to dsg3 in PV patients and establish the basis for evaluating new, antigen-specific therapeutic approaches in PV.

P072 (V16)

Homeostatic proliferation drives the accumulation of 'antigen-experienced' CD3+CD4-CD8- (DN) T cells in CD18−/− mice

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Beta2 integrins (CD11/CD18) importantly participate in leukocyte transmigration and lymphocyte activation supporting firm intercellular adhesion and signalling e.g. during antigen presentation. We reported previously that CD18-deficient mice, suffering from severe primary immunodeficiency and autoimmunity, show an impaired homing of lymphocytes in the periphery due to absent CD18 expression, changes in lymphocyte maturation and phenotype. Compared to wild-type (WT) mice, CD18−/− mice harbor a >30-fold increase in CD3+CD4-CD8- double-negative (DN) T cells with an apparently antigen-experienced phenotype in peripheral lymphoid and non-lymphoid tissues, whereas single-positive (SP) T cells are reduced. To elucidate the reason for the high increase in CD18−/− DN T cells in the periphery, we hypothesized that the transition from the DN to the double-positive (DP), and hence, to the SP state may be impaired during intra thymic T cell development. Surprisingly, neither a correlating increase in DN nor decrease in DP or SP thymocytes was detected by FACS analyses of thymi from CD18−/− mice ($n \geq 6$). Thus the high prevalence of DN T cells in the periphery was not reflected by an analogous shift in thymic T cell populations. Therefore, we next hypothesized unconventional DN T cells can be expanded primarily in the periphery of CD18−/− mice. Regarding the marked decrease in peripheral naïve SP T cells in CD18−/− mice, T cells may be stimulated to expand homeo statically under these lymphopenic conditions. Key cytokines known to drive homeostatic T cell expansion are IL-7 and IL-15. Indeed, when purified from peripheral lymph nodes by MACS sorting and subjected to CFSE proliferation assays, only unconventional DN T cells but not conventional CD4+ or CD8+ SP T cells could be expanded by exogenous IL-7 *in vitro*. This demonstrated DN T cells were highly susceptible to proliferate upon a key homeostatic stimulus. However, no significant proliferation was observed uponIL-15 alone. We conclude that unconventional DN T cells can be generated byhomeostatic expansion in the lymphopenic periphery of CD18−/− mice. We conclude that unconventional DN T cells can be generated by homeostatic expansion in the lymphopenic periphery of CD18-/- mice [abstract was shortened, because it exceeded space limitations].

P073

Mature mast cells induce dendritic cell maturation resulting in T cell proliferation and polarization to TH1 response

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Mast cells (MCs) have been recently shown to link innate immune responses and long lasting adaptive immunity resulting from pathogen infection. However, the underlying mechanisms of this novel mast cell function remain to be elucidated indetail. Here, we asked whether MCs can induce the maturation of dendritic cells (DCs), which is required for the generation of effective adaptive immune responses.We found that mature murine connective tissue type mast cells (CTMCs) can undergo cell-to-cell interactions with immature murine bone marrow derived DCs. The cellular contact of CTMCs to immature DCs induced the expression of the costimulatory signals CD80, CD86 and CD40 on the cell surface of DCs suggesting the induction of DC maturation. Moreover, the interaction between CTMCs and immature DCs induced the expression of the chemokine receptor CCR7 by DCs, which is required for the initiation of DC migration to T cell areas of draining lymphnodes. Furthermore, we could detect high amounts of IFN-γ?and IL-2 in supernatantsof DC/MC co-cultures. Most interestingly, DCs matured by previous interaction with mature MCs induced strong proliferative responses in naïve CD4+ T cells (more than LPS stimulated mature DCs). Therefore, we analyzed the pattern of cytokines released by CD4+ T cells which had interacted with DCs matured by the contact to mature MCs. Surprisingly, we found that the crosstalk between naïve CD4+ T cells with mast cell primed DCs resulted in the release of high levels of IFN-γ, but no significant secretion of IL-4 and IL-5, thus suggesting a polarization to TH1 response. In conclusion, our data demonstrate that cellular contact of immature dendritic cells to mature mast cells resulted in the maturation of dendritic cells, which subsequently induced naïve CD4+ T cell proliferation and the polarization of their cytokine responses towards the TH1 subtype. Thus, mast cells may initiate and modulate the adaptive immune response by inducing the maturation of dendritic cells and regulating their function.

P074

IL-4 and IL-15 promote immature mast cell adhesion to skin endothelial cells and chemotaxis

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Mast cell numbers in peripheral tissues are relatively stable under physiological conditions, but markedly increased in many pathological disorders associated with chronic inflammation or long standing immune responses. This mast cell hyperplasiais presumably due to a modified regulation of proliferation, survival and/or the migration of mast cells. The aim of this study was to investigate the regulation of immature mast cell recruitment from the vasculature to peripheral tissues. We could demonstrate that immature mast cells exhibit a strong adhesion to microvascular skin endothelial cells followed by directed transendothelial migration. The adhesion of immature mast cells to endothelial cells was markedly enhanced by previous stimulation with IL-4 or IL-15, both described to be released by mature connective tissue type mast cells upon stimulation. However, there was no significant regulation of immature mast cell adhesion to endothelial cells by the leukocyte chemo at tractants RANTES, MIP-1α?or MCP-1. Furthermore, chemotaxis of immature mast cells was induced by IL-4 and IL-15, but not by RANTES, MIP-1α?or MCP-1. In contrast, the chemotaxis of mature connective tissue type mast cells was induced by a wide spectrum of chemokines and cytokines including IL-4 and IL-15 but also RANTES, MIP-1α?and MCP-1. Thus, our data show that the adhesion of immature mast cells to the endothelium as well as their subsequent chemotaxis is promoted by IL-4 and IL-15, but not by leukocyte chemo attractants suggesting a selective recruitment of immature mast cells from the vasculature independent of leukocyte infiltration. These findings may explain, why some chronic inflammatory conditions are associated primarily with the accumulation of mast cells and not other leukocytes.

P075

Cytokines and TLR ligands exert different effects on B7H1 expression by dendritic cell (DC) subpopulations

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Expression of regulatory molecules of the B7H family by DCs plays an important role in the regulation of immune responses. However, their function(s) as well as regulation of their expression during DC maturation is not completely understood. To test how DC maturation affects expression of these molecules, we stimulated *in vitro* prepared monocyte derived DCs (MoDCs) as well as genuine DCs, isolated from peripheral blood of healthy donors (gDCs). Stimulation of MoDCs with a cytokine cocktail resulted in increased expression of CD83 and CD86 molecules as well as increased T-cell stimulatory capacity. In parallel we observed upregulation of B7H1.Similar results were observed using genuine DCs. While MoDC represent a homogenous population of myeloid origin (mDCs), genuine DCs consist of a mixed population (mDCs and plasmacytoid DCs (pDCs)) with different TLR receptor repertoires. To determine the responding subpopulation, genuine DCs were stimulated with various TLR ligands and assessed by FACS analysis and functional assays. LPS, poly I:C, as well as cytokines enhanced expression of B7H1 to different extent directly in mDCs. In contrast CpG was effective in direct affecting of expression in pDCs. The B7-H1 upregulation induced by cytokines and TLR stimulation was accompanied by activation of the MAPK kinase pathway in MoDCs and blocking of ERK/MAPK phosphorylation with a specific inhibitor reduced increased B7H1 expression. Thus our results indicate that the expression of B7H1 is regulated by the MAPK kinase pathway and that different stimuli induce differential expression of B7H1 on DCs. This differential regulation may have an input on DC function in healthy persons and in pathological conditions.

P076

Viral chemokines antagonist vMIP-II reduces metastasis formation

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Chemokines and their receptors play an essential role in pro-inflammatory processes as well as metastasis formation. Viral MIP-II is a broad spectrum chemokine receptor antagonist, that binds different chemokines receptors regardless of subfamily. Ininflammation vMIP-II was shown to have potent inhibitory functions by blocking specifically pro-inflammatory chemokines receptors and preventing immune cells from infiltrating the tissue. The role of vMIP-II in metastasis and cancer formation is not clear so far. To study the *in vitro* effects of vMIP-II on metastasis formation we used murine B16F10 melanoma cells. First we analyzed the chemokines receptor profile of these cells via RT-PCR and flow cytometry and then we investigated the effects of different chemokines alone or on combination with vMIP-II on chemokine receptor internalization and migration. No ligand-induced internalization was seen for CCR2, CCR5, CCR10 and CXCR3 on B16F10 cells. Viral MIP-II inhibited the ligand-induced migration of B16F10 cells mediated by CCR2, CCR5 and CXCR3. Using the *in vivo* lung metastasis model, we could show that after 11 days C57Bl/6 mice treated with 50 μg vMIP-II each developed significantly less lung metastasis than untreated mice. These data indicate that vMIP-II may also affect metastasis formation via chemokine receptor blockade or modulation.

P077 (V18)

Glucocorticoids induce regulatory monocytes that control adaptive immuneresponses

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Glucocorticoids (GC) are still the most widely used immunosupressive 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 Glucocorticoids (GC) promote survivalof anti-inflammatory monocytes that influence T cell responses in antigen-specificways. Thus, GC-treatment does not lead to global suppression of monocytic effect or functions it rather leads to induction and differentiation of monocytes to become regulatory cells. We show that GC- induced regulatory monocytes upregulate surface molecules like CD163, and CD80 while others e.g. CD86, and MHC II are downregulated. Upon GC- treatment transmigration and motility of these cells is up-regulated while adherence is reduced. Furthermore, co-culture of regulatory monocytes and T cells leads to inhibition of T cell activation as shown by diminished proliferation and cytokine production in antigen- dependent and antigen- independent ways. We examined the mechanisms of regulatory function of monocytes, and show that they produce increased amounts of immunosuppressive cytokine IL-10. In addition we determined cell-cell contact dependent inhibition of T cell responses. In conclusion, GC treatment generates regulatory monocytes that are capable to controlongoing T cell responses, and thus have a high potential to become valuable tools inimmunotherapy against inflammatory diseases.

P078

The role of the peripheral tissue microenvironment in T cell homing receptor imprinting

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The migration of effector and memory T cells to peripheral tissues depends on the expression of tissue specific combinations of adhesion molecules and chemokine receptors. For T cell homing to the skin E-selectin ligands, CCR4 and CCR10 arerequired, T cells homing to the gut express α4β7 integrin and CCR9 on their surface. Our recent finding that epidermal Langerhans cells potently induce skin homing receptors on T cells led us to the hypothesis that environmental factors of the tissue of origin of the dendritic cells (DC) are important for homing receptor imprinting. These factors might license DC to imprint homing receptors on T cells upon activation in draining lymph nodes. To test this we have set up co-culture experiments using either dermal fibroblasts or small intestinal epithelial cells (SIEC), bone marrow derived (BM-) DC pulsed with a peptide derived from LCMV (GP33) and splenocytes from T cell receptor transgenic CD8+ P14 mice specifically recognizing the GP33 peptide. Co-cultures including dermal fibroblasts revealed an upregulation of E-selectin ligands on P14 T cells whereas T cells incubated with BM-DC and SIEC upregulated CCR9 and α4β7 integrin. DC re-isolated from co-culture with SIEC showed marginally up-regulated CD103 (αE integrin) and induction of retinal dehydrogenases. Thus, we suggest that the BM-DC had acquired features of gut-specific DC. In summary, our data suggest an important role for peripheral tissue stromal and epithelial cells in licensing DC for homing receptor imprinting on T cells in the lymph node.

P079

Interferon-alpha partially reverses tolerance mechanisms induced by tolerogenic dendritic cells

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In malignant diseases, the induction of tolerance by tumour-derived factors is one critical mechanism involved in tumour progression. Tumour-derived soluble mediators such as IL-10 can provoke immunosuppression by inhibition of dendritic cell function and T-cell activation. The beneficial effect of Interferon-alpha (IFN-α) adjuvant therapies for patients suffering from stage II/III malignant melanoma has been evaluated in several studies. Multiple activities of IFN-α?on immune cells have been described but, to date, no information is available on the effect of IFN-α?on tolerance promoting mechanisms. In this study, we investigated effects of IFN-α?on IL-10 modulated tolerogenic DC (IL-10 DC) and their respective stimulatory capacity on Tcells. Tolerogenic IL-10 DC are known to induce a population of anergic regulatory Tcells, thus we further analyzed the effects of IFN-α?on induction of anergy. Intolerogenic IL-10 DC, the expression of maturation markers and co-stimulatory molecules was reduced as compared to mature DC. IFN-α-treatment of these tolerogenic DC provoked a significant increased expression of CD80, CD83 and CD86 molecules. Furthermore, CD4+ as well as CD8+ T-cells showed enhanced proliferation when stimulated with IFN-a-treated IL-10 DC in contrast to T-cells activated with control IL-10 DC, indicating the restoration of the antigen presenting capacity of human tolerogenic DC in the presence of IFN-α. However, addition of IFN-α?did not induce full maturation of IL-10 DC as triggered by a maturation cocktail (IL-1β, TNF-α, IL-6, PGE2). In addition, T-cells primed with IFN-α-treated IL-10 DC exhibited a significantly impaired anergic phenotype in anti-CD3/CD28 restimulation assays compared to IL-10 DC, as demonstrated by proliferation assays and cytokinepattern (IFN-γ). Furthermore, we analyzed effects of IFN-α?on primary culture ofT-cells with IL-10 DC. Treatment of CD4+ T-cells with IFN-α?stimulated by IL-10 DC did not influence the reduced stimulatory capacity of tolerogenic DC. However, restimulation of these T-cells revealed that IFN-α?partially prevented the induction of anergy by IL-10 DC and thus restored the T-cell response. Taken together, we show that IFN-α?can influence tolerance induction at priming of T-cells as well as via imparting the tolerogenic function of IL-10 DC.

P080

Cytokines are critically involved in a PMN-dependent and TLR4-mediated protective mechanisms against oral *C. albicans* infections

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Immune responsiveness to many microbial pathogens depends on a family of innaterecognition molecules known as Toll-like receptors (TLRs). We could previously demonstrate that addition of polymorphonuclear leukocytes (PMNs) to a Candida-infected three dimensional organotypic epithelial model (RHE) strongly up regulates epithelial TLR4 expression, which correlates directly with protection against fungal invasion and cell injury. This interaction does not need a direct physical contact of PMNs to epithelial cells indicating that all communication between the two cell types must be due to soluble factors – most probably chemokines and cytokines. IL-8 and GM-CSF are keycytokines in recruiting and stimulating PMNs to sites of microbial infection which are rapidly activated by exposure to proinflammatory cytokines including IL-1a, IL-1b, IL-6, and TNF-a. Together with the observation that MIP-1b and MCP-1 production of epithelial cells was increased after PMNs supplementation of the *C. albicans*-infectedoral RHE, we investigated whether blocking of these cytokines with neutralizing antibodies could prevent epithelial TLR4 upregulation and subsequent protection against fungal infection. In a first approach the oral RHE but not PMNs were treated with neutralizing antibodies. However, no discernible changes in epithelial cell damage were evident, and a strong TLR4 upregulation was still observed after PMN addition. Interestingly, additional incubation of PMNs with neutralizing antibodies led to strongly reduced TLR4 mRNA levels for all cytokines except GM-CSF when assessed using real-time RT-PCR. The strongest effect was observed after neutralization of TNF-a. Analysis of TLR4 protein expression by confocal microscopy showed a similar decrease. Furthermore, neutralization of TNF-a led to increased epithelial damage compared with control antibodies and restored the protective effect of PMNs as indicated by increased release of lactate dehydrogenase (LDH) after addition of PMNs. Our results point out that activation of PMNs and/or PMN-mediated cytokine release are crucial for the upregulation of epithelial TLR4 and the subsequent protection.

P081

Blockade of ICOS-ICOS-ligand interaction prevents tolerance induction by immature dendritic cells

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Type and maturation state of dendritic cells (DC) determine the differentiation of resting CD4+ T cells. While activated mature DC induce their development into IFN-gamma-producing effector cells, immature DC induce anergic, IL-10-producing T cells with suppressive properties. Thus, tolerogenic DC play a crucial role in peripheral T cell tolerance and subsequently have an important impact on various human diseases including autoimmunity and allergy. The Inducible Cost imulator (ICOS), an activation induced member of the CD28 family on T cells, is involved in the induction of IL-10 in T cells. Expression of ICOS-Ligand on mature DC contributes to the induction of suppressive T cells in an inflammatory environment indicating a major role for ICOS in promoting tolerance and limiting autoimmune pathology. Therefore, we analyzed the functional role of ICOS in the differentiation of human CD4+ T cells upon their interaction with mature versus immature DC comparing the functional properties of resting CD4+ T cells from healthy volunteers to T cells from ICOS-deficient patients after stimulation with DC. We report here that the induction of T cell anergy by immature DC is completely blocked after knock down of ICOS expression in T cells as well as after blocking of ICOS-ICOS-L interaction in DC-T cell co cultures. Moreover CD4+ T cells from ICOS-deficient patients were completely resistant to anergy induction and differentiation into suppressive T cell seven after addition of IL-10 to the culture. In contrast to CD4+ T cells from healthy volunteers, those ICOS-deficient CD4+ T cells expanded in the presence of allogeneic immature DC and differentiated into effector T cells with increased IL-4and reduced but still significant IL-10 production. Additionally, the induction of regulatory T cells by repetitive stimulation with immature DC was completely blocked in the absence of ICOS. Taken together, these results indicate a crucial role for ICOS in the regulation of peripheral T cell tolerance after crosstalk with immature DC and designate ICOS as a promising target for immunomodulation strategies in allergic and autoimmune diseases.

P082

Differentially expressed molecules during the maturation of dendritic cells

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Background: All known and implicated functions of the adaptor molecule Bam32 are related to the signalling of the B-cell receptor for antigen. However, during the maturation of dendritic cells we found Bam32 to be induced. Since DC do not express receptors specific for antigen, but still induce the expression of the adaptor molecule Bam32 during maturation, in this study we aim to characterize the role ofBam32 in DC. We hypothesize a regulatory role for this adaptor molecule in one of the functions of DC aquired during maturation, e.g. migratory behaviour and/or T-cell activating capacity.
Methods: 1. To verify the induction of Bam32 during the maturation of DC we applied real time PCR, western blots and confocal microscopy for Bam32 on monocytederived immature and mature DC, Langerhans cells and dermal dendritc cells derived from skin explant cultures. 2. To localize the molecule within the dendritic cell we stained DC-T-cell co-cultures and DC treated with antibodies against different surface markers with a Bam32 specific monoclonal antibody. Monitoring was with confocal microscopy. 3. To identify the signalling pathway in which Bam32 could be involved in DC we searched fo a putative interaction partner using an yeast-2-hybrid aproach.
Results: 1. We verified the induction of Bam32 expression during maturation of DC both on the mRNA and protein level. 2. Bam32 localizes to the contact point of DC with T-cells and DC treated with an MHC class I specific antibody relocalize Bam32 to the membrane. 3. Using Bam32 as a bait we could isolate Galectin-1 from a cDNA library derived from mature DC cell.
Conclusion: The preliminary results on the localization of Bam32 to the immunological synapse during DC-T-cell co-culture, the relocalization of Bam32 to the membrane upon treatment with an MHC class I antibody and its interaction withgalectin-1 indicate a role for the adaptor molecule Bam32 in the signalling of mature DC, possibly in the signalling of MHC class I.

P083 (V19)

Desmoglein 3 (Dsg3)-specific T regulatory 1 (Tr1) cells consist of twosubpopulations with differential expression of Foxp3

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Pemphigus vulgaris (PV) is an autoimmune bullous skin disorder associated with auto-antibodies against demoglein (Dsg)3. An imbalance of type 1 regulatory (Tr1) T cells and T helper 2 (Th2) specific for Dsg3 may be critical for the loss of tolerance against Dsg3 in PV because the ratio of Dsg3-specific Tr1/Th2 cells is much lower inPV than in healthy individuals. Within the population of Dsg3-responsive, IL-10-secreting Tr1 cells that were isolated by MACS cytokine secretion assay, two major subpopulations were identified and sorted by FACS based on their size (FSC) and granularity (SSC). Upon *in vitro* culture, the larger subpopulation differentiated back into the two former subpopulations, while the smaller subpopulation died within 2 weeks. The smaller subpopulation of Tr1 cells was characterized by the expression of Foxp3, the secretion of IL-10, TGF-b and IL-5 upon stimulation with Dsg3, a proliferative response to IL-2 but not to Dsg3 or mitogenic stimuli and a marked inhibitory effect on the proliferative response of Dsg3-responsive Th clones in an Ag-specific (Dsg3) and cell number-dependent manner. In contrast, the larger subpopulation showed a Th2-like phenotype with no Foxp3, CTLA-4 and GITR expression, proliferative response to Dsg3 and mitogenic stimuli and IL-2 secretion. Both Tr1 subpopulation showed expression of identical TCR Vß chains which varied among the PV patients studied. Noteworthy, upon Foxp3 antisense treatment, the smaller Tr1 subpopulation developed a proliferate response to Dsg3 and mitogenic stimuli, did no longer suppress Dsg3-specific Th cells, lost expression of GITR and CTLA-4 and secreted higher levels of IL-2. Our observations suggest a distinct relationship between Dsg3-specific Tr1 and Th2 cells which may be critical for the continuous generation and survival of the Dsg3-specific Tr1 cells.

P084

The anti-CD20 monoclonal antibody, rituximab, acts in pemphigus vulgaris (PV) by decreasing pathogenic auto-antibodies against the NH2-terminus of the desmoglein 3 (Dsg3) ectodomain

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In PV, IgG autoantibodies against distinct regions of the ectodomain of Dsg3 arethought to be directly responsible for the loss of keratinocyte adhesion leading to blister formation. The aim of the present study was to study the effect of the B cell depleting monoclonal antibody, rituximab, on IgG recognition of Dsg3 epitopes in 20PV patients which were followed up clinically by ABSIS score and serologically by ELISA with Dsg3 recombinants that represented the five extracellular subdomains (EC1 to EC5) of Dsg3. Prior to rituximab treatment, 12/20 patients showed IgG reactivity against Dsg3EC1, 5/20 patients against Dsg3EC2, 6/20 patients against Dsg3EC3, 8/20 patients against Dsg3EC4, and 1/20 patients showed IgG against Dsg3EC5. On rituximab, IgG titers against the entire Dsg3 ectodomain markedly decreased. Moreover, a good clinical response to rituximab was associated with reduced IgG reactivity against the targeted Dsg3 regions, i.e. Dsg3EC1 and/orDsg3EC2, Dsg3EC3 and Dsg3EC4. In the 3 PV patients with a clinical relapse during the 24 month observation period, IgG titers against the Dsg3 ectodomain rose again and preferentially targeted the NH2-terminus of Dsg3, i.e. Dsg3EC1. Our findings demonstrate that rituximab acts by decreasing pathogenic IgG auto-antibodies against the NH2-terminus of Dsg3 (i.e. Dsg3EC1, EC2, EC3). Patients with clinical relapses showed IgG against the Dsg3EC1 suggesting that rituximab treatment did not completely abolish pathogenic B cells specific for the NH2-terminus of Dsg3.Thus, screening for IgG reactivity against Dsg3 subdomains may help to monitor the clinical response to rituximab treatment.

P085

Mutation of trex1, a sle-associated dnase, in a patient withlupus tumidus (LT)

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IntroductionLT is a rare variant of cutaneous LE, characterized by succulent deep urticaria-likeplaques devoid of epidermal changes with high photosensitivity. Autoantibodies areinfrequent (<10% of patients). DLE lesions and arthralgia can coexist. Genetic susceptibility is unknown, apart from a case with lesions following Blaschko-lines.
Case historyA 58-year-old female nurse presents with succulent bright red urticarial-appearing plaques on upper arms, shoulders and a single discoid plaque on her temple. Skin changes have appeared in bouts and healed over the past 12 years, often accompanied by arthralgia. She tests highly sensitive to UVA. Histology reveals superficial and deep periappendageal and perivascular lymphocytic infiltrates of T cells admixed with sparse B cells and plasma cells. ANA <1: 100, ENA negative. Therapy with hydroxychloroquine and sun-screen induces remission. Genetic analysis reveals a mutation in TREX1.
DiscussionGenetic susceptibility, autoimmunity, immune dysfunction and environmental trigger factors have been implicated to interact in LE pathophysiology. TREX1 is an intracellular mammalian DNAse, mutations of which we have linked in a previous paper to monogenic chilblain lupus and demonstrated to also exist in outbred SLE-patients included in DNA banks. As LT is triggered by UV and chilblain by cold, defining this mutation may reveal insight in the interaction between genetic susceptibility and environmental triggers.

P086

Mast cells control toxicity of bacterial toxins

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Mast cells (MCs) are well known for their key role in the induction of protective immune responses to bacteria. Most of our knowledge on MC-driven host defense against bacteria comes from models of septic peritonitis. Far less is known about the relevance of MCs in bacterial skin infections. Here, we tested the role of MCs in regulating skin inflammation following i.d. injection of the bacterial toxins streptolysinO (SLO) from Streptococcus pyogenes or α-Toxin (αT) from *Staphylococcus aureus*. Both toxins potently induced the degranulation of MCs ex vivo, indicating that MCs may contribute to the skin inflammation associated with bacterial infections, and/or the regulation of toxicity of the bacterial toxins. To test this hypothesis, we first assessed the inflammatory potential of the toxins by measuring ear swelling after i.d. injection. While SLO induced a potent dose-dependent inflammatory response which quickly resolved within a few hours, the injection of αT resulted in long lasting ear swelling. To investigate the role of MCs in the induction and maintenance of the toxin-induced skin inflammation, we injected SLO or αT into the ears of MC-deficient KitW/KitW-v mice (W/Wv). SLO injection failed to induce ear swelling in W/Wv mice, whereas MC-competent wild type (WT) and W/Wv mice, which were locally reconstituted with MCs, exhibited strong inflammatory responses indicating that SLO-induced skin inflammation is MC-dependent. In contrast, injection of αT induced rapid and potent skin inflammation in WT and reconstituted W/Wv mice and only slightly reduced ear swelling in W/Wv within the first hours after i.d. injection (136 ± 10 μM vs 177 ± 7 μM in WT mice at 4 h, $P < 0.01$). Most interestingly however, ear swelling in WT and reconstituted W/Wv mice reached a maximum after 2 days after which the inflammation slowly resolved, while the inflammatory response in W/Wv mice failed to resolve and reached a maximum after 11 days (543 ± 48 μM vs 252 ± 62 μM m in WT, $P < 0.01$). These surprising findings indicate a negligible role of MCs in the acute inflammatory response following αT injection but point towards an important role of early MC degranulation in resolving the toxin-induced long lasting inflammation.

These data reinforce the important role of MCs in innate immune responses to bacteria and show that, depending on the stimulus, MCs can either induce and/or limit skin inflammation.

P087

Nanotechnology to unravel quantitative and spatial requirements for mast cell degranulation using allergen bound to nano structured surfaces

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It is well known that FcεRI mediated degranulation of mast cells requires receptor cross linking, however, the requirements for the spatial distribution of allergen epitopes and receptors to elicit cross linking are not known. Multivalent antigens with several epitopes in proximity can mediate cross linking and thereby mast cell degranulation. Haptens, usually representing one epitope, must bind to carrier proteins and may be a suitable model to investigate spatial epitope requirements for FcεRI cross linking. We used the method of block-copolymer nanolithography to create artificial full antigens by coupling haptens to glass plates in different distances on a nano scale basis. Nanolithography allows depositing gold nano dots on glass and these provide anchor points to which haptens can bind mediated by polyethyleneglycol (PEG) linkers. Glass surfaces were created with either monovalent haptend initrophenol (DNP) bound to gold dots with a distance of exactly 70 nm between each anchor point or with DNP bound to a homogenous gold layer without nanostructure. In addition, long chain PEG 3000 (>70 monomers) and a short chain PEG 6 (6 monomers) linkers were used to investigate the impact of epitope motilityon the efficiency of cross linking. Mast cells were passively sensitised by anti-DNPIgE before incubation on differently coated glass surfaces or with soluble DNP bound to human serum albumin (HSA) as control. Mast cell degranulation was detected by measuring β-hexosaminidase release. Release of β-hexosaminidase in response to DNP bound to a homogenous gold layer by PEG 3000 (27 ± 8%) and using the short PEG 6 as linker (38 ± 4%) was well detectable and comparable to soluble multivalent DNP-HSA (42 ± 11%). Controls such as unsensitised cells on PEG 3000 or PEG 6with or without DNP as well as sensitised cells on PEG 3000 or PEG 6 without DNP showed only background release (8–11%). Most interestingly, the same results were obtained using nanostructured DNP with an epitope distance of 70 nm coupled to PEG 6. Thus, our data demonstrate that a monovalent allergen like DNP can mediate effective cross linking of FcεRI on mast cells with an epitope distance of up to 70 nm.

P088

Herpes simplex virus type 1 induces CD83 degradation in mature dendritic cells with immediate-early kinetics via the cellular proteasome

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Mature dendritic cells (DCs) are the most potent antigen-presenting cells within the human immune system. However, Herpes simplex virus type 1 (HSV-1) is able to interfere with DC biology and to establish latency in infected individuals. In this study, we provide new insights into the mechanism by which HSV-1 disarms DCs by the manipulation of CD83, a functionally important molecule for DC activation. Fluorescence-activated cell sorter (FACS) analyses revealed a rapid down modulation of CD83 surface expression within 6–8 h after HSV-1 infection, in a manner strictly dependent on viral gene expression. Soluble CD83 enzyme-linked immunosorbentassays, together with Western blot analysis, demonstrated that CD83 rapidly disappears from the cell surface after contact with HSV-1 by a mechanism that involves protein degradation rather than shedding of CD83 from the cell surface into the medium. Infection experiments with an ICP0 deletion mutant demonstrated an important role for this viral immediate-early protein during CD83 degradation, since this particular mutant strain leads to strongly reduced CD83 degradation. This hypothesis was further strengthened by cotransfection of plasmids expressing CD83and ICP0 into 293T cells, which led to significantly reduced accumulation of CD83. In strong contrast, transfection of plasmids expressing CD83 and a mutant ICP0defective in its RING finger-mediated E3 ubiquitin ligase function did not reduceCD83 expression. Inhibition of the proteasome, the cellular protein degradation machinery, almost completely restored CD83 surface expression during HSV-1infection, indicating that proteasome-mediated degradation and HSV-1 ICP0 play crucial roles in this novel viral immune escape mechanism.

P089 (V35)

HIV/gp120 activates human CD4+CD25+ regulatory T cells and prevents autoreactive T cell responses *in vivo*

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We have recently shown that stimulation of human CD4+CD25+Foxp3+ regulatory Tcells (Tregs) by crosslinking of CD4 surface molecules results in functional activation of Tregs *in vitro*. Since gp120, the major envelope glycoprotein of HIV-1 also binds to CD4, we analyzed the potential of HIV/gp120 to induce suppressive activity in human Tregs in a T cell receptor independent manner. We show here that HIV/gp120 efficiently induces suppressive activity in human Tregs resulting in a strong inhibition of co-cultured CD4+ and CD8+ effector T cells. More importantly, development of lethal graft-versus-host disease (GvHD) in NOD/SCID mice transferred with human peripheral blood cells can be prevented by either increasing the frequency of human Tregs or a single dose of gp120. This single dose of gp120 completely prevents all detectable GvHD effects, including colitis, inflammatory cellular lung infiltration, and loss of body weight up to 100 days after xenogenic transplantation. These data strongly suggest a novel anti-inflammatory activity of HIV/gp120 through functional activation of human Tregs. Besides prevention of lethal graft rejection, additional experiments are ongoing to evaluate the anti-inflammatory potential of HIV/gp120 *in vivo* for the modulation of effector T cell responses in humanized mouse models of asthma, allergy and autoimmune diseases.

P090

HIV-1 Viremia is associated with the upregulation of apoptosis-inducing TRAIL on Plasmacytoid dendritic cells

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Background: Plasmacytoid dendritic cells (pDCs) can exert innate effector functionsin viral disease. We recently found that pDCs stimulated with toll-like receptor-7(TLR-7) ligands are induced to express tumour necrosis factor-relate dapoptosis-inducing ligand (TRAIL) and mediate death in responsive cancer cells. It has been suggested that HIV-1 interacts with TLR-7 on pDCs and drives the iractivation.

Hypothesis: pDCs display TRAIL in HIV-1 viremia and trigger death in permissive T cells.

Methods: The surface expression of TRAIL and its pro-apoptotic receptors TRAIL-R1 and 2 as well as anti-apoptotic TRAIL-R3 and 4 were determined on subsets of circulating dendritic cells (DCs), T lymphocytes, NK cells and monocytes gained from viremic HIV-1-infected patients. Comparatively, such analyses were performed inHIV-1 patients on HAART with a stable viral load below the limit of quantification and healthy controls.

Results: TRAIL was found to be expressed in moderate amounts on monocytes and myeloid DCs (mDCs) but not on pDCs from healthy donors or treated recipients. In contrast, TRAIL expression was significantly upregulated on pDCs of untreated viremic patients, whereas no differences in TRAIL expression were found on monocytes, mDCs or T cell subsets when compared to healthy controls or patientson treatment. Importantly, the percentage of TRAIL+ pDCs as well as the mean amount of TRAIL molecules expressed per pDC correlated well with the individual plasma HIV-1 loads. Apoptosis-inducing TRAIL-R1 was significantly upregulated onCD4+ T cells of viremic HIV-1 patients as compared to those of healthy subjects, while antiretroviral therapy induced the anti-apoptotic TRAIL-R4 on these cells. CD4-T cells did not express any TRAIL receptors in significant amounts. In contrast to T cells, pDCs and monocytes not only expressed TRAIL-R1 but also the anti-apoptotic receptors TRAIL-R3 and TRAIL-R4, making them less susceptible to TRAIL-mediated death.

Conclusion: Our data provide evidence that pDCs have the potential to kill CD4+ Tcells in a TRAIL-dependent manner and, thus, could contribute to T cell depletion in HIV-infected patients.

P091

Functional properties of DC derived from Myosin 9b−/− mice

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Myosins are mechanoenzymes that convert chemical energy, liberated by AT Phydrolysis, into mechanical force along actin filaments. Based on homologous myosin head domain sequences, myosins can be subdivided into >18 different classes. The class IX myosin Myo9b can move along actin filaments without dissociation, although it has only a single motor domain. Functionally, class IX myosins are negative regulators of the small G-protein Rho that regulates the organization of the actin cytoskeleton. Because mature DC have a high cytoskeletalactivity which depends, amongst other factors, on actin organization, we analyzed the functionality of bone marrow derived Dendritic cells (DC) from Myo 9b−/− mice.

Although Myo9b is involved in the organization of the actin cytoskeleton, the DC morphology in fluidic cell cultures is indistinguishable between wt and Myo9b−/− DC. In contrast, when DC are cultured in a 3D-collagen gel the Myo9b−/− DC develop fewer dendrites and show a round cell shape in contrast to wt DC which form long dendrites and continuously change their cell shape. Moreover, the velocity of Myo9b−/− DC in a 3D-collagen gel is significantly reduced in comparison to wt DC. Further functional analysis shows that Myo9b−/− DC are less efficient inducers of T cell proliferation in a mixed lymphocyte reaction model, although the surface expression of costimulatory molecules is unaltered when compared to wt DC. These results demonstrate that Myo9b is of fundamental importance for the regulation of the actin cytoskeleton in DC and that a Myo9b−/− dependent loss of cytoskeletal activity results in reduced immunostimulatory capacity.

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Induction of IL-12 secretion and migration of human dendritic cells by adenoviral gene transfer of CD40L and combined IFN-gammatreatment

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In Dendritic cell (DC)- based vaccination trials often monocyte-derived DCs (moDCs) matured with a cocktail composed of IL-1beta, TNF-alpha, IL-6 and prostaglandineE2 (PGE2) have been used. It has been shown that PGE2 is indispensable for DC-migration but it is also known to inhibit IL-12p70 expression. Moreover other labs reported that these two essential functions of DCs are frequently not linked. Here we describe a new and interesting method to overcome this problem and to generate mature moDC capable of both migrating and producing biologically active IL-12p70.This could be achieved by a combined treatment with recombinant human interferongamma (rh-IFN-gamma) and adenoviral gene transfer of the trimeric CD40L. Immature moDCs were infected with an adenovirus serotype five coding for the trimerichuman CD40L (Ad5hCD40L) in an autologous monocyte-conditioned medium supplemented with GM-CSF, IL-4, IL-1beta, TNF-alpha, IL-6 and PGE2. Afterwards (1.5 h later) rh-IFN-gamma was added to the infected cells. Twenty-four hours later cells were checked for their migratory capacity, their IL-12 production as well as their CD40L expression and maturation status. Interestingly, moDCs infected with Ad5hCD40L and subsequently treated with rh-IFN-gamma fully matured, showed a high IL-12p70 secretion (approx. 700 pg/ml) as well as high migratory capacity. Even when the migrated cells were cultured for an additional time period of 24 h they were still able to secrete notable amounts of IL-12p70 (approx. 160 pg/ml). In summary, using this new maturation protocol, we could generate mature DCs which are able to both (i) migrate and (ii) secrete high levels of IL-12p70 providing a potentially new therapeutic tool for DC-based vaccination trials.

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Differentiation of human monocytes by glucocorticoids reveals uniquephenotypic and functional features that cannot be mimicked by regulatory cytokines such as IL-4, IL-6, and IL-10

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Glucocorticoids (GC) are still the most widely used immunosupressive agents inclinical 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. We have previously shown that in an *in vitro* model human monocytes treated with GC show a distinct phenotypic and functional profile resulting in an immunosuppressive phenotype rather than in deactivation of monocytes (1).GC-treatment resulted in secretion of regulatory cytokines such as IL-10. Thus the GC-induced phenotype may be influenced by autocrine cytokine action. To clarify these mechanisms and to further characterize the GC-induced phenotype we compared GC-induced monocytes with monocytes treated with both activating and deactivating cytokines.

Therefore we stimulated monocytes with regulatory cytokines IL-4, IL-6, or IL-10, respectively. The resulting monocyte populations were compared with GC-induced monocytes. We measured a range of phenotypic markers (e.g. CD11b, 14, 16, 64,163, CX3CR1), and tested monocyte-specific functions such as adherence, migration, apoptosis and phagocytosis. Summarizing, no tested interleukin was able to mimick the steroid-induced monocytes, neither phenotypically nor functionally. Hence, it can be emphasized that monocytes treated by GC develop distinct phenotypic and specific functional features. In addition, we examined the capacity of monocytes to influence T cell responses both in an antigen-dependent and -independent fashion. In antigenic T cell proliferation assays with steroid-induced monocytes as antigen presenting cells we showed an antigen-specific reduction in immune stimulation as measured by T cell proliferation and cytokine production. In conclusion, GC treatment generates regulatory monocytes that are capable to control ongoing T cell responses, and thus have a high potential to become valuable tools in immunotherapy against inflammatory diseases.

1: Ehrchen J et al. (2007) Blood Feb 1;**109** (3):1265–74.

P094

Regulation of chemokines and c-myc by interferon gamma-producing, antigen-specific Th1 cells

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Transgenic RIP1-Tag2 mice express the T antigen (Tag) of the simian virus 40 undercontrol of the rat-insulin-promotor (RIP) leading to development of endogenously growing ß-cell tumours of the pankreatic islets. Treatment of mice with interferon γ?(IFNγ)-producing, antigen-specific Th1 cells leads to reduction of the tumour size and significantly prolongs the life of treated animals as compared with sham-treated controls. However, neither tumour-infiltrating cytotoxic T cells nor significant apoptosis were detected *in vivo* in the tumour tissue of treated mice. Instead, reduced angiogenesis was demonstrated by immunohistochemistry using an anti-CD31antibody, a marker for endothelial cells. Furthermore, BrdU labelling revealed a decreased proliferation rate in tumours of Th1-treated RIP1-Tag2 animals. To elucidate the mechanisms underlying the therapeutic efficacy of antigen-specific Th1 cells, we analyzed the influence of Tag-specific Th1 cells on antiangiogenic chemokines, i.e. CXCL10 (IP10) and CXCL11 (MIG), and on c-myc regulation in an *in vitro* approach. After restimulation with antigen-presenting cells (APCs) and Tag-peptide, the polarization of Th1 cells was verified by enzyme-link edimmunosorbent assays (ELISA) of interleukin-4 (IL-4) and IFNγ. The transcriptional activity of the APCs and Th1 cells after restimulation was determined by RT-PCR ofIP10, MIG and c-myc. The phosphorylation status of the transcription factor c-mycwas examined by Western blot. Restimulation of Th1 cells indeed led to the expected cytokine profile, i. e. an increase of IFNγ?whereas IL-4 was detected at background levels. IP10 and MIG were highly upregulated after restimulation, but c-myctranscripts remained on a constant level. However, Western blot analysis showed adramatic and fast decline of c-myc phosphorylation. To unravel molecular changes inAPCs by Th1 cells, we depleted the Th1 cells after restimulation, and subsequently analyzed c-myc signalling. First results demonstrated regulation of c-myc by dephosphorylation in purified APCs. In conclusion, Tag-specific Th1 cells reduce the tumour growth of endogenous in sulinoma, presumably by inhibition of angiogenesis and proliferation. In accordance with the *in vivo* data, *in vitro* restimulation of Tag-specific Th1 cells by APCs leads to induction of antiangiogenic chemokines and regulation of c-myc signalling.

P095

Langerhans cells are crucial for early expansion of CD8+ T cells in experimental leishmaniasis but dispensable for resolving the infection

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Epidermal Langerhans cells (LCs) represent a subset of dendritic cells (DCs), which appear in the outer compartment of the skin. In the experimental model of leishmaniasis LCs were discussed to be crucial for the induction of cutaneous immune reactions. However, recently it was documented that dermal-derived DCs rather than epidermal LCs might represent the key players in experimental leishmaniasis. To answer the question whether LCs are indeed involved in T cell mediated immune response against *Leishmania (L.) major*, knock-in mice expressing the human diphtheria toxin receptor under control of the langerin gene were used toconditionally ablate epidermal LCs. Following depletion, mice were infected subcutaneously into the footpad with *L. major* parasites and the immune response in the footpad-draining lymph node (LN) and at the site of infection was analyzed overtime. Investigation of i) the course of infection, ii) the production of L. major-specific immunoglobulins and iii) the development of a memory T cell response revealed no differences between LC-ablated and control mice. In addition skin-draining LNs cells were isolated and re-stimulated with soluble Leishmania antigen to address the question whether *L. major* specific T cells are primed after LC-ablation. We detected comparable proliferation of antigen specific CD4+ T cells in LC-ablated and control mice. In contrast, the proliferation of CD8+ T cells was significantly decreased in LC-ablated mice. In agreement with this result we observed reduced numbers of activated CD8+ T cells at the site of infection whereas the number of activated CD4+T cells was not affected in the absence of LCs. Therefore, we assume that LCs interact transiently with CD8+ T cells, which are known to play a minor role in the high dose infection model of experimental leishmaniasis, but do not interact with CD4+ T cells. In conclusion, this is the first study demonstrating that epidermal LCs are dispensable for the induction of a protective immune response against Leishmania parasites.

P096 (V22)

Induction of IL-10 producing suppressor T cells by non-pathogenic gram-negative bacteria clears atopic dermatitis

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Non-pathogenic bacteria are part of the physiological flora on skin and gut epithelium without inducing inflammation. Some strains have even been used to treat allergic inflammation. We therefore performed a prospective, double-blind, placebo controlled, randomized clinical trial with 80 patients to investigate the therapeutic potential of non-pathogenic, gram-negative, thermal water bacterium Vitreoscillafiliformis (Vf) applied to atopic patient skin and Vf mediated reduction of SCORAD and pruritus was highly significant compared to placebo. To understand the underlying mechanisms, Vf activated DC were analyzed in detail. Several Vf preparations led to maturation of DC as monitored by upregulation of CD80, CD86 and MHC II. However, marked differences were seen in regard to cytokine production. As expected, isolated Vf LPS induced high levels of IL-12p70 in DC via aTLR4-dependent pathway and IL-10 levels remained low. Investigating the complete Vf bacterial extract revealed that the effects of LPS were only minor and IL-12 levelsin DC remained low. In contrast to IL-12, DC stimulated with Vf bacterial extract secreted large amounts of IL-10. Consequently, DC from several knock-out and wildtype mice were analyzed demonstrating that IL-10 production induced by Vf was almost completely dependent on TLR2 suggesting a hitherto unknown but dominantTLR2 ligand with immuno-modulatory activity. Indeed, co-cultures of Vf stimulated DC with naïve T helper cells resulted in the induction of remarkable levels of IL-10 in Th cells. To investigate functional consequences, co cultures with Th1 effector cells were carried out demonstrating strongly suppressed proliferation of T effector cells by the addition of these IL-10+ Th cells. Thus, effective treatment of atopic dermatitis by application of Vf may be the result of Vf induced, TLR2 dependent IL-10 induction inDC orchestrating the induction of IL-10+ suppressor T cells. Our data demonstrate not only a new therapeutic strategy for inflammatory skin diseases like atopic dermatitis, but also a possible general principle of how non-pathogenic bacteria stabilize the immune barrier of surface organs such as the skin or the gut.

P097

CD83 knockdown in monocyte-derived dendritic cells by small interfering RNA leads to a diminished T cell stimulation

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Mature human dendritic cells (mDCs) are the most powerful APCs known today,having the unique ability to induce primary immune responses. One of the best known surface markers for mDCs is the glycoprotein CD83, which is strongly up-regulated during maturation, together with costimulatory molecules such as CD80and CD86. When CD83 surface expression was inhibited by interference with the messenger RNA export or by infection with certain viruses, DCs showed a dramatically reduced capability to induce T cell proliferation. However, in these casesside effects on other cellular functions cannot be excluded completely. In this study we present an efficient method to specifically influence CD83 surface expression by the use of RNA interference. We used small-interfering RNA targeted against CD83 and carefully evaluated an electroporation protocol for the delivery of the duplex into the cells. Furthermore, we identified freshly prepared immature DCs as the best target for the application of a CD83 knockdown and we were also able to achieve along lasting silencing effect for this molecule. Finally, we were able to confirm thatCD83 functions as an enhancer during the stimulation of T cells, significantly increases DC-mediated T cell proliferation, and goes hand in hand with clear changes in cytokine expression during T cell priming. These results were obtained for the first time without the use of agents that might cause unwanted side effects, such as low m.w. inhibitors or viruses. Therefore, this method presents a suitable way to influence DC biology.

P098 (V23)

MCS-18, a novel natural product isolated from Helleborus purpurascensinhibits DC activation and prevents autoimmunity

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MCS-18, a novel natural product isolated from Helleborus purpurascens, is able to inhibit the expression of typical molecules of mature DC such as CD80, CD86, and especially of CD83 subsequently leading to a clear and dose dependent inhibition of the DC-mediated T cell-stimulation. Furthermore, MCS-18 impeded the formation of the typical DC/T cell clusters, which are essential to induce potent immune responses. Interestingly, MCS-18 also inhibited CCR7 expression on DC which subsequently lead to a dose dependent block of the CCL19-mediated DC-migration.MCS-18 not only inhibited the DC-mediated T cell stimulation but also the anti-CD3/anti-CD28 mediated T cell-stimulation. Strikingly, MCS-18 also strongly reduced the paralysis associated with the experimental autoimmune encephalomyelitis (EAE) -which is a murine model for human multiple sclerosis in a prophylactic as well as in a 'real' therapeutic setting. Even when the EAE was induced for a second time, theMCS-18-treated animals were still protected, suggesting that MCS-18 induces a long lasting suppressive effect. In addition, and very important for the potential practical application in humans, MCS-18 was also active when administered orally. MCS-18-treatment almost completely reduced leukocyte infiltration in the brain and in the spinal cord. In conclusion, using *in vitro* as well *in vivo* assays we were able to show that MCS-18 exerts a strong immunosuppressive activity with remarkable potential for the therapy of diseases characterized by a pathologically over-activated immune system.

P099

IL-4 potently regulates peripheral blood derived conventional dendritic cell function

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Peripheral blood contains three distinct subsets of myeloid human conventional dendritic cells (cDC) called MDC1, MDC2 and slanDC. Like other leukocytes, cD Center peripheral tissues and are among the first cells to encounter and recognized iverse danger signals. In contrast to the *in vitro* model of monocyte-derived DC, pre-existing and readily available human peripheral blood cDC are less well characterized. Interleukin (IL)-4 is an important cytokine dominating inflammatory responses in atopic individuals, however, the precise impact of IL-4 on newly recruited cDC has not been analysed. To this end, immature cDC were isolated from peripheral blood. Toll-like receptor (TLR) expression was then determined by quantitative real-time PCR as well as confocal fluorescence microscopy and cDC were activated with the respective TLR ligands. Among others, lipopolysaccharide (LPS) activation of slanDC (M-DC8pos/CD11cpos/CD86low/CCR5pos) via TLR4 ledto vigorous upregulation of MHCII and CCR7, while CCR5 was no more detectable. Morphology and maturation of cDC were not altered by the combination of signals via TLRs and IL-4R. However, the presence of IL-4 markedly altered the cytokine profile of all three cDC subsets and strongly enhanced IL-12p70 production. In the absence of IL-4 an IL-12/IL-10 ratio <1 was detected, whereas the combination of TLR ligands and IL-4 upregulated IL-12 and almost completely abolished IL-10 production. Investigating underlying mechanisms we found that enhanced IL 12p70 levels depended on IL-4 mediated IL-10 reduction. Co-cultures of IL-4 treated cDC with naïve autologous T cells revealed markedly enhanced IFN-γ?and even reducedIL-4 secretion upon T cell restimulation. Our data demonstrate that fully differentiated peripheral blood cDC are still susceptible to regulation and that one cytokine like IL-4 leads to massive upregulation of IL-12p70 production. Our findings are of general importance demonstrating functional plasticity of peripheral blood cDC and highlighting a new role for IL-4 in directly regulating cDC function. Moreover, these studies are of relevance for the understanding of immune responses in atopic diseases like atopic dermatitis that display the so called 'Th2 to Th1 shift# within peripheral tissues such as lung and skin.

P100

A fast and robust method to clone and test the function of T cell receptors

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The identification and cloning of T cell receptor (TCR) alpha- and beta-chains from specific T-cell clones is often required in immunological research and inimmunotherapy. However, a simple PCR to identify the TCR chains is not possible, because, although the sequence of the 3' constant domain of a TCR chain is known, the sequence of the 5' variable domain is not. Up to now, the TCR chain subtype was identified by performing single PCRs for each subtype; i.e. 40 PCRs for the alpha chain, and 60 PCRs for the beta chain. Therefore, a universal PCR-based protocol was developed, which allows amplifying and cloning of the TCR alpha- and beta chainmRNAs from very small numbers of cells, without previous knowledge of the TCR variable domain subtype. This protocol was based on the amplification of total mRNA of the T cells, during which a defined flanking sequence was attached to the 5'site of the cDNA. To acquire specificity for the TCR α- and β-chains cDNA, a second PCR was performed with primers that bind to the constant regions of either the α- or the β-chain, and to the introduced 5' flanking sequence. The obtained PCR product was sequenced with an additional primer binding further within the constant region of the cDNA fragment. This revealed the TCR subtype, and allowed the design of specific primers to amplify the complete coding sequence from the amplified total cDNA, which was then inserted into a plasmid. With this protocol, TCR specific forHLA-A2/MelanA, HLA-A2/HIVgag, and the TCR from Jurkat T cells were cloned. To test the function of the cloned TCR, the amplified cDNAs were cloned into *in vitro* transcription vectors and were transcribed back into RNA. This RNA was co-electroporated with an NFAT-luciferase reporter construct into Jurkat T cells. These cells were stimulated with peptide-loaded target cells, and via a luciferase assay, the TCR function was detected. This new protocol represents a rapid and low-cost tool for the identification and functional testing of TCR chains of T-cell clones, which can then be transferred to bulk T cells for application in immunotherapy.

P101

Making T cell independent of MHC - electroporation of RNA encoding chimeric T cell receptors

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The adoptive transfer of T cell receptors (TCR) to bulk T cells represents an innovative way of treating cancer. However, the use of wild-type TCR requires presentation of the antigen in an MHC context, and many tumours conceal themselves from the immune system by malfunctions in the processing and presentation machinery. An approach to circumvent the necessity of MHC-restricted antigen presentation is the use of chimeric TCR (cTCR), which consist of the antigen-binding moiety of an antibody against a cancer surface antigen, fused to signalling domains of the CD3-signalling complex and/or the signalling domains of co-stimulatory receptors such as CD28. Until recently, the transfer of these receptors required retroviral transduction, which harbours hazards like insertional mutagenesis and stable genetic alterations. Therefore, we introduced a cTCR into ex-vivo-generated T cells by RNA transfection. Since RNA can not integrate into the genome, this method excludes the risk of insertional mutagenesis and limits any unexpected autoimmunity to a few days. A cTCR, specific for ErbB2/Her2-neu was used. Although this tumour-associated surface antigen was originally found on breast cancer cells, we found expression on melanoma cell lines as well. The transfected CD8+ T cells did recognize ErbB2+ but not ErbB2- cells, and responded with production of IL-2, TNFα?and IFNγ.Experiments with another cTCR, specific for CEA, are under way, and T cells transfected with these receptors will be tested for specific cytokine production and cytolytic capacity. This approach is easily transferable into the clinic, as transfection with RNA is classified not to be gene therapy, and is therefore well suitable to complement immunotherapeutic therapy of cancer.

P102

Providing specific T-cell help by electroporation of TCR-encoding RNA intoCD4+ T cells

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Immunotherapy of melanoma has mainly concentrated on the generation of tumour-specific T cells, with emphasis on CD8+ T cells. For a lasting cytotoxic Tlymphocyte (CTL) response, however, help by CD4+ T cells is required, although the exact mechanism by which CD4+ T cells modulate the priming and expansion of CTLs is not yet fully understood. We used RNA-electroporation to adoptively transfera melanoma-antigen-specific TCR into ex-vivo-generated CD4+ T cells to examine the mode of action of T cell help in an antigen-specific manner, with the final goal of clinical application of these transfected cells in tumour therapy. In these experiments, a gp100/HLA-A2-specific TCR was used. This receptor is of high affinity and is therefore not depending on the binding of the co-receptor CD8. Electroporation of CD4+ T cells with RNA coding for this receptor resulted in antigen-specific production of the cytokines IL-2, IL-8, TNF-alpha and IFN-gamma in substantial amounts, while IL-4 and IL-10 were produced in much smaller quantities after stimulation with peptide-loaded DC. The production of cytokines was not limited to the T cells, because intracellular staining revealed that both DC and T cells produced TNF-alpha upon stimulation. Moreover, phenotypic changes of the T cells and DC were determined by FACS after the stimulation. An increase in expression of the maturation markers CD25, CD40, CD80, CD83, and CD86 on the DC was observed, and on T cells the expression of the activation marker CD25 was increased. Transwell experiments revealed that the activation of the T cells was completely cell-cell-contact dependent, while the maturation of the DC was in part mediated by soluble factors. These data indicate that TCR-transfected CD4+ T cells can induce DC maturation, and can therefore be used to provide T-cell help. Accordingly, this method for transient TCR transfer using RNA electroporation into CD4+ T cells can form a new strategy to induce more efficient CD8+ T-cell responses for the immunotherapy of cancer.

P103

Activation of mast cells via TLR2 results in strong cytokine production and release, but not degranulation

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Recent data indicate that activation of mast cells (MCs) via Toll-like receptor 2(TLR2) is crucial to initiate protective host defense responses against gram-positive bacteria, which rely on MC degranulation. To better characterize the effects of TLR2 mediated activation on MCs we assessed the release of preformed and de novo synthesized mediators released from different MC populations in response to variousTLR2 ligands. Surprisingly, we found that murine peritoneal MCs fail to degranulate *ex vivo* after stimulation with the TLR2 ligand lipoteichoic acid (LTA), the TLR2/1ligand Pam3Cys, or the TLR2/6 ligand MALP-2. The same results, i.e. absence of peritoneal MC degranulation, were seen *in vivo* as assessed in mice challenged intraperitoneally with these TLR2 ligands. In contrast, TLR2 activation strongly induced the production and release of various pro-inflammatory cytokines by peritoneal MCs (IL-6 > IFN-gamma > TNF-alpha > IL-1 > GM-CSF). Interestingly,TLR2-mediated production and release of cytokines from immature bone marrow-derived cultured MCs was limited to IL-6, indicating that murine MCs only acquire full responsiveness to TLR2 ligands once they have differentiated to mature tissue residing MCs. In support of this hypothesis we found that TLR2 expression is significantly higher in peritoneal as compared to immature MCs (average percentage of TLR2 positive cells 15% vs 4%, $P = 0.042$). Taken together, our data suggest that MC degranulation observed during infections with gram-positive bacteria is not due to bacteria-derived TLR2 ligands. In contrast, MC activation via TLR2 may be important for the local increase of pro-inflammatory cytokines and the prolongation of inflammation triggered by the exposure to bacteria harbouring TLR2 active components.

P104 (V24)

Reprogramming CD8+ T cells with a HIV-1-specificity by electroporation of TCR-encoding RNA

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In patients, HIV-1 establishes a persistent infection and destroys the CD4+ immune cells, which finally leads to a paralyzed immune system that is unable to defend the body against opportunistic diseases. The currently applied treatment, the highly active antiretroviral therapy (HAART) only suppresses the virus, but does note liminate the virus infected cells. However, an immune reaction against the virus takes place, and high levels of HIV-1-recognizing CD8+ cytotoxic T lymphocytes (CTL) with a widespread specificity, especially against conserved epitopes, are considered to play a critical role for long-term control of HIV-1 replication. Unfortunately, most HIV-infected patients are incapable of generating such a powerful immune response. A possible immunotherapy is, therefore, the adoptive transfer of T cells, which are reprogrammed by introduction of an HIV-specific T cell receptor (TCR). Until now, these HIV-specific CTL were generated by retroviral transduction of TCR-encoding cDNA. However, this strategy harbours the threat of stable genetic alteration of autologous cells, and the development of a life-long autoimmunity through a mispairing of the induced TCR and the endogenous TCR. Therefore, we studied TCR transfer by RNA electroporation into CD8+ T cells. AnHIVpol-specific TCR, which recognized the HLA-A2 restricted peptide ILKEPVHGV, was used. T cells, reprogrammed with this receptor, obtained the ability to produce the pro-inflammatory cytokines IL-2, TNFα, and IFNγ?after stimulation with peptide-loaded target cells, and were able to efficiently and specifically lyse these targets, even after cryopreservation. The cytolytic function of the reprogrammed T cells persisted for at least 72 h after transfection, a time-span long enough for the transfected T cells to migrate into the lymph nodes, where HIV-infected targets are present. A peptide-titration assay showed that the lytic avidity of theTCR-RNA-electroporated CD8+ T cells was in the same range as that of the parental CTL clone. Taken together, this technology represents an innovative, secure, and easy method to produce virus-specific T cells, and may represent a new tool in the fight against HIV infection.

P105

Anergic induced regulatory T cells exhibit strongly impaired nuclear translocation of NFAT

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Previously, we have characterized a population of human induced regulatory T cells (iTreg), generated *in vitro* by stimulation of T cells with tolerogenic IL-10 modulated dendritic cells (IL-10DC). These iTreg are anergic and display a MAP kinasep38-dependent G1 cell cycle arrest, mediated by increased expression of the cd kinhibitor p27Kip1. The anergic state, associated with impaired production of IL-2, is a prerequisite for the regulatory function of iTreg and both, high amounts of exogenousIL-2 or specific inhibition of p38 can prevent or break anergy. NFAT has been linked to anergy induction in clonal anergy. In this study, we analysed the expression and subcellular distribution of NFAT transcription factors to characterize intracellular mechanisms involved in mediating the anergic state in iTreg. As controls, effector Tcells (Teff) generated by stimulation with mature DC were used. In both, iTreg andTeff, we observed no differences in the expression levels of NFAT c2 after primary culture with mDC and IL-10DC, respectively, as determined in Western blots of total cell lysate. After restimulation with anti-CD3/anti-CD28, Teff exhibited a strong nuclear mobilization of NFATc2 which peaked after 2 h as determined in kinetic studies (2–96 h) by use of nuclear extracts. In contrast, in iTreg nuclear translocation of NFATc2 was severely impaired. Furthermore, we observed a reduction in total NFATc2 protein expression over time (up to 96 h after restimulation) in iTreg as compared to Teff. Thus, unlike situation of clonal anergy, where nuclear mobilization of NFAT in the absence of other factors such as AP-1 contributes to anergy, inhibited activation of NFAT may play a crucial role in anergy induction in human iTreg.

P106 (V25)

DC cancer vaccines: development of new strategies for antigen loading

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Although dendritic cell (DC)-based vaccination has shown encouraging responses in melanoma and renal cell carcinoma, further improvement of the efficiency of these vaccines is needed. To find the most efficient DC antigen-loading technique, differentmethods, such as direct peptide loading, electroporation of tumour-antigen RNA, orthe use of antibody-antigen constructs need to be thoroughly analyzed and compared. Therefore, the cancer-testis antigen MAGE-A3, which is expressed in many tumours including malignant melanoma and multiple myeloma, was chosen as a tumour model. As a functional read-out system for the antigen-presentation efficiency, we transfected CD4+ T cells with RNA encoding T cell recptors (TCR) recognizing MHC-presented MAGE-A3 epitopes. After co-incubation with the DC, cytokinerelease was quantified. As one possible loading strategy, we electroporated the DCwith MAGE-A3-DCLAMP RNA. The DCLAMP sequence targets the antigen to lysosomes, which leads to MHC class II presentation. Indeed, electroporation of mature (m)DC with MAGE-A3-DCLAMP RNA resulted in HLA-DP4 class II-restricted presentation of the MAGE-A3 peptide KKLLTQHFVQENYLEY (KKL, aa 243–258)antigen and induction of cytokine release (i.e. INFγ?and IL-2) by autologous MAGE-A3/DP4-specific CD4+ T cells. For a different loading strategy, we targeted DEC-205, an endocytosis receptor expressed on the surface of DC. We designed antibody-antigen constructs, consisting of a single-chain variable fragment (scFv) directed against DEC-205, genetically linked to different parts of the MAGE-A3antigen (i.e. the KKL, and EVDPIGHLY (aa 168–176) peptides). The fusion proteins were expressed in 293T cells and were purified via a HIS-tag. They were detected in western blot analysis and displayed binding to immature (i) DC and mDC, and specific-binding to DEC-205-transfected CHO cells. We could show in preliminary experiments that loading of DC with the anti-DEC-205scFv-MAGE-A3-KKL constructled to antigen presentation, whereas incubation of DC with the heat-inactivated construct or a control construct did not. Taken together, we have established tools to thoroughly compare different antigen-loading strategies of DC, which hopefully will contribute to the future generation of better DC-based anti-cancer vaccines.

P107

Murine mast cell proliferation and apoptosis is regulated by activation of Proteinase-activated receptors

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Proteinase activated receptors (PARs) are activated via proteolytic cleavage by several serine proteases such as thrombin, trypsin, tryptase, and cathepsin Gthereby contributing to inflammatory skin reactions. As mast cells (MCs) can importantly induce and contribute to skin inflammation, we sought to investigate the expression and function of PARs on mast cells. Interestingly, we found that all four PARs 1–4 are expressed on both immature murine bone marrow derived mast cells (BMMCs) and mature murine connective tissue type mast cells (CTMCs), as assessed by PCR analyses. We also examined the influence of the activation of mastcells via PARs on mast cell-dependent passive systemic anaphylaxis by determining the body temperature of mice injected i.p. with anti-DNP-IgE and DNP. Surprisingly, the serine proteases thrombin, trypsin, and cathepsin G neither effected the decrease in body temperature observed in mast cell-mediated passive systemic anaphylaxis nor did they result in a body temperature decrease by themselves. In contrast, activation of CTMCs with the PAR agonist combination thrombin, trypsin, and cathepsin G *in vitro* resulted in reduced proliferation and enhanced apoptosis compared to unstimulated cells. This was also true in MCs subjected simultaneously to an IgE-dependent activation. These findings show that mast cell proliferation and apoptosis may be modulated by PAR/PAR agonist-interactions, whereas IgE-dependent mast cell-mediated anaphylaxis is not modulated by PARs. Thus, skin mast cell numbers may be under the control of serine proteases via paracrine or autocrine effects.

P108

Identification and role of carboxypeptidase D in lupuserythematosus

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Transforming growth factor-beta (TGF-beta) is a multifunctional cytokine that mainlyacts as an inhibitor of immune reactions, e.g. by inhibiting macrophage functions. Alack of functional TGF-beta in animal models leads to autoimmune disease, and dysregulated TGF-beta signalling is implicated in human autoimmune diseases suchas lupus erythematosus (LE). In order to define target genes that mediate the inhibitory signals of TGF-beta, we analyzed genes expression in macrophages upon stimulation by TGF-beta using DNA microarray analysis. One of the TGF-beta regulated genes was identified to be carboxypeptidase D (CpD), a 180-kDa type I membrane protein. CpD showed regulation by TGF-beta in various cell types of both, murine and human origin. Based on the involvement of TGF-beta target proteins in autoimmune diseases, we examined 24 blood samples of patients with cutaneous and systemic LE. Interestingly, CpD was significantly downregulated in CD14-positive cells isolated from LE patients. Small interfering RNA analysis showed, that downregulation of CpDled to downmodulation of TGF-beta itself, suggesting a role for CpD in a positive feedback loop. Further, initial data suggested that downregulation of CpD also led to dysregulation of Toll-like receptor (TLR)-7 and TLR-9 mRNA levels in macrophages, suggesting across talk between CpD and the innate immune system, thus providing further evidence for a role of this enzyme in recognition of circulating self-DNA in LE pathogenesis. This is the first report that demonstrates CpD as a TGF-beta target gene that is implicated in the pathogenesis of LE.

P109

(Cross)-presentation of ovalbumin by langerhans cells (LC) and dermal dendritic cells

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We recently demonstrated that murine LC are capable of cross-presenting proteinantigen. Though it is not yet clear among which premises LC themselves presentantigen to T cells *in vivo* in the lymph node, they may provide specific advantages for immunisation via the skin. We further studied this capacity of LC in relation to dermaldendritic cells (dDC). Ovalbumin was applied epicutaneously. After 6 h epidermal and dermal explants were placed in culture. Migratory DC cells took up the protein in the epidermis and dermis. They processed and (cross)-presented Ovalbumin toantigen-specific T-cells (OT-I and -II) *in vitro*. Interestingly, in contrast to LC, dermal DC proved to be poor stimulators for MHC II-restricted OT-II cells. Similar findings were made when Ovalbumin was injected intradermally. Furthermore, the impact of endotoxin-free Ovalbumin was measured comparatively. Responses were in principle unchanged, whereas the magnitudes of T cell proliferation were lower. This made it feasible to study the influence of systemic versus topical TLR ligands *in vivo* on the antigen-presenting capacity of skin DC. In doing so, systemically applied LPS downregulated cross-presentaion *in vivo*. Our findings emphasize an immungenic (rather than inert) role of LC and a superiority of LC over dermal DC with regard to MHC II-restricted responses emanating from the skin.

P110

Slan-dendritic cells (6-Sulfo LacNAc-expressing dendritic cells) in contrast toCD1c+ dendritic cells express high levels of functional TLR7- and TLR8-receptors

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The family of Toll like receptors (TLR) enables the recognition of specific microbial components widely expressed in bacteria, fungi, protozoa and viruses. Individual dendritic cell (DC) subtypes largely differ in their repertoire of expressed TLR which critically determines their functional role during the initiation of specific immune responses against individual pathogens. We previously described a highly proinflammatory type of myeloid DC called slanDC (6-Sulfo LacNAc-expressing dendritic cells) that produced particularly high levels of TNF-a as well as IL-12 in response to stimulation by the bacterial TLR4-ligand lipopolysaccharide. However, the exact expression pattern of TLR by slanDC remained unknown. Here we demonstrate that slanDC express high mRNA encoding for TLR2, 4, -5, -6, -7 and -8 as revealed by qualitative and quantitative RT-PCR. In contrast, CD1c+ myeloid DC express TLR3, but failed to express significant levels of TLR2, -4, -6 and -8, and plasmacytoid DC (pDC) expressed preferentially TLR7 and -9. In our functional studies we focussed on the relevance of the TLR7 and -8 expression by slanDC, whose natural ligands are single stranded viral DNA. When slanDC were stimulated with R848, a ligand of TLR7 and TLR8 we observed a by far higher IL-12p40 andIL-12p70 production among slanDC compared to CD1c+ DC and pDC. Specificligation of TLR7 (3M-001) and TLR8 (3M-002) revealed that slanDC, but not other DC subsets, can respond to both ligands with a strong production of IL-12 as well as TNF-a. The expression of functional TLR7 and TLR8 by slanDC uncovered in this study may have practical implications when designing therapeutic vaccinations to induce prominent Th1-responses specific for tumour antigens.

P111

Low doses of denileukin difitox (ONTAK) induce survival of CD4+CD25+regulatory T cells *in vitro*

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Denileukin diftitox (ONTAK™) is composed of interleukin 2 (IL-2) and diphtheria toxin, designed to direct the cytocidal action of the toxin to cells that overexpress the IL-2receptor (IL-2R). *Ex vivo* studies have shown that ONTAK™ interacts with the high-affinity IL-2R and undergoes internalization, resulting in rapid cell death. ONTAK™ is approved for treatment of persistent or recurrent cutaneous T-cell lymphoma (CTCL) expressing the CD25 component of the IL-2R. Additionally, some groups described that ONTAK™ was able to kill CD4+CD25+ regulatory T cells (Treg). Treg play a major role in suppressing anti-tumour immunity and are significantly increased in tumour bearing individuals. Because of our interests in immunotherapy of melanoma and since so many controversial data exist about the killing effect of ONTAK™, we performed *in vitro* studies with this drug. We investigated the influence of ONTAK™ on either resting or activated Treg. BecauseCD4+CD25- conventional T cells (Tcon) upregulate CD25 after activation, we additionally examined the effect of ONTAK™ on these cells. Indeed we found that Tcon are only killed by ONTAK™ when the cells were preactivated by anti-CD3/anti-CD28 stimulation. ONTAK™ treatment led to a clear, dose-dependent increase of apoptosis in Treg that where activated simultaneously, even in low doses, while resting and preactivated Treg showed no apoptosis. Therefore activation of Treg seems to be necessary for ONTAK internalization. Low doses of ONTAK™ even improved survival of resting and preactivated Treg. Possibly because ONTAK™ was not internalized and remained on the cell surface where it delivered an IL-2R signal to the cells. In fact, we found a strong increase of STAT-5 phosphorylation upon ONTAK™ treatment which designates that intracellular signalling occurs. Caspase-3 activation, but seemed Bcl-2 independent. Our findings indicate that low concentrations of ONTAK™ can induce survival of Tregin stead of depletion. This survival effect appears due to binding and intracellular signalling in the absence of significant internalization. The described phenomenonmay explain the discrepant *in vivo* effects regarding depletion of T regs in patients by ONTAK™, and has to be taken into account in the design of future clinical trials.
*equal contribution

P112 (V28)

Homotypic interactions mediated by Ly108 and SLAM control NKT lineage development

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NKT cells are distinct from conventional T cells in a number of ways. They are CD1restricted, have an invariant TCR, express activation markers such as CD44 andCD122 as well as NK receptors, and rapidly secrete large amounts of both Th1 and Th2 cytokines upon stimulation. T and NKT cells share a common alpha beta T cell receptor (TCR) expressing precursor. Lineage commitment is determined during interactions with ligand-expressing cells in the thymus. Whereas mainstream thymocyte precursors recognize Major Histocompatibility Complex (MHC) ligands expressed by stromal cells, NKT precursors interact with CD1d ligands expressed by cortical thymocytes. The alternative differentiation pathway of NKT cells relies on signalling mediated through both SAP (Slam associated Protein) and the Src kinaseFyn. In our work we investigated the exact stage of the NKT cell development block seen in Fyn and SAP deficient mice. In addition, we analyzed the surface expression of receptors known to signal through SAP and Fyn. Of the six receptors of the SLAM family, both Ly108 and SLAM were shown to have the highest expression levels on double positive (CD4+CD8+) thymocytes, which are crucial for mediating selection of NKT cell precursors. When analyzing both Ly108 and SLAM deficient mice, we found only minor effects on NKT cell development. However, when forced to compete with wildtype bone marrow in a mixed bone marrow chimera setting, there was a mild but clear defect. In order to show the effect of a loss of both receptors, we generated mixed bone marrow chimera, where in a 'pseudo-double knock out' situation thymocytes being selected to the NKT cell lineage could receive neither a SLAM nora Ly108 signal (Ly108−/−CD1−/−CD45.2+SLAM−/−CD45.1), we saw a very significant developmental loss of NKT cells. We thus demonstrate that homotypic T-Tinteractions generate 'second signals' mediated by the cooperative engagement of the homophilic receptors Ly108 and SLAM and downstream recruitment of the adaptor SAP and the Src kinase Fyn, which are essential for the lineage expansion and differentiation of the NKT lineage.

P113 (V29)

Lympho-Epithelial-Kazal-Type-Inhibitor (LEKTI)-2: a novel epithelial peptide antibiotic

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We recently purified several human peptide antibiotics from psoriatic scale and stratum corneum extracts. Following the hypothesis that healthy skin is protected from infection by producing a panel of antimicrobial peptides, we systematically analysed heel stratum corneum extracts for human antimicrobial peptides. As a result, we purified an *E. coli*-cidal 7-kDa peptide, a novel protein as revealed by N-terminal sequencing. Based on the N-terminal 30-amino-acid sequence, we cloned the full-length cDNA sequence for this protein and found that it encodes a protein of 86 amino acids, which fits with the characteristics of the serine protease inhibitors Kazal type (SPINKs) family, a putative N-terminal signal peptide domain and a typical Kazal domain. Due to its location on chromosome 5p33.1, telomeric to Spink7 and Spink5, the corresponding gene was designated as Spink9. Meanwhile, the mature peptide was termed 'LEKTI-2' as its Kazal domain is highly similar to domains 2 and15 of the multi- 'Lympho-Epithelial-Kazal-type-Inhibitor (LEKTI)'. RT-PCR analyses revealed Spink9 to be expressed in thymus, tonsils, adenoids, bronchial epithelial cells and cultured skin keratinocytes. To get insight into the biological function ofLEKTI-2, we generated in *E. coli* - to avoid putative suicidal activity – an antimicrobially inactive LEKTI-2-fusion protein. The 61-residue form of LEKTI-2 was liberated after cleavage with enterokinase and then purified by HPLC. Interestingly, this LEKTI-2 form lacked *E. coli*-cidal activity. To address this surprising observation, we re-analysed our natural LEKTI-2 preparations by additional HPLCs and found by ESI-MS-analyses that they contained both, N-terminally extended as well as N-terminally truncated LEKTI-2 forms. Among them, the 62-residue pyroglutamylvariant and 63-residue-variant of LEKTI-2 were found to be major contaminants ofthe dominant 61-residue LEKTI-2 form. We therefore generated further the 63-, 62-,60-, 59-, 58- and 57-residue LEKTI-2 variants recombinantly. Antimicrobial assays revealed the N-terminally extended 62- and 63-residue LEKTI-2 variants to exhibit antimicrobial activity, whereas all other variants lacked that activity. In conclusion, LEKTI-2 represents a novel skin-derived antimicrobial peptide.

P114

CD11c+ dermal DC but not epidermal Langerhans cells are required for efficient induction of CD8+ CTL responses in the skin following gene gun immunization

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Particle-bombardment of the skin with plasmid DNA using the gene gun leads to antigen expression *in vivo* mostly in keratinocytes of the epidermis. Recently, it has been debated which type of antigen-presenting dendritic cells (DC) in the skin is critically involved in transporting the antigen to the draining lymph nodes and presenting it to naïve CD8+ CTL. Here we used mice which express the human diphtheria toxin receptor (DTR) under the control of either the Langerin (Lg-DTRmice) or the CD11c promoter (CD11c-DTR mice) to address this question. In these mice cell type-specific DTR expression mediates transient selective ablation of either epidermal Langerhans cells (LC) or CD11c+ dermal DC after injection of recombinant diphtheria toxin (DT). Lg-DTR mice and CD11c-DTR mice were either treated with DT or left untreated and then bombarded with plasmid DNA using the gene gun. Antigen expression and elimination of LC or CD11c+ DC in the skin was verified by immunofluorescence staining and RT-PCR. Antigen-presentation to CD8+ CTL in the draining lymph nodes was assessed using adoptively transferred oval bumin-specific TCR-transgenic CD8+ T cells (OT-1). Expansion and induction of cytotoxic activity ofOT-1 T cells in the draining lymph nodes *in vivo* following cutaneous gene gun bombardment was not altered in DT-treated compared to untreated Lg-DTR mice despite efficient ablation of LC in the skin. In contrast, expansion and induction of cytotoxic activity of OT-1 T cells was significantly reduced in DT-treated compared to untreated CD11c-DTR mice, although ablation of CD11c could only be achieved incompletely for a short period of time. Taken together, our experimental dataindicate that effective stimulation of CD8+ CTL *in vivo* following cutaneous gene gunbombardment largely depended on the presence of CD11c+ dermal DC and did not require Lg+ epidermal LC.

P115

New genetic tools for the investigation of mast cell biology

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Mast cells are important effector cells in type I allergy, but were recently also shown to play key roles in host defence against pathogens. In addition, mast cells were implicated in tissue remodelling, wound healing and transplant tolerance. Investigation of this important cell type, however, was severely hampered by the scarcity of mast cells in the various tissues. Until today, *in vivo* analysis of mast cell-specific functions of individual genes relied on the reconstitution of genetically mast cell-deficient mice with mast cells differentiated *in vitro* from bone marrow of mice deficient for the gene of interest. This system yielded important information in the past, but suffers severe limitations. Herein, we use the Cre/loxP recombination system for conditional mast cell-specific mutagenesis *in vivo*. In a bacterial artificialc hromosome (BAC) transgene containing the entire mouse mast cell protease 5(Mcpt5) locus, the coding region of exon 1 was replaced by a Cre-cassette.Mcpt5-Cre mice showed highly efficient Cre-mediated recombination in mast cells, but not in other cell types and are presently used for mast cell-specific inactivation of various genes. The new transgenic line also allows inducible ablation of the mast cellline age *in vivo* by crossing to the iDTR line, which expresses a human diphtheriatoxin receptor upon Cre-mediated deletion a loxP-flanked stop element. In a pilot experiment, the population of peritoneal mast cells was no longer detectable 24 h after a single i.p. injection of diphtheria toxin, indicating efficient line ageablation. In addition, we have generated mice carrying a modified transgene encoding a Cre-estrogen receptor (ERT2)-fusion protein allowing *in vivo* induction of Cre-mediated gene inactivation by administration of tamoxifen. The new Cre-transgenic mouse lines will be useful tools for the investigation of mast cellbiology.

P116

A C-terminal ifapsoriasin fragment is a selectively pseudomon as aeruginosa-killing peptide

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Healthy skin forms a 'chemical barrier' consisting of several antimicrobial peptides(AMPs), while the known AMPs preferentially kill Gram-negative and -positive gutbacteria. To address the question whether healthy skin produces also AMPs that in particular protect us from infection by soil bacteria, e.g. *Pseudomonas aeruginosa* (P.a.), heel stratum corneum extracts were screened for P.a.-killing activity. Upon HPLCa peptide fragment of ifapsoriasin (IFPS), a novel and skin-specific 248 kDa S100 fusion type protein (SFTP), could be identified by mass spectrometry analyses in P.a.-killing fractions. The IFPS protein is structurally closely related to profilaggrin andhornerin, two prototypical members of this family. Like other SFTPs, IFPS contains apart from the S100 domain an EF-hand, a spacer, and two glycine/serine-richtandem repeat domains. To raise antibodies and investigate the possible function of IFPS three recombinant 7.5–15.5 kDa fragments representing different parts of the whole molecule were generated in *E. coli*. Immunohistochemistry of various skin locations revealed IFPS staining in the uppermost cell layer of stratum granulosum, in the stratum corneum as well as in all eccrine sweat gland parts, in particular in the acrosyringium. An IFPS antibody directed against the C-terminus stained also the sweat gland lumen, which suggests that the IFPS C-terminus is secreted. Western blot analyses revealed diffuse 60 kDa bands upon extraction of stratum corneum only at reducing conditions and also in sweat, which might be due to cleavage and partial covalent cysteine-mediated linkage of IFPS fragments to stratum corneum and sweat proteins. To follow the hypothesis that IFPS fragments have antimicrobial activity the recombinant fragments were tested for microbicidal activity in the plate diffusion and microdilution assay system. Antimicrobial activity could be detected neither against *S. aureus* nor against *C. albicans* and only moderate against *E. coli* only in the C-terminal fragment. Interestingly, the C-terminal fragment was highly active against different strains of *Pseudomonas aeruginosa* and some other soil bacteria. Our results suggest a role of IFPS in preventing skin infection by soil bacteria, inparticular *P. aeruginosa*, a moisture-requiring soil bacterium that would optimally grow in humid skin areas such as sweat glands.

P117

Down-regulation of constitutive NF-κB activation in macrophages by acetyl-keto-b-boswellic acid alleviates skin inflammation in a murine psoriasis model

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The CD18 hypomorphic (CD18hypo) PL/J mouse model clinically resembling human psoriasis is characterized by reduced expression of the common chain of β2 integrins(CD11/CD18) to only 2–16% of wild-type levels. Previously, we found that this chronic psoriasiform skin inflammation depends on efficient recruitment and activation of macrophages with sufficient release of TNF-α. Here, we addressed the question whether the NF-κB pathway is causally involved in the pathogenesis of psoriasiform skin disorder of CD18 hypo mice, and whether the psoriasiform skin inflammation could be improved by blocking this pathway using newly analyzed natural compounds. Using specific antibodies against phosphorylated IκB-α?Ser32 and p65Ser536 we have detected that NF-κB signalling is activated in macrophages in the lesional skin of CD18hypo mice. Up-regulation of NF-κB activity correlates with enhanced expression of NF-κB-dependent genes, such as TNF-α, K14, Ki67 and EGF. Acetyl-keto-β-boswellic acids are cytotoxic compounds that can be isolated to chemical homogeneity from the gum resins of various Boswellia species, and have been used in traditional medicine and recently in small clinical trials for the treatment of chronic inflammatory diseases such as rheumatoid arthritis and inflammatory bowel disease. In this study we demonstrate that inhibiting NF-κB activation with acetyl-keto-β-boswellic acid decreases expression of NF-κB-dependent genes, and administration of acetyl-keto-β-boswellic acid significantly improves the psoriasiform skin disease in CD18hypo mice. These data suggest that activation of NF-κB plays a significant role in the pathogenesis of the psoriasiform skin disease, and that a potential mechanism of action for TNF-targeting agents is down-regulation of NF-κB transcriptional activity. Targeting activation of NF-κB by acetyl-keto-β-boswellic acid may be a potential therapeutic strategy for autoimmune diseases, such aspsoriasis.

P118 (V31)

Autoantibodies of the IgG1 and IgG3 subclasses mediate the main pathogenic effects in epidermolysis bullosa acquisita

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EBA is a subepidermal blistering disease caused by autoantibodies against type VII collagen at the dermal-epidermal junction (DEJ). The binding of autoantibodies induces complement activation as well as recruitment and activation of neutrophils which then release inflammatory mediators leading to DEJ separation. Autoantibodies of the IgG1 and IgG4 subclasses are found in the skin of most of EBA patients, but IgG2 and IgG3 may also be present. The specific contribution of the four different IgG subclasses to the pathogenesis of EBA has not been elucidated yet. To address this question, we generated a scFv phage display library from mice immunized with recombinant fragments of the immunodominant NC1 domain of human type VII collagen. scFv antibodies specific for type VII collagen were selected by phage panning; their specificity was demonstrated by indirect immunofluorescence microscopy on salt-split skin, immunoblotting of dermal extracts and ELISA with recombinant NC1. To obtain a complete human antibody, variable domains of the scFvs were fused with constant domains of the four human IgG subclasses. Mammalian expression yielded, in great amounts, chimeric human-mouse monoclonal IgG1, IgG2, IgG3, and IgG4, which all possessed the same binding properties as the original scFvs. These four different antibodies were then tested for their pathogenicity using a previously established cryo section assay of human skin. In this assay, only IgG1 and IgG3, but not IgG2 and IgG4, were able to activate complement at the DEJ. When co-incubated with leukocytes of healthy donors, IgG1 and IgG3 were also capable to induce DEJ separation of the cryosections, while IgG2 and IgG4 were not. These results clearly demonstrate the major importance of IgG1 and IgG3 in 2 crucial steps of the pathogenesis of the inflammatory phenotype of EBA, namely complement activation and activation of neutrophils at the DEJ. The generation of recombinant monoclonal autoantibodies not only provides us with a powerful novel tool. Our data also indicate that the use ofimmunomodulatory drugs aimed at class-switching autoreactive B cells to produce IgG subclasses with lower pathogenic activity may provide a new therapeutic principle for the treatment of EBA and other autoimmune bullous dermatoses.

P119 (V07)

In experimental leishmaniasis T cell specific GATA-3 transgenic C57Bl/6 mice develop a Th-2 immune response but remain resistant to *L. major*

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Resistance in cutaneous *L. major* infection crucially depends on the development of a L. major specific Th1 response while Th2 differentiation in Balb/c mice results insusceptibility. While the decisive role of T cells for the infection is generally accepted there is growing evidence that cells of the innate immune system are critically involved in initiating and controlling Th-cell differentiation. In T cells GATA-3 is a major transcription factor responsible for Th-2 differentiation. Thus GATA-3transgenic T cells develop a robust Th-2 response after *in vitro* activation. To test there lative importance of T cell versus non T cell compartments for Th-differentiation *in vivo* we analysed *L.major* infection in genetically resistant Th1-prone C57Bl/6 mice with a T cell specific over-expression of the GATA-3 transcription factor. As expected GATA-3 transgenic animals developed a Th-2 type immune response asCD4 positive T cells isolated from infected GATA-3 transgenic mice produced significantly more antigen specific IL4 and less IFNγ?than T cells isolated from wild type mice. In agreement with these data GATA-3 transgenic mice developed significantly larger footpad lesions. However, GATA-3 transgenic mice eventually were able to cure the disease as did wild type mice, demonstrated by reduction of footpad swelling and clearance of parasites in limiting dilution assays of footpads, local lymphnodes and spleen during the late phase of infection. We conclude that over-expression of the transcription factor GATA-3 in T-cells is sufficient to induce Th-2 differentiation in genetically resistant Th1-prone mice, but that GATA-3 mediated Th2 differentiation is not sufficient to maintain this Th2response and that thus non-T cell compartments or other transcriptional factors are able to counteract these mechanisms and finally induce resistance towards the parasite.

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Influences of toll like receptor ligands on the immunstimulatory capacity of human dendritic cells

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We and others have shown that terminal differentiated dendritic cells (DC), pulsed with tumour associated antigens, are very efficient in the induction of anti-tumour T cell responses in melanoma patients. However, this cellular therapy did not lead to permanent defense against the tumour but only a transient resistance for just a few months. Since patients suffering from severe melanoma show a stage-dependent increased frequency of CD4+CD25+Foxp3+ regulatory T cells (Tregs) we suggest that the increased numbers of these cells might inhibit adequate and long-lasting anti-tumour T cell responses. Therefore, DC used for vaccination have to exhibit a superior abilty to activate naïve T cells in order to break self tolerance leading to adequate tumour immunity subsequently. The aim of this study was the generation of 'conditioned' DC, able to overcome the suppressive activity of Tregs and to induce long-lasting anti-tumour T cell immunity. Therefore, we combined different toll like receptor (TLR) ligands with proinflammatory cytokines for conditioning of monocyte-derived DC. Stimulation of human DC with TLR-ligands alone leads to a partial maturation of DC with an intermediate potential to activate resting T cells in an allogeneic MLR whereas maturation of DC with a cocktail of proinflammatory cytokines showed an increased T cell stimulatory capacity compared to the stimulation with TLR-ligand treated DC. Furthermore, synergistic effects on maturation by combining different TLR-ligands were not observed. Currently, we are testing different combinations of TLR ligands with proinflammatory cytokines in the capacity to induce conditioned DC that can induce T effector cell activation even in the presence of activated Tregs to establish improved DC protocols for anti-tumour therapies.

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Immunisation with different adjuvants alters the disease progression in mice with experimental epidermolysis bullosa acquisita (EBA)

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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 can lead to the establishment of autoimmune diseases. An example is the deposition of autoantibodies against type VII collagen at the dermal-epidermal junction which leads to blistering of the skin, a disease called epidermolysis bullosa acquisita (EBA). In EBA, a dominance of CD4+ T cells, which preferentially produce IFN-g (T-helper1 cells; Th1), has been reported. We have previously established an active disease model of EBA by immunizing mice with recombinant murine type VII collagen. In the present study, we addressed the question, whether a shift from Th1to Th2 cells (preferentially producing IL-4) would ameliorate the course of diseased mice. In this active mouse model of EBA, Th2 cells were induced *in vivo* using alumas an adjuvant. Three major observations were made: 1) The course of the disease was drastically improved as shown by the significant reduction of the clinical score. 2) However, in the blood, the concentration of type VII collagen-specific antibodies ofthe Th1 subtype (IgG2a) and the Th2 subtype (IgG1) were identical among the two groups. 3) Interestingly, in the spleen of alum-treated mice, the number of type VIIcollagen-specific B cells was altered. These cells were increased in the red pulp and decreased in the follicles of the spleen. Our results indicate that the affinity of antibodies to type VII collagen in the alum-treated group is reduced thereby causing a milder disease. This conclusion is supported by the observation that the localization of the antibody producing cells in the spleen is different in alum-treated compared to non-treated animals and by reports that the affinity of antibodies is influenced by the localization of the antibody producing cells. Our results may provide a basis for novel strategies to treat EBA, i.e. by the use of appropriate adjuvants inducing anti-type VII collagen antibodies which are not pathogenic. Ultimately, these findings may also have implications for the treatment of other autoantibody-mediated diseases.

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Scavenger receptor stabilin-1 links endocytosis and intracellular sorting in alternatively activated macrophages

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The multifunctional scavenger receptor stabilin-1 is expressed on macrophages inplacenta and adult tissues as well as on sinusoidal endothelial cells and macrophages in lymph nodes. *In vitro* induction of stabilin-1 on macrophages requires IL-4 and glucocorticoids. We showed that stabilin-1 mediates endocytosis of extracellular ligands acLDL, regulator of ECM-remodelling and cell adhesion SPARC, and hormone placental lactogen (PL). Both SPARC and acLDL 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 theendosomal/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 lectin-type Glyco_18 domain, which lack senzymatic activity, similarly to YKL-40 and Ym1/Ym2. YKL-40 and Ym1/Ym2 are secreted by macrophages, possess cytokine activity and are associated withTh2-type inflammations and allergies. We demonstrated that endogenous SI-CLP is over expressed in human macrophages stimulated with IL-4 and dexamethasone and is transported via stabilin-1 from biosynthetic to the secretory pathway. High levels ofSI-CLP were detected in bronchoalveolar lavage samples from patients with chronicbronchitis. We propose that alternatively-activated macrophages use stabilin-1 1) to coordinate ECM remodelling, angiogenesis, and tissue turnover via endocytosis and degradation of SPARC; 2) to regulate extracellular concentration of PL in placenta; 3) to regulate delivery of chitinase-like protein SI-CLP into the secretory pathway.

P123

Effects of pollen-associated lipid mediators (PALMs) on maturation and cytokine secretion of human dendritic cell subsets

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Pollen associated lipid mediators (PALMs) such as PPE1 license human monocyte-derived dendritic cells (MoDC) for Th2 polarization of naïve T cells. This is due to a reduced capacity of MoDC to secrete bioactive IL-12. Since MoDC represent an artificial cell-type, we analysed the effects of PALMs on cytokine secretion and maturation of native DC precursors from human peripheral blood. SlanDC, myeloid (MDC) and plasmacytoid DC (PDC) were isolated from human PBMC using antibodies against MDC8, CD1c and BDCA-4, respectively. The cells were incubated with medium, aqueous birch pollen extracts (APE) or PPE1 alone or in combination with maturation stimuli (LPS, R848, CpG-A or CpG-B). After 24 h, cells were analysed for the expression of maturation markers by flow-cytometry. Supernatants were analysed for IL-12 (slanDC, MDC) or IFNα?(PDC). APE, but not PPE1, dose-dependently inhibited the LPS-induced IL-12 production of slanDC, as well as the LPS/R848-induced IL-12 production of MDC and the CpG-A-induced IFN-α?secretion of PDC. IL-6 secretion remained unaffected by APE or PPE1 in slanDC and MDC. Furthermore, APE reduced the surface expression of CD40, CD86, CD80, CD83 and HLA-DR on MDC. The inhibition of CD40, CD86 and HLA-DR was also seen in PPE1-treated MDC. In PDC matured in the presence of CpG-B, the expression of CD80 and CD83 was significantly reduced by APE and PPE1, while the expression of CD40, CCR7 was only inhibited by APE. Expression of CCR7 and HLA-DR remained unaffected by either stimulus. In the case of slanDC, the LPS-induced up-regulation of CD80, CD83, CD40 and CCR-7 was inhibited by APE but not by PPE1. Our data suggest the presence of receptors for PALMs on multiple subsets of native DC precursors. Since all the DC subsets analyzed in our study play distinct roles in the initiation and maintainance of allergic immune responses, we conclude that PALMs, especially PPE1, might influence the outcome of an immune reaction not only in terms of T cell polarization, but also in terms of co-stimulation.

P124

Myeloperoxidase is required for antibody-induced tissue damage in experimental epidermolysis bullosa acquisita

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Type VII collagen, the main component of the anchoring fibrils, is the autoantigen of epidermolysis bullosa acquisita (EBA), a subepidermal autoimmune blistering disease. We have recently shown that antibodies against type VII collagen, when passively transferred into mice, cause a subepidermal blistering skin disease reproducing the findings in the skin of EBA patients. In addition, autoantibodies to type VII collagen induce dermal-epidermal separation in cryosections of human skin when co-incubated with human granulocytes. In these models, reactive oxygen species (ROS) were shown to be critically involved in granulocyte-mediated antibody-induced tissue injury. Trying to further characterize the ROS species directly involved in tissue destruction in EBA, we demonstrate in the present study that myeloperoxidase (MPO) is of importance in antibody-induced blistering both in the passive transfer mouse model of EBA and *ex vivo*. Mice deficient in MPO (B6.129X1-Mpotm1Lus/J; $n = 8$), in contrast to wild type controls ($n = 8$), developed a delayed and significantly less severe blistering induced by injection of antibodies against type VII collagen. In addition, specific inhibition of MPO, released upon activation from normal human granulocytes, by treatment with salicyl hydroxamic acid resulted in a dose-dependent abolishment of dermal-epidermal separation *ex vivo*. Our results demonstrate that blister induction by antibodies against type VII collagen depends on MPO and facilitate the development of ROS-targeted therapies in EBA and related diseases.

P125

A protective role of complement component 3 (C3) in allergic contact dermatitis

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Recent studies described the role of complement component C3 in T cell mediated immune responses. Keratinocytes synthesize C3 constitutively and increased expression of C3 has been described upon stimulation with pro-inflammatory cytokines. In this study, we investigated the role of C3 in allergic contact dermatitis (ACD). Here, we observed that C3 deficient (C3KO) mice showed a substantial and significant increase in contact hypersensitivity responses to haptens inducing eitherTh1 (2,4-dinitrofluorobenzene: DNFB and dinitrochlorobenzene: DNCB) or Th2 (Fluoro-isothiocynate: FITC and toluene-2,4-diisocyanate: TDI) cytokine mediated skin inflammation as compared to their wild type (WT) controls. Stimulated splenocytes from hapten (DNCB) immunized C3KO mice secreted higher amounts ofTh1 (IFN-γ) but not of Th2 (IL-4, IL-5, IL-10) cytokines or IL-17 as compared to their WT controls. The addition of purified C3 or C3a to stimulated splenocytes from C3KO mice did not reduce the increased IFN-γ?expression *in vitro*. A higher secretion of IL-12 from splenocytes of C3 KO mice as compared with WT was observed after TLR-4 ligand (LPS) stimulation. The increased expression of the Th1 polarizing cytokine IL-12 and of IFN-γ?in C3KO mice might be responsible for the increased skin inflammation in C3 deficient mice. These findings provide a new insight into a novel anti-inflammatory role of the complement component C3 in skin inflammation.

P126

Consequences of PPAR-alpha deficiency on the *in vivo* phenotype of Langerhans cells and T cells.

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We have previously shown that PPAR-alpha activation inhibits Langerhans cell (LC) function *in vivo*. In this study, we assessed whether epidermal LC function in PPAR-alpha deficient mice was affected. LC isolated from skin-draining lymph nodes of PPAR-alpha deficient mice exhibited higher CD86 and B7-H2 (ICOS ligand) expression after sensitization of skin with a contact allergen. These cells produced more IL-12 than wild type LC, but the amount of TFN-alpha was unchanged. The percentage of CD4+ T cells was higher in the skin-draining lymph nodes of PPAR-alpha deficient mice after application of the same contact allergen. While CD4+ T cells expressed a higher amount of the cell surface activation marker CD25, the percentage of CD25+IL-10+CD4+ T cells (Treg) was decreased in PPAR-alpha deficient mice compared to wild types. Finally, we observed an increased contact sensitivity reaction in PPAR-alpha deficient mice compared to wild types after challenge with a contact allergen. In conclusion, both PPAR-alpha deficient LC and T cells displayed an exaggerated activation state that may explain the increased skin inflammation of PPAR-alpha deficient mice compared to wild types.

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Dendritic cell differentiation state and their interaction with NKT cells determines Th1/Th2 differentiation in the murine model of Leishmania major infection

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Recent reports demonstrated that dendritic cells (DC) sense inflammatory and microbial signals differently, redefining their classical subdivision into an immature endocytotic and a mature antigen-presenting differentiation stage. While both signals induce DC maturation by upregulating MHC II and costimulatory molecules, only Toll-like receptor signals are able to trigger proinflammatory cytokine secretion by DC, including Th1-polarising IL-12. Here, we explored the murine Leishmania major infection model to examine theCD4+ T cell response induced by differentially matured DC. Initial experiments showed that pulsing DC with L. major lysate did not affect DC maturation with TNFα or LPS+anti-CD40. When partially matured TNF-DC were injected into Balb/c mice prior to infection, the mice failed to control L. major infection and developed a Th2 response which was dependent on IL-4Rα?signalling. In contrast, injections of fully matured LPS+CD40-DC induced a Th1 response controlling the infection. Furthermore, when the expression of different Notch ligands on DC was analyzed, we found increased expression of Th2-promoting Jagged2 in TNF-DC whereas LPS+CD40-DC upregulated the Th1-inducing Delta4 and Jagged1 molecules. TheTh2 polarization induced by TNF-DC required interaction with CD1d-restricted NKT cells. However, NKT cell activation by L. major lysate-pulsed DC was not affected by blockade of the endogenous glycolipid suggesting exchange with exogenous parasite-derived CD1 glycolipid antigen. In sum, the differentiation stage of DC as well as their interaction with NKT cell determines Th1/Th2 differentiation. ¥ These results have generic implications for the understanding of DC instructed Th cell responses and the development of improved DC vaccines against leishmaniasis.

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T-cell responses in stage IV melanoma patients vaccinated with dendritic cells electroporated with defined RNA

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Objective: In this study we investigated the immunogenicity of a tumour vaccine consisting of autologous monocyte-derived dendritic cells (DC), which are electroporated with defined RNA encoding for the tumour antigens MageA3, MelanA and Survivin, and in half of the patients in addition with a RNA encoding for E/L-Selectin which is designed to redirect the injected mature DC from blood to lymph nodes via highen dothelial venules.

Patients and study design: Stage IV melanoma patients with progressive disease after at least one systemic therapy are to be included in this study. DC are electroporated with the three tumourantigens, and in half of the patients in addition with E/L-Selectin. Patients are apheresed at the beginning of the study as well as after four vaccinations to providecells for DC generation and immunomonitoring. The vaccine was given at day 1, 14, 42 and day 70 as intravenous infusion over 30 min.

Monitoring of T-cell responses: T-cell responses were monitored on day 1 and on day 70 using freshly isolated PBMC and overlapping peptides covering the whole sequence of MageA3, MelanA and Survivin. PBMC were stimulated with pools of peptides together with IL-2 and IL-7 for 7 days. Peptide specific responses were detected using an Interferon gamma elispot assay.

Results: Up to now four patients reached the second leukapharesis after four vaccinations. In three patients a strong induction or expansion of T-cell responses could be detected. Two of the patients had received a vaccine with ELS; one patient received a vaccine without ELS.

Conclusion: In contrast to previous reports the intravenous route appears to be unexpectedly effective. It is possible that this is a unique property of the DC-RNA vaccine used here. A more detailed study of the immune responses and trafficking will show whether the introduction of the E/L-Selectin RNA induces as expected a more diverse homing pattern of induced T cells by redirecting the intravenously injected DC from blood to lymph nodes.

P129

Th2-biased de novo immune response to keyhole limpet hemocyanin in humans

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Allergen-specific T helper 2 (Th2) cells play an important role in the pathogenesis of atopic disorders. To date, no model system for such an imbalanced immune response exists in humans that would allow the monitoring of a developing immune response *in vivo*. To address this issue, we used Keyhole Limpet Hemocyanin (KLH) as a neo antigen coupled to aluminum hydroxide for immunization of healthy and atopic subjects. The immunization protocol was well tolerated and highly specific and efficient, as shown by KLH-specific proliferation of PBMC. Antigen-specific production of Th2-cytokines (mainly IL-5 and IL-13) suggested a Th2-pattern of the immune response, as did the presence of KLH-specific IgG4 in sera of KLH-immunized but not non-immunized subjects. Intra-dermal KLH challenge induced an immediate type of response in atopic subjects followed by a late phase skin response. This reaction was accompanied by a strong upregulation of IL-4 transcripts in biopsies taken from the site of injection. In conclusion, the tested immunization protocol allowed the monitoring of a Th2-skewed de novo immune response in humans, and should be a valuable tool for measuring the therapeutic efficacy of anti-allergic candidate compounds.

P130

High frequency of activated slanDC (6-sulfoLacNAc+ dendritic cells) in psoriasis

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Targeting of TNF alpha as well as IL-23/IL12-p40 has proven highly effective in the treatment of psoriasis. According to studies by Krueger et al., an iNOS-expressing and TNFa-producing dermal proinflammatory myeloid dendritic cell population is of central importance in the immunopathogenesis of psoriasis. However, the nature of these DC is ill defined. We previously identified the subset of slanDC in human blood which stand out by their high-level production of TNF alpha and IL-23/IL12-p40. We here asked whether slanDC may be relevant to the pathogenesis of psoriasis. A total of 37 specimens of a total of 27 donors with the diagnosis of psoriasis were studied by immunohistochemistry and immunofluorescence. These studies included different clinical subgroups of psoriasis as well as early and late lesions. Over all we found high numbers of slanDC in the dermis. The frequency was comparable to that of CD1a positive DC in epidermis and dermis. SlanDC were mainly located in the upper dermal papillary compartment and interspersed between T cells. As signs of activation slanDC were shown to be able to express TNF alpha as well as iNOS. SlanDC were found in plaque type psoriasis as well as in other forms of psoriasis:inversa, guttata and pustulosa . Interestingly, slanDC were readily found in early psoriatic lesions. The frequency of slanDC in blood of psoriatic patients was comparable to that of healthy donors. This demonstration of activated slanDC in the inflammatory dermal infiltrate of psoriasis together with the previously described high proinflammatory and T cellstimulatory capacity of slanDC implicates that slanDC are highly relevant for the immunopathogenesis of psoriasis.

P131

Agonist of Proteinase-Activated Receptor-2 (PAR2) enhances effects of IFNγ on human monocyte functions

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Recent findings suggest a crucial role of PAR2 in inflammation and innate immunity. However, the ability of PAR2 agonist to affect monocyte function or to modulate the effects of certain cytokines remains unclear. PAR2 belongs to a family of G protein-coupled receptors activated by serine proteases via proteolytic cleavage. Tryptase, trypsin, and bacterial proteases are known to activate PAR2. The data of our study indicate that PAR2 agonists enhance the ability of IFNγ to upregulate $\alpha v\beta 3$and FcγRI expression on monocyte cell surface. The upregulation of these molecules is associated with initial events of immune complex diseases such as Arthus reaction in skin, for example. Moreover, PAR2 agonists enhance the effects of IFNγ?at monocyte production of IP-10, which plays role during development of allergic dermatitis via modulation of T-cell migration. Additionally, we found that co-application of PAR2 agonist and IFNγ?suppresses influenza A (H7N7) replication in human monocytes. The last finding makes promising further investigation of PAR2 agonist application at replication of other viruses, for example Herpes simplex virus, in humans. Together, our results indicate that PAR2 enhances immunomodulatory and anti-viral effects of IFNγ?at human monocytes.

P132

Dendritic cell survival during differentiation and maturation depends on anti-apoptotic effects of TNF

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TNF is well known as cytokine that induces maturation of human monocyte derived dendritic cells (DC) and, in combination with GM-CSF, the differentiation of CD1a+Langerhans Cell like DC from human CD34+ progenitors. In the mouse, TNF has been described as survivial factor which maintains the viability of epidermal Langerhans cells in culture, but in contrast to granulocyte/macrophagecolony-stimulating factor, without inducing their functional maturation (Koch F et al. J Exp Med 1990; **171** (1): 159–71). Our initial observation which pointed towards a crucial role of TNF as survival factor also in human DC, was a minute concentration of TNF in monocyte cultures with GM-CSF and IL-4 from certain donors, who repeatedly yielded very low DC numbers. Addition of recombinant TNF normalized DC yields in these patients. Conversely, blocking of TNF during the early days of monocyte culture with TNF-R2-Fc generally resulted in markedly reduced yields of immature DC in all donors. We now show, that the viability maintaining function of TNF is also required during maturation of human monocyte derived DC. Maturation induced via the TLR3 agonist polyI:C or TLR4 agonist LPS, despite the presence of GM-CSF and IL-4, resulted in pronounced apoptotic cell death of maturing DC, as evidenced by annexin staining and DNA fragmentation. DC apoptosis was paralleled by low TNF concentration, could be prevented by addition of TNF and, most interestingly, was not seen using the TLR7/8agonist R848 as maturation stimulus, which induced autocrine TNF production in DC. Biochemical analysis of apoptosis pathways provided first evidence for TNF dependent regulation of bcl-2 expression in DC, whereas IAP family members or the signalling pathways of other death ligands (TRAIL, CD95L) were not markedly affected. Our results identify TNF as a principal survival factor for DC differentiation and maturation in human monocyte-derived DC that inhibits apoptotic cell death. Current work concentrates on a refined analysis of signalling pathways in DC that are involved in 1) anti-apoptotic effects of TNF and 2) induction of autocrine TNF production in DC and monocytes.

P133

Amino peptidase activity impairs the recognition of melanoma cells by Melan-A/MART-1(26–35)-specific T cells

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The knowledge of tumour antigen epitopes recognized by cytotoxic CD8+ Tlymphocytes (CTL) is currently exploited in different clinical trials of active and passive immunotherapy. However, tumour cells are barely characterized for the processing and presentation of these epitopes. We therefore asked whether beside the proteasome other cellular proteases influence the generation of theMelan-A/MART-1(26–35) epitope, a frequent target in melanoma immunotherapy. When melanoma cells were treated with leucinethiol, an inhibitor of metalloproteases which does not affect proteasome activity, presentation of Melan-A/MART-1(26–35)was strongly enhanced suggesting that cytosolic and/or endoplasmatic reticulum(ER)-resident pepetidases interfere with efficient epitope generation. Indeed, by *in vitro* epitope digestion experiments, we could demonstrate that a proteolytic activity located in the ER is responsible for Melan-A/MART-1(26–35) degradation. Knock down of ER-aminopeptidase 1 (ERAP1) expression by siRNA strongly enhanced tumour cell recognition by specific CTL, demonstrating that ERAP1 destroys the Melan-A/MART-1(26–35) epitope in melanoma cells. Our observations suggest that data on the generation of tumour antigen epitopes should have an impact on the design of active and passive immunization strategies.

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The immunoprivileged bulge: regeneration pool and immunological 'Achillesheel' of the hair follicle?

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Recent gene expression evidence suggests that, besides the anagen hair bulb, the epithelial stem cell region of the outer root sheath (ORS) of normal human scalp hair follicles (HFs), the bulge, may represent an area of relative immune privilege (IP).Sites of IP may serve to sequester autoantigens from immune recognition and autoaggressive inflammation. By using immuno histological staining methods like EnVision®, tyramide signal amplification, immuno fluorescence and ABC methods, our *in situ*-protein expression data from normal human scalp skin sections complement previous work on human HF immunology and close important gaps of knowledge in the field of IP. We demonstrate that the bulge region of the ORS of human scalp HF displays absence or prominent downregulation of MHC-Ia and MHC-II expression during anagen VI, associated with a lower expression level of MHC-I associated proteins (β-2 microglobulin). Besides the recognized upregulation of immunosuppressive CD200, the potent 'IP guardian', α-MSH, was upregulated in the keratin 15+ zone of the ORS. TGF-β?2 immunoreactivity (IR) was prominently in the bulge (though not restricted to it), whereas macrophage migration inhibitory factor (MIF) and indoleamine dioxygenase (IDO) IR were upregulated in the bulge and below.CD200+ bulge cells also co-expressed a non-classical MHC-Ib molecule, HLA-E (= component of feto placental IP). Interestingly, however, the key pro-inflammatory adhesion molecule ICAM-1, was constitutively expressed in the bulge region. Stimulation of organ-cultured, full-thickness human scalp skin with interferon-gamma, a key stimulant of IP collapse, caused significant ectopic MHC Ia expression in the bulge. The bulge response of other parameters to interferon stimulation (e.g. MIF,IDO, MHC-II) is currently being assessed. Thus, the bulge region of human HFs likely represents an additional, though morelabile site of relative IP in human skin, designed to protect the HFs epithelial stem cell reservoir. However, our data also suggest that the bulge is susceptible to attracting destructive autoimmune responses (e.g. constitutive ICAM-1 and base-line MHCIa/β2 microglobulin

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Priming of Leishmania-reactive CD8+ T cells occurs primarily in Leishmania-resistant C57BL/6 mice and is not immunoproteasome-dependent

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Protective immunity against infection with the intracellular parasite L. major is dependent on the generation of IFNγ?producing CD4+ Th1 cells and CD8+ Tc1 cells. We have previously shown that in physiological low dose infections with <1.000 *L.major* - mimicking natural transmission by the sand fly - mice deficient in CD8 cells and/or MHC I do not heal. In addition, adoptive transfer of IFNγ+ L. major-specific CD8+ T cells protected from disease, whereas IFNγ−/− CD8 cells did not. We now show that in physiological infections of C57BL/6 mice, Leishmania-specific IFNγ production was comparable in CD4+ and CD8+ T cells, whereas both T cells subsets produced significantly less (~three to fivefold) IFNγ in Leishmania-susceptible BALB/c mice($P < 0.002$). In parallel, the percentage of proliferating CD4 and CD8 BALB/c T cells was significantly lower as compared to C57BL/6 T cells, especially at early time points post infection (wk2 and wk4, $P < 0.005$). Beginning in wk4 post infection, CD4+T cells accumulated slowly in lesions and more CD4+ cells were found in infected ears of C57BL/6 as compared to BALB/c mice (24.2 ± 6.6 vs $8.6 \pm 4 \times 10E4$/ear, wk6).Interestingly, despite of impaired Th1/Tc1 priming in BALB/c mice, no difference was found in the number of CD8+ cells recruited to infected BALB/c ears over time as compared to C57BL/6 ears (4.8 ± 2.1 vs $5.9 \pm 0.9 \times 10E4$/ear, wk6). The IFNγ-induced immunoproteasome plays a major role in generating MHC I presenting peptides in APC. To determine the dependence of CD8 priming on immuno proteasomes, we utilized C57BL/6 mice deficient in genes for the inducible immuno proteasome subunit Lmp7. Over 3 months, both lesion sizes as well as lesional parasite loads were comparable between wild type and Lmp7−/− immunoproteasome-deficient mice; the cytokine profiles in lymph nodes showed no difference with regard to antigen-specific IFNγ, IL-4 or IL-10. Finally, infected Lmp7−/− DC and wild type DC exhibited comparable stimulatory capacity of antigen-specific CD8+ T cells. In summary, priming of CD8+ T cells is essential for protection against *L. major*, defects in CD8priming may contribute to disease susceptibility of BALB/c mice. Surprisingly, however, Leishmania-specific Tc1 induction is not dependent on a functional immunoproteasome.

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Limited role of *in vivo* expanded regulatory T cells for psoriasiform skin disorder in TGF beta transgenic mice.

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Skin targeted over expression of TGF beta via keratin five promoter driven transcriptionin transgenic mice results in a chronic inflammatory phenotype that mimics some features of psoriasis. To investigate the role of T cells in the pathogenesis of this psoriasiform skin disorder *in vivo* we employed different antibody-based approaches targeting T cells. First, we explored the role of regulatory T cells (Tregs), a T cell subtype that convey stolerance against autoreactive T cell clones. To this end, transgenic mice were treated with a super agonistic anti-CD28 mAb, known to activate and preferentially expand Treg cells over conventional T cells *in vitro* and *in vivo*. In fact, a single i.p. administration of the super agonistic anti-CD28 mAb resulted in a threefold increase of Tregs within the peripheral lymphocyte pool after three days. Based on the hypothesis that this therapy would either down-modulate the chronic psoriasiform inflammation or prevent disease onset, transgenic mice were either treated in an advanced disease state ('therapeutic' setting) or early before disease onset ('prophylactic' setting). However, in the 'therapeutic' setting neither was the phenotype reversed nor could the disease progression be stopped within 6 weeks of observation. Likewise, administration of the super agonistic anti-CD28 antibody before the disease onset could not prevent the inflammatory reaction, although the phenotype in female mice was delayed as compared to animals treated with a 'conventional' anti-CD28 mAb or an isotype control, respectively. To analyse the overall contribution of CD4+ T cells to the inflammatory phenotype in TGF beta transgenic mice, animals were repeatedly treated with a CD4+ depletingm Ab resulting in a 75- 100 % decrease of total CD4+ T lymphocyte counts after 4 weeks. However, no alleviation of the inflammation was macroscopically or histopathologically observed. Finally, we generated different bone-marrow chimeras to elucidate the role of cells derived from the hematopoietic progenitor stem cell lineage in the inflammatory process.

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Are TLR agonists better maturation stimuli for clinically applicable dendritic cell vaccines ?

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The induction of immune responses generally depends on the immunostimulatory capacity of dendritic cells (DC), which is governed by their state of maturation induced by danger signals such as inflammatory cytokines, pathogen derived Toll receptor agonists (e.g. LPS, bacterial DNA, viral RNA) or T cell derived signals(CD40L). Cytokine cocktail (TNF alpha, IL-1, IL-6, Prostaglandin E2) matured DC are routinely used in clinical trials. They exert high migratory capacities, but lack the capacity to produce significant amounts of IL-12p70 *in vitro*. Despite of this deficiency, cytokine matured DC have been proven to induce antigen specific CTL and TH1 responses *in vivo*. So far the clinical efficacy of vaccinations with cytokine matured DC in tumour patients has not been satisfying and better maturation stimuli might be crucial for the induction of effective cellular immune responses. We have analyzed TLR agonists supplied by the company 3M for their capacity to induce DC maturation. We found that TLR8 agonists, but not TLR7 agonists were inducers of DC migration and IL-12 production. We now have thoroughly compared TLR8 stimuli and combinations of TLR8 with TLR3 and TLR4 agonists, which have been shown to be extremely potent in their IL-12p70 inducing capacity. To address the use in DC vaccination trials we established a model that mimicks the *in vivo* situation and analyzed IL12p70 production prior and after migration using a transwell assay. Thereby we made the interesting observations that i) TLR matured DC are significantly inferior compared to cytokine cocktail matured DC in terms of migratory capacity, which cannot be corrected by addition of Prostaglandin E2 ii) following migration the IL12p70 production by TLR matured DC signficantly drops and, upon CD40 ligation, even cytokine cocktail matured DC consistently produce some IL12p70 iii) using the MelanA analog peptide, TLR and cytokine cocktail matured DCs exert comparable capacity to prime tumour antigen specific CD8 T cells *in vitro* in terms of quantity and quality. Our data do not provide a clear cut answer on the putative best maturation stimulus for DC based vaccination but warrant *in vivo* comparisons in small two armed trials, as soon as TLR agonists of GMP quality are available.

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Antigen specific abrogation of immunity using Indium111 labelled dendritic cells

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The immune system is a dynamic organ not only regulated by quality and quantity ofits receptors, but also by localisation of its cellular components. Thus bioimaging, i.e. tracking of immune cells and studying their interaction *in vivo*, has gained enormous interest in recent years. One routine technology applied by several groups to analyze migration of dendritic cells (DC), is labelling with indium111. Prior using indium for tracing lymphnode homing of our newest DC generation, i.v. applied and functionally modified by transfection with RNA encoding a chimeric E/L-Selectin, we studied the effect of indium on DC migration and T-cell interaction *in vitro* and made remarkable observations. In terms of migratory capacity, which was analyzed using a transwell assay adressingCCR7-mediated migration of DC towards MIP3-?, we could not find any inhibition by labelling of DC with indium111 up to 4 MBq (100 μCi). In contrast, priming and expansion of MelanA specific nave CD8 T-cells by DC loaded with MelanA analogpeptide or MelanA encoding RNA, was markedly affected by indium labelling in adose dependent manner. Whereas MelanA specific T cell expansion was only slightly reduced using DC labelled with 1 MBq of indium111, 2 MBq already completely abrogated specific T cell expansion, assessed via tetramer staining and flow cytometry, and labelling with 4 MBq even resulted in death of MelanA specific T cells.In mixing experiments using DC ± antigen loading and ± Indium labelling we evendetected antigen unspecific inhibition of T cell expansion by Indium labelled by standerDC, which, however, was less pronounced compared to antigen specific inhibition by antigen loaded and Indium labelled DC. In aggregate our *in vitro* results disclose inhibitory effects of Indium111 labelled DC, that have not been payed the duly attention to. Indium labelled DC should not be loaded with specific peptides if applied in tumour patients, which may result in loss of specific immune responses. In contrary, Indium labelled DC could intentionally be loaded with antigen to downregulate unwanted immunity in patients suffering from cellular autoreactive diseases.

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Reactive oxygen species (ROS) inhibit IL-6 production *in vitro* and *in vivo* through induction of the negative regulating transcription factor ATF-3

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Interleukin 6 (IL-6) is an immediate type cytokine that is rapidly induced under conditions of sepsis or during sunburn reactions. Together with IL-1 and TNF, IL-6 is responsible for acute phase reactions, such as fever, hypotonia and shock. ATF-3 (activating transcription factor 3) has recently been described as central transcription factor, activated in response to TLR4-mediated signalling; ATF-3 seems to initiate the negative feedback loop. As we found that increased oxidative stress may inhibit the production of inflammatory cytokines, namely IL-6, we analyzed the effects of ROS on the regulation of ATF-3. We generated bone marrow-derived DC (BMDC) and incubated the BMDC with various inducers of ROS. Stimulation of BMDC under conditions of low ROS through TLR-4 with Lipopolysaccaride (LPS) resulted, as described, in moderate induction of ATF-3 and the induction of IL-6. High ROS levels did not directly modulate ATF-3 mRNA or protein. Yet, under conditions of high ROS levels, LPS induced a three to fourfold increased expression and production ofATF-3. This increase in ATF-3 was associated with a significant suppression of IL-6mRNA expression, while IL-10 production by BMDC was slightly increased. To determine, whether the inhibitory effect was AFT-3-dependent, we stimulated BMDC from ATF-3.KO mice under conditions of either low or high ROS with LPS. ATF 3.KOBMDC showed increased IL-6 mRNA and production compared to wild-type mice as described. Importantly, increased IL-6 mRNA induction was not suppressed by high ROS levels in ATF 3.KO-BMDC. Furthermore, high ROS levels promoted LPS-induced ATF 3-induction and suppression of IL-6 also *in vivo* in wild-type mice; again, ATF-3.KO mice were resistant to ROS-induced suppression of IL-6. However, our data also suggest that the bulge is susceptible to attracting destructive autoimmune responses [abstract was shortened, because it exceeded space limitations].

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Successful treatment of refractory Behet's disease with TNF-α?blocker infliximab

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Behet's disease (BD) is a chronic-relapsing, multisystemic, autoimmune, inflammatory disorder, classified among the vasculitides. The pathogenesis of BD is not completely understood. As far as there is no pathognomonic laboratory parameter the diagnosis is entirely based on the combination of typical clinical manifestations such as oral ulcerations plus any of two of the following: recurrent genital ulceration, ocular lesions (uveitis), typical skin lesions (erythema nodosum, pseudofolliculutis), positive pathergy test and/or organ involvement (lung, joints, brain and/or GI tract). Conventional therapies include high dose prednisone with/without other immunosuppressive agents such as azathioprine, cyclosporine, cyclophosphamide, colchicine, interferon-α?or thalidomide. Unfortunately many BD patients do not respond sufficiently to these therapies or the therapy is accompanied with considerable side effects. Here we present two patients diagnosed as BD with uveitis as their major complaint, who were refractory to conventional therapies and could be successfully treated within fliximab. The first patient was a 56-years-old man with oral ulcerations, uveitis, arthralgia, CNS involvement, chronically active HBV infection and irresponsive to previous treatments with prednisone, cyclophosphamide, thalidomide, chlorambucil, colchicine and highdose IVIG. The second patient was a 35-years-old man with oral ulcerations, uveitis, arthralgia and irresponsive to previous treatments with prednisone, thalidomide and methotrexate. Having excluded latent tuberculosis both patients received infliximab at a dose of 5 mg/kg body weight, at weeks 0, 2, 6 and every other 8 weeks, so far without anycomplications (follow up >30 & 8 months, respectively). The active HBV infection inthe first patient could be controlled (as measured by virus load and liver enzyme parameters) with simultaneous administration of tenofovir. This study shows that: a) infliximab can be an effective treatment for BD especially in those refractory to conventional therapies, b) concurrent use of anti-viral medications with infliximab can control active HBV infection.

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Transcutaneous PO2 imaging during tourniquet-induced forearm ischemia using planar optical oxygen sensors

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Background: Oxygen-dependent quenching of luminescence using transparent planar sensor foils was shown to overcome the limitations of the polarographic electrode technique in an animal model. This method was now transferred to a clinical setting to measure the transcutaneous pO2 (ptcO2).

Methods: In six healthy subjects, a cuff on the upper arm was occluded up to 20 mmHg above systolic pressure and released after 8 min. PtcO2 was measured at the lower arm every 30 s prior to, during, and up to 20 min after cuffocclusion (40°C applied skin temperature) by using luminescence lifetime imaging of platinum(II)-octaethyl-porphyrin immobilised in a polystyrene matrix. For validation, the polarographic Clark electrode technique was applied in close proximity, and measurements were conducted simultaneously.

Results: PtcO2 measurements prior to (70.8 ± 19.1 mmHg vs 66.2 ± 7.7 mmHg) and atthe end of ischemic (2.7 ± 1.2 mmHg vs 3.6 ± 1.7 mmHg) and reperfusion phases(72.2 ± 3.6 mmHg vs 68.4 ± 8.9 mmHg) did not differ significantly using the Clarkelectrode versus luminescence lifetime imaging. At both the initial ischemic and there perfusion phases the Clark electrode measured a faster decrease or increase respectively in ptcO2 because of the oxygen consumption occurring in this method.

Conclusion: The presented method provides accurate and reproducible ptcO2 values under changing microcirculatory conditions. The lack of oxygen consumption during measurement allows both a more realistic estimation of ptcO2 than compared to the gold standard and the permanent use in regions with critical oxygen supply.

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Differential value of the NC16a-ELISA in elderly patients with chronic pruritus

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Bullous pemphigoid (BP), the most common autoimmune blistering dermatosis affecting the elderly, is associated with tissue-bound and circulating autoantibodies against collagen XVII (BP180). Since intractable pruritus is a common initial symptom of BP, this dermatosis must be considered in elderly patients presenting with itch associated or not with skin manifestations. In this study, we have evaluated the prevalence of positive reactions in BP180 NC16a-ELISA and development of BP within 6 months in elderly patients with pruritus. Fifteen patients aged >63 years with pruritus of at least 6 weeks# duration, but noskin lesions, and 34 age-matched controls without pruritus were included. Presence of autoantibodies to basement membrane proteins was investigated byNC16a-ELISA and indirect immunofluorescence microscopy using salt-split skin (IIF).In addition, FBC count, total IgE, liver and kidney function tests and HbA1c were assessed. At the first visit, the NC16a-ELISA values were slightly positive in 4/15 (27%) patients(OD < 0.57 by a cut-off value of 0.3). Two of them demonstrated persisting low-titer autoantibodies (OD < 0.45) after 6 months, although they were free of pruritus at that time. Low-titer autoantibodies (OD < 0.45) were also observed in 2/34 controls (6%). IIF studies were negative in all patients and controls at all time points. None of the patients or controls developed BP during the observation period of 6 months. Clinically, the patients with positive ELISA did not differ from those with negative ELISA. The duration of pruritus varied from 6 weeks to several years, but nearly all patients were in clinical remission after 6 months. In conclusion, slightly positive NC16a-ELISA values are frequently found in elderly patients with chronic itch. Our study, limited by the relative small number of patients, indicates that the NC16a-ELISA test alone cannot be used to identify patients with BP or at risk for BP. Direct immunofluorescence studies remain essential for establishing a definitive diagnosis of BP.

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Correlation of disease activity and severity and IgG reactivity against BP180 in a cohort of 50 patients with bullous pemphigoid (BP)

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BP is an autoimmune bullous disease characterized by IgG auto-antibodies (auto-ab) against two components of the dermo-epidermal basement membrane zone, BP180 and BP230. The goal of this study was to determine whether there is a correlation of the auto-ab reactivity to distinct epitopes of BP180 and BP230 with disease activity. Our group established the retrospective autoimmune bullous skin disorder intensity score (RABSIS), which allowed retrospective assessment of disease activity and severity in BP patients. RABSIS divides the body surface area into 10 regions and attributes a weighing factor for disease activity (erosive/bullous versus crusty lesions) and severity (multiple versus single lesions) to each region. As a result, a RABSIS score reaching from 0 to 10 can be attributed to every patient. We then compared auto-ab reactivity of 50 patients with clinical activity of BP as assessed by RABSIS. There was a statistically significant association with IgG reactivity against the extracellular domain of BP180 and a high RABSIS score, whereas lack of IgG against BP180 correlated with a low RABSIS score. In addition, there was a statistically signifcant relation ($P < 0.05$) between IgG reactivity with the NH2-terminus of BP180 and disease activity while no correlation was found between disease activity or severity and IgG reactivity agagainst BP230. Our results suggest, that i) there is a statistically significant correlation of disease activity and severity and IgG reactivity against BP180. ii) the newly developed RABSIS scoring system is a valid tool in retrospectively assessing disease activity in BP. iii) RABSIS may be of great benefit for prospectively assessing disease activity and severity in BP.

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Analyses of Fussel-15, a new antagonist of TGF-ß/BMP signalling cascade which plays a role in skin sclerosis

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Fibrotic diseases like skin sklerosis is characterized by excessive scaring due to an increased production, deposition and contraction of extracellular matrix. There is evidence that transforming growth factor-beta (TGF-ß) is involved in sklerosisformation. Expression of TGF-ß, TGF-ß receptors or downstream mediators of TGF-ß signalling cascade are known to be disregulated in dermal fibrotic lesions of scleroderma patients. However, the underlying mechanisms are poorly understood. We identified a novel molecule with inhibitory function on bone morphogenic protein(BMP) signalling pathway. BMPs belong to the TGF-ß family. Due to its function we named this molecule Fussel-15, for Functional smad suppressing element on chromosome 15. We detected a specific expression pattern of Fussel-15 infibroblasts of early wound healing processes and a deregulation of this molecule infibroblasts of skin sclerosis. To analyse the expression of Fussel-15 and other TGF-ß/BMP members *in vitro*, we used a collagen contraction model. To determine the function of Fussel-15 *in vitro*, knockdown and overexpression experiments were established. In addition, we used immunohistochemistry to analyse the expression ofFussel-15 *in vivo*. All together, understanding the exact process of normal and abnormal scar formation will help to define better ways to successfully manage and potentially prevent abnormal healing like sclerosis. We could show that Fussel-15, a novel co-repressor of BMP signalling, is elongated expressed in sclerosis derived fibroblasts. Fussel-15 knockdown delayed and an overexpression enhanced collagen contraction *in vitro*. Further analysis of Fussel-15 in normal and abnormal scar formation must show how Fussel-15 is involved in this process and if Fussel-15 could be used as a therapeutic for wound healing.

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Biological relevance of GRO-α gene inhibition in human epithelial cells

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Introduction: GRO-α?(growth-related oncogene-alpha), a member of the CXC chemokine subfamily, plays a major role in inflammation, tumourigenesis and angiogenesis. Previous studies have demonstrated that GRO-α?is one of the chemokines which is involved in the complex process of wound healing. GRO-α?is expressed by keratinocytes at areas where epithelialization and neovascularization occur.

Materials and methods: Primary foreskin keratinocytes and HaCaT keratinocytes were transiently transfected by RNA interference with *in vivo*-tested cationic lipids (AtuPLEX®, 1–2 μg/ml) and synthetic ribonucleotides (AtuRNAi®, 20 nM). The cells were seeded 24 h before transfection in 24-well plates. Cell transfection was performed for 8–10 h in serum- and antibiotics-free medium followed by different recovery times (0–16 h) in the culture medium. The reduction of GRO-α?RNA level was shown by qRT-PCR. GRO-α?knock-down on protein level was detected by GRO-α-ELISA. To exclude that the cells undergo apoptosis the TUNEL assay was performed.

Results: Highest GRO-α?gene reduction was achieved by 8 h transfection of primary keratinocytes and 10 h transfection of HaCaT keratinocytes. The optimal recovery time was 14 h for primary keratinocytes and 16 h for HaCaT keratinocytes. Both cell types secreted GRO-α?protein in culture supernatant. The protein levels of GRO-α?were reduced about 30 % in primary keratinocytes and about 60 % in HaCaT keratinocytes compared with negative controls. No apoptotic effect of a GRO-α?siRNA was detected. GRO-α?siRNA had no influence on IL-6, IL-8 and activin levels. VEGF levels were reduced about 70 % in HaCaT keratinocytes.

Conclusion: Protein levels of GRO-α, a pro-angiogenetic chemokine, and VEGF strongly correlate, an evidence of the close involvement of both factors in wound healing.

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In vivo confocal laser-scanning microscopy of inflammatory skin diseases

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Background: *In vivo* confocal laser-scanning microscopy is a recently developed potential diagnostic tool, which provides a non-invasive window into living skin at nearly histologic resolution. The aim of our present study is to evaluate lesions of inflammatory skin diseases, such as psoriasis, mycosis fungoides, chronic discoid and subacute cutaneous lupuserythematosus (CDLE, SCLE), in order to define typical features and to compare with histopathological characteristics.

Methods: In our ongoing study we included 68 patients. We have evaluated >1000 confocal laser scanning images of 30 psoriasis, 11 contact dermatitis, 10 mycosisfungoides and 17 CDLE/SCLE lesions.

Results: In psoriatic lesions characteristics such as parakeratosis, elongated and increased papillae with dilated capillary loops were always present. Inflammatory cells widespread within the epidermis and upper dermis, focally forming microabscesses were identified in about 80%. Transepidermal migration of inflammatory cells, epidermal oedema with microvesical formation and dermal vasodilatation could be observed in contact dermatitis lesions. Even langerhans cells were visualized by CLSM imaging. The examination of mycosis fungoides lesions revealed an infiltration of the upper epidermal layers by roundish cells, distributed in nests or diffusely widespread throughout the epidermis. Skin lesions in patients with CDLE or SCLE showed inflammatory cells and blurred intercellular borders, focally with degeneration of keratinocytes and a loss of regular epidermal stratification.

Conclusion: The corresponding histopathological evaluation matches well with CLSM images. CLSM seems to be a promising method for examination of dynamic skin processes, but the limited depth of penetration and the disability to distinguish between white blood cells in detail awaits further development.

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Evaluation of disease activity in pemphigus patients by the autoimmunebullous skin disorder intensity score (ABSIS)

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Pemphigus represents a group of severe blistering skin diseases clinically characterised by flaccid blisters and erosions of the skin and mucous membranes.High dose immunosuppressive drugs are commonly applied, but long term control of disease activity remains challenging. Therefore, clinical studies evaluating the safety and effectiveness of new therapies are strongly needed. A principal requirement for performing multicenter trials in pemphigus is a clinical score to quantify disease activity. The aim of this study was to validate the recently introduced disease score ABSIS in a prospective setting. Scoring cutaneous involvement by ABSIS involves both the extent of affected skin area and the quality of the lesions. Involvement of the oral mucosa is quantified by the presence of lesions at defined areas and patients' discomfort during ingestion of food and beverages. A group of 17 pemphigus patients (15 pemphigus vulgaris, two pemphigus foliaceus) were scored using ABSIS for aperiod of 12 months. ABSIS values for cutaneous and mucosal involvement werecorrelated to desmoglein 1 (dsg1)- and dsg3-reactive autoantibody titres, respectivelyand to cumulative doses of systemic corticosteroids. Mean anti-dsg3-IgG titresdecreased from initially 648 ±? 930 PIV (protein index value) to 331 ±? 428 PIV 12 months later, correlating well with the decreased mucosal ABSIS scores (2.7 ± 3.3 at day 0 and 0.8 ±? 1.4 at 12 months). ABSIS values for cutaneous involvement droppedfrom 9.5 ±? 22.4 to 0 at 12 months, paralleling the decline of anti-dsg1-IgG titres (176 ±? 275 PIV at day 0 and a PIV < 20 12 months later). During the 12 months' follow-upperiod systemic prednisolone was gradually tapered as reflected by the decrease ofits mean cumulative dose (0.5 ±? 0.52 mg/kg/d at day 0 to 0.08 ±? 0.12 mg/kg/d at 12 months). Both cutaneous and mucosal ABSIS values correlated well with the mean cumulative doses of steroids . Moreover, there was an excellent correlation between the objective mucosal ABSIS and the patients' subjective score for discomfort during ingestion. Therefore, ABSIS holds great promise as a direct and comprehensiveclinical scoring system in both mucosal and cutaneous variants of pemphigus.

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High frequency of Corticosteroid and immunosuppressive therapy in patients with systemic sclerosis

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Systemic sclerosis (SSc) is a severe and life-threatening autoimmune disease, whereonly little data exist on the effectiveness of anti-inflammatory and immunosuppressivetherapy. Corticosteroids are still the mainstay of treatment for most autoimmune diseases. However, there is no controlled study available on the use of corticosteroids in SSc that demonstrates improvement of skin or organ involvement. Our objective was to evaluate the corticosteroid and immunosuppressive treatment in >1700 patients whose data were recorded in the registry of the German Network for Systemic Scleroderma (DNSS). Latest analyses of the data showed, that 40.8% received corticosteroids. Corticosteroid use was reported in 50.3% of patients with diffuse cutaneous (dcSSc), 31.5% of patients with limited cutaneous SSc (lcSSc) and 62.7% with over lapsyndromes. Internal organ involvement was associated with varying degrees of corticosteroid use. Immunosuppressive therapy was prescribed in 34.7% of SSc patients. 62.2% of patients with overlap syndromes were treated with immunosuppressants, compared with 47.1% of those with dcSSc and 22.1% with lcSSc. The most commonly used compounds included methotrexate (31.2%), azathioprine (22%), cyclophosphamide (20.8%) and chloroquine (8.8%) varying significantly in their use in different disease subsets. Despite lacking evidence for their effectiveness the study reveals a high frequency of corticosteroid and immunosuppressive therapy. This study therefore underlines the urgent need to develop treatment recommendations for SSc and constitute a basis to follow and compare the outcome of national and international developments of therapeutic strategies in daily practice in the future.

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Pili annulati associated with alopecia areata: coincidence or a new entity?

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The hair shaft disorder pili annulati (PA) is inherited in an autosomal dominant manner and was recently linked to chromosome 12q24.32–24.33. Clinically, PA is characterized by alternating light and dark bands. Interestingly, the manifestation of PA with concomitant alopecia areata (AA) has been reported on several occasions. However, no systematic evaluation of patients manifesting both diseases has been performed yet. Therefore, we compared clinical and epidemiologic data of 14 unrelated PA families (162 family members; 76 affected and 86 non-affected individuals) with that of six patients manifesting both diseases who were previously reported by other groups. Seven individuals (Six women; one man) from distinct PA families showed AA. Six of these patients were only diagnosed with PA after they initially presented to a dermatologist with complaints of AA. Although apparently rare, the simultaneous occurrence of AA in patients with PA is well documented and could be confirmed in our PA families. Based on the current data, however, a direct association between these two disorders seems rather unlikely. This notion is also supported by the recent reports about new susceptibility loci for AA that do not coincide with the candidate region for PA. Interestingly, however, the clinical course of AA observed in five of our PA patients was more severe than usually encountered. Thus, it is tempting to speculate that an as hitherto unknown genetic defect giving rise to PA might also confer a more pronounced manifestation of AA with involvement of larger scalp areas, longer lasting disease, and frequent recurrence. Since the total number of patients reported with both conditions is too low to draw far-reaching conclusions, prospective studies and elucidation of the molecular basis of PA might deliver valuable information as to a putative association with AA.

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Effects of 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin on SZ95 sebocyte proliferation and lipid synthesis

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Introduction: Chloracne is an acneiform skin disease, which is considered to be the most specific and sensitive clinical condition of dioxin intoxication. Sebogenesis is decreased and skin xerosis is one of the most prominent clinical characteristics compared with acne vulgaris. However, the mode of action of dioxin on these baceous glands is still unclear. We studied the effects of TCDD (2, 3, 7, 8-tetrachlorodibenzo-p-dioxin), which is the most active congemer of the polychlorinated dioxins, on proliferation and lipid synthesis (a marker of sebocyte differentiation) of human SZ95 sebocytes *in vitro*.

Materials and methods: Proliferation of SZ95 sebocytes after treatment with TCDD atvarious concentrations for 3 days was assessed by the XTT method. After pretreatment with and without linoleic acid (10^{-4} M for 3 days), SZ95 sebocytes were treated again with TCDD with and without linoleic acid 10^{-4} M for three additional days. Neutral lipids in SZ95 sebocytes were labelled with the fluorescence dye nile red and analyzed in flow fluoroscan reader.

Results: TCDD (10 nM) slightly increased sebocyte proliferation after 3 days of treatment ($P < 0.05$). The neutral lipid content in SZ95 sebocytes was markedly and concentration dependently inhibited, when SZ95 sebocytes were treated with linoleic acid and TCDD (10 nM, $P < 0.01$; 1 nM, $P < 0.05$), independently of the pretreatment with linoleic acid. SZ95 sebocyte lipid content was not affected by TCDD alone.

Conclusions: TCDD can markedly inhibit sebaceous lipogenesis and slightly increasesebocyte proliferation *in vitro*. These findings indicate that decreased sebaceous gland differentiation instead of altered proliferation is likely to be the major reason of decreased sebogenesis in patients with chloracne.

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Biphasic expression of stromal cell-derived factor-1 (SDF-1/CXCL12) during human wound healing

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Chemokines tightly regulate the spatial and temporal infiltration of invading leukocyte subsets during wound healing. Furthermore, they contribute to the regulation of epithelialization, tissue remodeling and angiogeneseis. In contrast to the majority of chemokines that is exclusively expressed under pathological conditions such as inflammation, the multifunctional CXC chemokine stromal cell-derived factor-1(SDF-1/CXCL12) is a homeostatic chemokine which is abundant in the skin. Its role during cutaneous wound healing, however, needs to be explored. To elucidate the expression of SDF-1/CXCL12 during human wound healing skin biopsies were obtained from 14 volunteers between 1 and 21 days after incisional wounding and processed for *in situ*-hybridization and immunohistochemistry. Analyzing the spatial and temporal distribution of SDF-1/CXCL12 after artificial wounding we detected a complete down-regulation at both the mRNA and protein level within the fibrous stroma that is replacing the initial wound defect. However, at the wound margins increased levels of SDF-1/CXCL12 were observed. Focusing on mediators regulating SDF-1/CXCL12 expression *in vitro* we realized that both tumour necrosis factor-α (TNFα) and interferon-γ (IFNγ) downregulated its expression in dermal microvascularendothelial cells and fibroblasts. Our data suggest that SDF-1/CXCL12 is tightly regulated during wound repair. Beyond its classical effects on leukocyte attraction SDF-1/CXCL12 may interfere with central events occurring during wound repair that include regulation of fibroblast proliferation and epithelialization. In addition, increased expression at the wound margin may contribute to the accumulation of endothelial progenitor cells resulting in accelerated neovascularization.

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The macrophage-activating lipopeptide-2 (MALP-2) which accelerates wound healing in mice is well tolerated after intracutaneous injection in a phase I trial in 12 patients

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Chronic skin ulcers, such as leg ulcers, pressure sores and diabetic foot ulcers are achallenge to physicians and medical personnel, and a cause of tremendous discomfort and ensuing loss of quality of life to the patients. Wound healing involves production and action of various growth factors. A novel approach, distinct from the application of single growth factors, is the administration of the macrophage stimulator MALP-2. The rationale is based on the finding that macrophages are the main source of several growth factors required for wound healing which are sequentially released during this process. MALP-2 has previously been shown to be effective in an established animal model with diabetic mice. The purpose of the present phase I study was to establish tolerability of MALP-2 when applied into small cutaneous wounds in human beings. Twelve patients (Six female and six male, mean age 66.8 years, range 52–87 years) with different diagnoses were enrolled into the study. An artificial wound was created with a 2 mm diameter skin biopsy punch and a volume of 100 μl MALP-2 (0.125 μg up to 1 μg) or vehicle control, respectively, was injected intracutaneously into the wound and closed with a water resistant transparent adhesive. Photos were taken daily from every patient up to 6 days and skin biopsies were performed after 1 week from six patients. We could show in the present study for the first time that MALP-2 was well tolerated up to a dose of 1 μg per wound in human beings. In healthy as well as in diabetic patients MALP-2 induced local inflammation which faded after 48 h. The effectiveness of MALP-2 in the healing of chronic wounds in humans, e.g. in chronicskin ulcers, such as leg ulcers, pressure sores and diabetic foot ulcers could now be addressed in further studies.

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Comparison of immune responses after vaccination with tumour-peptide-loaded dendritic cells with or without prior denileukin diftitox treatment in metastasized malignant melanoma

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Expansion of tumour-specific T-cells and tumour regression has been observed upondendritic cell (DC) vaccination. We recently presented a regular and up to 100-fold expansion of circulating tumour-antigen-specific CD8+ T cells as well as an increase of helper T cells in a previous trial employing tumour-peptide-loaded DC to treat melanoma patients ($n = 62$). To further enhance immune responses we conducted a trial relying on an identical vaccine and schedule except that denileukin-diftitox (ONTAK®) was administered on days -3,-2,-1 before the first vaccination at 5 μg/kg with the intention to deplete regulatory T cells. In contrast to what one might have expected from published data (e.g. Mahnke et al. Int J Cancer, June 2007) we neither found a significant decrease in regulatory T cells nor an enhanced immunity.In contrast, immune responses which had been regularly induced or expanded after DC vaccination in the preceding trial (as measured by ELISPOT and determination of precursor cell frequencies in mixed lymphocyte/peptide cultures) remained strikingly low and sometimes even decreased in these ONTAK®?pretreated patients. We are currently looking into the causes of this unexpected response to ONTAK®?pretreatment emphasizing a varying sensitivity of regulatory T-cells in different clinical settings and to different doses of denileukin-diftitox.

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Clinical images and therapy of cutaneous side-effects induced by inhibitors of the epidermal growth factor receptor

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Here we present the broad spectrum of cutaneous side-effects induced by inhibitors of the receptor of the epidermal growth factor (EGFR). Furthermore, we discuss novel clinical observations, that provide interesting new insights into the pathogenesis of this unique cutaneous toxicities. Finally, an algorithm for the management of EGFR-inhibitor (EGFRI) is given. Recently, EGFRI, like the tyrosinekinase inhibitors erlotinib (Tarceva®) and gefitinib (Iressa®), or the monoclonalantibody cetuximab (Erbitux®) have been established successfully as so called targeted cancer drugs in the clinical practice. Most frequent and severe side-effects of this class of drugs are cutaneous toxicities occurring in >50 percent of the patients: within the first weeks after initiation of EGFRI therapy patients develop inflammatory, papulopustular eruptions mainly located on the face and the upper trunk. Subsequent super infection of this rash is observed frequently. Additionally, many patients develop paronychias, xerosis cutis, itch, as well as a progressive atrophy of the skin. Interestingly, cutaneous lesions appear to be induced or aggravated by physical stimuli like mechanical trauma or UV-irradiation. Next to the skin also hair follicles are a target of EGFRI. Patients develop a variety of hair alterations like non-scarring alopecia, trichorhexis or trichomegaly. EGFRI related cutaneous side-effects represent a severe threat for patient compliance and their management is challenging. Nevertheless, most side-effects are reversible when EGFRI therapy is stopped or doses are reduced. However, own clinical experiences have shown, that side-effects can be managed effectively by applying combinations of anti-inflammatory and anti-microbial agents according to a specific management algorithm. An effective treatment can help to manage these challenging toxicities and avoid a reduction of dose or even cessation of EGFRI therapy in the most cases.

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Toward the pathogenesis of cutaneous side-effects induced by inhibitors of the epidermal growth factor receptor

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Here we analyze the function of the receptor of the epidermal growth factor (EGFR) for the cutaneous expression of chemokines and antimicrobial peptides. Recently, inhibitors of the EGFR (EGFRI), like the small molecule tyrosine kinase inhibitors erlotinib (Tarceva®) and gefitinib (Iressa®), or the monoclonal antibody cetuximab (Erbitux®) have been established successfully as so called targeted cancer drugs in the clinical practice. Most frequent and most severe side-effects of this class of drugsare cutaneous toxicities occurring in >50 percent of the patients. Patients treated with EGFRI develop a characteristical inflammatory papulopustular rash, xerosis cutis, as well as hair and periungual alterations. In the course, superinfections of the rash with *Staphylococcus aureus* occur frequently. These clinical observations assume, that the EGFR critically regulates cutaneous inflammation and infection. Nevertheless, the pathogenesis of EGFRI induced cutaneous side-effects has yet to be elucidated. Here we analyze the *in vitro* expression of chemokines and antimicrobial peptides in primary human keratinocytes treated with EGFRI using quantitative real time PCR. Moreover, we examine the *in vivo* expression of chemokines in skin samples of patients treated with erlotinib. Our results demonstrate that the EGFR in fact controls the cutaneous expression of chemokines and antimicrobial peptides. In particular, we show that the blockade of the EGFR induces an overexpression of proinflammatory chemokines as well as a suppression of antimicrobial peptides in human keratinocytes. In line with these findings, cell supernatants of EGFRI treated keratinocytes demonstrated a reduction of the cytotoxic activity against *Staphylococcus aureus* as compared to medium controls. Our results present interesting new insights into the pathogenesis of cutaneousside-effects associated with the use of EGFRI.

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Withdrawn.

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Differences in intercellular cytokine release of lesional versus non lesional skinin patients with atopic dermatitis determined by cutaneous microdialysis and flow cytometric bead-array analysis

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Objective: Release of cytokines are of interest in skin inflammation. Cutaneous microdialysis is a novel method to analyze the kinetic of inflammation markers *in vivo*. We addressed the issue if microdialysis can be used to detect differences in cyto kineamount from intercellular space in lesional as well as non lesional skin of patients with atopic dermatitis.

Methods: Five patients with moderate atopic dermatitis (SCORAD <50) at least at both arms were included. Microdialysis was performed at lesional as well as non lesion alatopic skin at the forearms of each patient. For cutaneous microdialysis, 20 kD cutoff membranes of 2 cm with a flow rate of 0.5 μl/min were used and perfused with sterile solution containing Dextran 60 by mobile CMA 107 microdialysis pumps. After microdialysis membrane placement, membrane was flushed with perfusate solution at 5 μl/min for 2 h. Then microdialysate samples were collected at 4 h intervals up to 8 h. Up to 18 cytokines in microdialysates were determined with a flow cytometric bead array-kit (Becton Dickinson).

Results: Cutanous microdialysis was well tolerated by all five patients with atopic dermatitis without any side effects over 10 h. Insertion of microdialysis catheters resulted in an increase of cytokine release. In various but not all patients differences in cytokine amount released from lesional skin compared to non lesional skin could be detected.

Conclusion: Microdialysis may have the potential to detect differences in the cytokine amount from intercellular space in skin diseases such as atopic dermatitis and for analysis of skin pathology *in vivo* at least up to 10 h.

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Serum concentrations of IP-10 in patients with metastasising melanoma during chemotherapy

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Various chemokines have been shown to play an important role in melanomaprogression and metastasis. Among these, the angiostatic effects of inferferon γinducible peptide of 10 kD (IP-10) may be of special importance. First results of acorrelation of IP-10 serum levels and responses to chemotherapy in individual patients were further scrutinized in this study. Twenty-nine patients with metastasising melanoma were followed up during systemic chemotherapy (BELD, BHD, dacarbazin and fotemustin) by taking sequential serum samples and testing IP-10 levels in a specific in house ELISA. Elevated chemokine levels were found in correlation tometastases, not, however, to S100 levels, with a decrease of IP-10 concentrations following tumour progression. No correlation was found with the clinical response to chemotherapy. Though IP-10 expression is a sensitive parameter for inflammatory and neoplastic processes, serum levels are influenced by diverse mechanism of binding and inactivation. Major effects may only be exerted at the local level of the tumour. Altogether, despite earlier results, IP-10 serum concentrations do not allow clinical monitoring nor estimating prognosis of metastasising melanoma.

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Pimecrolimus but not betamethasone preserves antimicrobial protein expression in atopic dermatitis

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Previously, reduced expression in antimicrobial proteins (AMP) in atopic dermatitis(AD) in comparison to psoriasis has been described and this may be a reason for the increased rate of bacterial infection. We asked whether topical drugs affect AMP expression in AD. In a randomized, double-blinded left-right comparison study, 15 patients with AD were treated on one upper limb with pimecrolimus and on the other with betamethasone twice daily for 3 weeks. Immunohistology was performed with specific antibodies for AMPs: psoriasin, RNase7, human beta-defensin 2 (HBD2), human beta-defensin 3 (HBD3), cathelicidin (LL-37), and the α-defensins HNP1-3. We found, as recently reported, a pronounced expression of psoriasin in AD before treatment in the upper spinous, the granular, and the horny layers. Bethamethasone treatment led to a significant reduction in psoriasin expression in most of the patients, whereas pimecrolimus led to a slight reduction of psoriasin expression, only. HBD2, HBD3, and RNase7 showed a weak to moderate staining in the epidermis in AD before treatment. After treatment with betamethasone, staining intensity was reduced or disappeared, whereas pimecrolimus treatment led to slightly reduced staining, only. For LL37 and HNP1-3 there was no clear immunoreactivity in AD before treatment and staining intensity did not significantly change after treatment with either treatment regime. We conclude that treatment of AD by betamethasone, in contrast to pimecrolimus, results in reduced protein expression of several AMPs in most of the patients. The reduction of AMPs is probably related to a broad reduction in protein synthesis by the potent corticosteroid, which further increases the risk of skin infections. In contrast, pimecrolimus does not seem to significantly affect the skin's ability in AMP expression and preserves antibacterial defence.

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ECE-1 regulates receptor recycling-independent resensitization

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Substance P, is localized in unmyelinated sensory nerve fibers in skin. Release of Substance P causes redness, wealing and itching. Phosphorylation of Gprotein-coupled receptors plays an important role in regulating their function. Endothelin-converting enzyme 1 (ECE-1) inactivates internalized SP within early endosomes thereby accelerating resensitization of these cells. The time course of degradation of internalized SP by ECE-1 is independent from the concentration of the ligand. Confocal laser scanning microscopy shows that intracellular degradation of SP by ECE-1 allowed dissociation of beta-arrestin-1 from early endosomes and trafficking of beta-arrestin-1 to the cell membrane. Resensitize of NK1R to a second challenge of SP is not mediated by recycling of the internalized NK1R but rather by dephosphorylation of cell membrane-located receptors by a fostriecin-senitive proteinphosphatase 2A (PP2A). Surface receptor binding assays demonstrate that SP binding sites did not recover 2h after challenge of cells with SP, a time point where cells are normally fully resensitized. The stimulation with SP induced association ofPP2A on cell membrane-located NK1R. We have thus identified that resensitization of NK1R-expressing cells is mediated by reactivation of cell membrane-associated NK1R by PP2A.

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Immunogenicity of HLA-A2-restricted peptide epitopes of the tumourstroma-associated antigen FAPα

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Fibroblast activation protein α (FAPα, seprase) is a cell surface protease which is hardly expressed on normal adult tissue but is upregulated in the tumour micromilieu, i.e. on cancer-associated fibroblasts. Thus, FAPα is a promising target for cancer immunotherapy. Consequently, we identified several HLA-A2-restricted peptide epitopes derived from FAPα by means of reverse immunology. Notably, spontaneous T cell responses to these epitopes were detectable in melanoma patients; (i) FAPα-reactive T cells were detected by FCM with HLA-A2/FAPα peptide-multimers; (ii) their functional capacity was revealed by IFN-γ, and granzyme B ELISPOT assays as well as their capacity to lyse both peptide pulsed target cells as well as HLA-matched *in vitro* activated FAPα-positive fibroblasts. Further experiments revealed that FAPα-reactive T cells can not only be induced by stimulation with there spective FAPα peptides, but also by stimulation with FAPα mRNA transfected DC, indicating that the identified peptide epitopes are indeed generated by processing of endogenously expressed FAPα. Subsequent optimisation of the anchor amino residues of these HLA-A2-restricted peptide epitopes enhanced their binding to theHLA-A2 molecule. Immunisations of HLA-A2/kb transgenic mice using such immunodominant epitopes induced FAPα-specific T cell responses. Hence, FAPαdirected vaccination may be used to target the immune system to the tumour stroma to treat cancer.

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T cell-mediated immunity differs significantly in patients with herpes zoster incomparison to age- and gender-matched controls

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The incidence and severity of herpes zoster and postherpetic neuralgia increase with age in association with a progressive decline in cell-mediated immunity to VZV. Immunological studies should focus on both, serologic testing and evaluation of cell-mediated immunity. The objective of our experiments was to compare cellular immunity of patients with herpes zoster in comparison to age- and gender-matched controls with positive VZV-serology. Lymphocyte subpopulations were analyzed performing four-colour flowcytometric analyses. VZV-specific CD4 and CD8 cell frequencies were measured byIFN-g Enzyme-linked Immunospot (Elispot) assay. Blood was drawn at the beginning of shingles and after a time period of 4 weeks (tp4). Statistical analysis was done by Wilcoxon-, Mann–Whitney-, and Friedman–test. At tp0, the median frequencies of total lymphocytes, T cells (CD3), B cells (CD19), cytotoxic T cells (CD8), T helper cells (CD4), activated T cells (CD3, HLA-DR), NK cells (CD16+CD56+), and NKT cells (CD3+CD16+CD56+) were significantly lower in 21 patients with shingles as compared to 18 controls. At tp4, the median frequencies of total lymphocytes, CD4 cells, NK cells, and NKT cells remained significantly lower. Between tp0 and tp4, in the patients with herpes zoster, the percentage of the median frequencies of T cells and CD4 cells decreased, whereas CD8 cells and NK cells increased significantly. There were no significant changes of the controls between tp0 and tp4. At the beginning of shingles, the median number of CD8 cells and CD4 cells reactive to VZV were significantly higher, 6-fold and 14-fold, respectively, as compared to the controls. After 4 weeks the responses to VZV declined significantly, but the number of VZV-specific CD4 cells remained high. In summary, T cell-mediated immunity during the course of herpes zoster was surprisingly suppressed in comparison to the controls. However, the patients with shingles developed a boosted VZV-specific immune response in both T cell subsetsCD4 and CD8, which remained detectable after 4 weeks by the Elispot assay. Further immune monitoring and long-term surveillance studies in VZV and in VZV/zoster vaccination are needed.

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Peroxisome proliferator-activated receptor (PPAR)- and vitamin D receptor(VDR)-signalling pathways in melanoma

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Peroxisome proliferator-activated receptors (PPARs) are members of the nuclearreceptor superfamily of transcriptional regulators that regulate lipid, glucose, andamino acid metabolism. In recent studies it also has been shown that these receptor sare implicated in tumour 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 (LightCycler), we characterized expression of PPARa, d and g in primary cultured normal melanocytes and in melanoma cell lines. 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 now 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- signalling pathways and our data support the concept that PPARs may be of importance for pathogenesis, progression, and therapy of malignant melanoma.

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Neuroendocrine cross-regulation in human scalp skin: ACTH and TRHstimulate prolactin and prolactin receptor expression in human hairfollicles

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The pituitary hormone prolactin modulates hair growth in different species. We have recently shown that prolactin induces premature catagen development by up-regulation of apoptosis and down regulation of proliferation in organ-cultured human hair follicles (HF). In addition, we could show that the hair follicle itself is an important source for prolactin synthesis in the skin. The regulation of prolactin expression and secretion is very complex and differs in the periphery from the pituitary gland. Therefore, this study aimed at testing whether recognized stimulators of prolactin synthesis and secretion in the pituitary gland like TRH, thyroid hormones(T3, T4), CRH and ACTH have similar effects on prolactin expression in the HF. HF in the anagen VI stage of the hair cycle were cultured in the presence of TRH (1 ng/ml, 10 ng/ml, 1 μg/ml), T3, T4 (100 pM), ACTH (10^{-7} M) or CRH (10^{-8} M) for 6 days. After 6 days hair follicles were harvested and cryosectioned. Only hair folliclesin early catagen were used, since prolactin expression is up regulated in catagen. Prolactin and prolactin receptor expression were assessed by quantitative immunohistochemistry. Immunoreactivity (IR) was prominently noted in the outer roots heath (ORS), while the ligand was seen in the ORS and matrix keratinocytes in all HF. Prolactin IR was significantly increased in the ORS in TRH- or ACTH- treated HF, while treatment with CRH did not show marked changes in prolactin IR. The stimulating effect of TRH was dose-dependent: While TRH 1 ng/ml showed no difference in the staining compared to the control, TRH 10 ng/ml and TRH 1 μg/ml showed increasing expression in the ORS of early catagen HF. In some TRH-treated HF, both prolactin and prolactin receptor IR could also be detected in the dermal papilla, where it is usually not expressed. In T3, T4 treated HF prolactin expression also appeared to be up regulated. In conclusion, these pilot data suggest the existence of a novel intracutaneousneuroendocrine cross-regulatory loop, with TRH, T3, T4 as well as ACTH as apparent stimulators of prolactin synthesis in human scalp skin, whose functional significance for human skin and HF biology must now be systematically explored.

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Recovery of cytokines can be improved *in vitro* and *in vivo* using oncoticactive perfusates in microdialysis

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Objectives: Microdialysis is a method to study release of inflammation markers inhuman tissue as well as human skin under *in vivo* conditions. Release of cytokines to the extra cellular fluid is of high interest to study skin pathology and tissue micro environment but when using micro dialysis it has several limitations as very low recovery by the micro dialysis catheters and ultra filtration. We investigated if the use of oncotic active agents such as colloid solutions and plasma expanders for perfusate solution may improve recovery of cytokines by the catheter.

Methods: 800 pg/ml of different cytokines with various size (IL8 (8 kD), IL2 (15.4 kD),TNF-alpha (17.5 kD), IL6 (20.5 kD), VEGF (39 kD) and IL12p70 (70 kD)) were injected in excised human skin and added to human blood, human plasma and water. Microdialysis was then performed using a mobile CMA107 microdialysis pumps at flow rates of 2 μl/min, 1 μl/min, 0.5 μl/min and 0.2 μl/min and catheters of 100 kD cut off (CMA71, 20 mm) for all cytokines and catheters of 20 kD cut off (CMA70, 20 mm) for IL8, IL2 and TNF-alpha. For microdialysis different perfusate solutions were used: NaCl 0.9%, Ringer Lactate, Glucose 10%, Dextran 40, Dextran 60, HES 200,35 kD gelatine and human serum albumin 20%. Furthermore retrodialysis was performed in excised human skin, human blood, human plasma and water *in vitro* using the same setup but adding cytokines to the different perfusates this time. Recovery determined by retro dialysis was calculated: 100-(%cytokine indialysate/%cytokine in perfusate). Cytokine recovery was done *in vivo* with retrodialysis using pig skin of anaesthesized pigs and CMA71 catheters, Dextran 60 as perfusate solution at a flow rate of 0.5 μl/min. Cytokines were determined by a colorimetric ELISA (RayBio inc) measured at 450 nm.

Results: *In vivo* recovery of several cytokines with different size were determined for the first time by retrodialysis. Differences in cytokine recovery were observed inhuman skin in comparison to human blood, human plasma and water. Using oncoticactive agents as colloid solutions and plasma expander could improve recovery of cytokines.

Conclusion: The use of colloid active agents as colloidal solutions may improve recovery of cytokines in microdialysis for the measurement of intercellular fluid in skin pathology.

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The relevancy of molecular weight and pH-value on the antimycotic activity of chitosan hydrochloride and carboxymethyl chitosan against *Candida albicans* -Comparative measurements via nephelometry and fluorescence

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Chitosan hydrochloride is a water soluble derivate from chitosan. Chitosan is produced via deacetylation of the amino groups of chitin. Another water soluble derivate is carboxymethyl chitosan, which is won by integration of a hydrophiliccarboxymethyl group. For determination of the molecular weight measurement of viscosity is one possible method. We compared antifungal activity of chitosanhydrochloride with viscosities of 5 mPas (low molecular weight) and 100–150 mPas(high molecular weight). Measurements were done with microplate readers detecting scattering (nephelometer) and fluorescence of cultures from *Candida albicans* (DSM11225). Fluorescence stain was Alamar BlueTM. The conditions of incubation were due to NCCLS-M27 A2 (neutral medium RPMI, incubation for 48 h, inoculumsize 0.5–2.5 × 10e3 cells/ml). For comparison of the activity of chitosan hydrochloride and carboxymethyl chitosan measurements were done under weak acid conditions for only 24 h (Sabouraud-Glucose-Bouillon). The results were expressed via calculating the minimal concentrations, which inhibit the yeasts about 80% (minimalinhibitory concentration, MIC80). Calculations were arranged with SPSS statistical programme. The most effective substance is high molecular weight chitosanhydrochloride with MIC80 between 0.186% and 0.877%. Both low and high molecular weight chitosan showed more inhibition under acid conditions (pH5) than in neutral medium. After incubation in neutral medium the high molecular substance featured a better antifungal activity than the low molecular weight chitosan hydrochloride. In contrast the latter substance advanced growth of *Candida albicans*. The results from nephelometry and fluorescence measuringaccording to NCCLS were similar. Carboxymethyl chitosan only showed little influence on *Candida albicans* compared with high molecular weight chitosan hydrochloride. The MIC80 of carboxymethylchitosan was superior to the highest tested concentration. Thus neutral medium and low molecular weight are predictors of worse antifungalactivity. Integration of a carboxymethyl group decreases antifungal activity, too.

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Fusariosis in a peripheral blood stem cell transplant (PBSCT) recipient with acute lymphoblastic leukaemia - Comparative diagnostics with MALDI-TOF and PCR

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Infections with Fusarium spp. occur seldom. Mostly patients suffer from underlying immune deficiency and prognosis decreases after infection with this mould. Fusarium spp. more often associated with cutaneous involvement than Candida spp. or Aspergillus spp. in immuno-compromised patients. We describe one patient with acute lymphoblastic leukaemia, who was treated firstly with chemotherapy after GMALL protocol 07/03. After relapse the patient was successfully transplanted with aCD34+ allogenic HLA-matched peripheral blood stem cell graft. Ten months later, after a new relapse the patient died from neutropenic fever and respiratory insufficiency. Mycological serology was positive. Antifungal therapy with liposomal amphotericin did not lead to success, in contrast disseminated papules appeared. Histology showed hyphens, conventional cultural diagnostics, polymerase chain reaction and determination of the genus with MALDI-TOF (Matrix Assisted Laser Desorption/Ionisation - Time Of Flight) resulted in Fusarium proliferatum.

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Immunization with a dominant-negative recombinant Herpes simplex virus(HSV) type 1 protects against HSV-2 genital disease in guinea pigs

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The best option to prevent primary infection with herpes simplex viruses (HSV) and subsequent recurrences would be a prophylactic vaccine that is safe and effective against both HSV-1 and HSV-2. CJ9-gD is a novel dominant-negative recombinantHSV-1 that can inhibit its own replication as well as that of wild-type HSV-1 and HSV-2 in co-infected cells. CJ9-gD is completely replication-defective, does not establish detectable latent infection *in vivo*, and expresses high levels of the majorHSV-1 antigen glycoprotein D immediately following infection. In mice CJ9-gD induces strong and protective immune responses both against HSV-1 and HSV-2 genital disease. Because of the close resemblance to HSV infections in humans, guinea pigs remain the ideal animal model to study HSV disease and its prevention. Previously, we found that immunization with CJ9-gD in guinea pigs effectively protects against primary HSV-1 skin disease and reduces the extent of latent infection. In this report we investigated the effectiveness of CJ9-gD as a vaccine against HSV-2 genital disease in guinea pigs. Animals immunized with CJ9-gD developed at least 700-fold higher titers of HSV-2-specific neutralization antibodies than mock-immunized controls. After challenge with wild-type HSV-2, all 10mock-immunized guinea pigs developed multiple genital lesions with an average of21 lesions per animal, whereas only 2 minor lesions were found in two of 8CJ9-gD-immunized animals, representing a 40-fold reduction on the incidence of primary genital lesions in immunized animals ($P < 0.0001$). The amount and duration of viral shedding from the vagina after challenge was significantly reduced in immunized guinea pigs compared to mock-immunized controls. Immunization ameliorated symptoms, prevented systemic involvement and provided 100% survival, whereas 90% of mock-immunized animals succumbed due to the severity and extent of disease. After recovery from primary infection immunized animals showed no signs of recurrent disease. Collectively, we demonstrate that vaccination with the HSV-1 recombinant CJ9-gD elicits strong and protective immune responses against primary and recurrent HSV-2 genital disease.

P169

Dermcidin - derived peptides show a different mode of action against *Staphylococcus aureus* than the cathelicidin LL-37

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Dermcidin (DCD) is an antimicrobial peptide, which is constitutively expressed in eccrine sweat glands. By postsecretory proteolytic processing in sweat the Dermcid inprotein gives rise to anionic and cationic DCD-peptides with a broad spectrum of antimicrobial activity. Many antimicrobial peptides induce membrane permeation as part of their killing mechanism, which is accompanied by a loss of the bacterial membrane potential. We could show that there is a time-dependent killing by anionic and cationic Dermcidin-derived peptides which is followed by bacterial membrane depolarization. However, Dermcidin-derived peptides do not induce pore formation of the membranes of Gram-negative and Gram-positive bacteria. This is in contrast to the mode of action of the cathelicidin LL-37. Interestingly, LL-37 as well as Dermcidin-derived peptides inhibit bacterial macromolecular synthesis, especially RNA- and protein synthesis without binding to microbial DNA or RNA. In our previous studies using immune electrone microscopy we could show that anionic and cationic DCD peptides interact with the bacterial cell envelope. In order to identify the target to which DCD peptides bind, we performed binding studies with components of the cell envelope of Gram-positive and Gram-negative bacteria and with model membranes. These studies indicated that Dermcidin-derived peptides show only a weak binding to lipopolysaccharide (LPS) from Gram-negative bacteria as well as top eptidoglycan (PGN), lipoteichoic acids (LTA) and wall teichoic acids (WTA) isolated from *S. aureus*. In contrast, LL-37 binds strongly in a dose-dependent fashion to these components. These data indicate that the mode of action of Dermcidin-derived peptides is different from that of the cathelicidin LL-37 and probably most other pore-forming antimicrobial peptides, hence mode of antimicrobial activity of Dermcidin-derived peptides awaits further elucidation.

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Differential induction of antimicrobial peptide expression in human keratinocytes by microorganisms

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Human skin is selectively colonized, especially by *Staphylococcus epidermidis*,whereas *Staphylococcus aureus* is only rarely found on healthy human skin. However, patients with atopic dermatitis often suffer from skin infections with *S. aureus*. In this study, we asked whether human keratinocytes respond differentially with respect to the induction of antimicrobial peptide expression upon contact with *S. aureus* and *S. epidermidis*. This would explain the selective microbial skin colonization. We analyzed by real-time PCR in primary keratinocytes and in the immortalized keratinocyte cell line HaCaT the expression of the beta-defensinshBD-1,-2,-3 and RNase7 after incubation with *S. aureus* or *S. epidermidis*. Interestingly, both viable and the supernatant of *S. aureus* were able to induce the expression of hBD-3 in keratinocytes up to 10-fold higher than S. epidermidis. Furthermore, in Ca2+- differentiated primary keratinocytes *S. aureus* upregulated the expression of RNase7 and strongly hBD-3. This indicates that the differentiation state of the keratinocytes influence the antimicrobial peptide expression in response to *S. aureus* and *S. epidermidis*. Finally, we could show that both – the direct bacterial contact as well as secreted bacterial components - are responsible for the induction of hBD-3 and RNase7 expression. These data indicate that commensal and pathogenic staphylococci are able to differentially induce antimicrobial peptide expression in primary human keratinocytes.

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Murine β-Defensin 14 is an antimicrobial active and inducibleprotein

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The skin needs to be protected against infection because it is constantly exposed toa variety of microbial challenges. Beta-defensins, which are produced by epithelial cells, are most important for the skin's antimicrobial defense. A potent antimicrobial protein of human skin is β-defensin 3 (hBD3), which is antimicrobial active against many potentially pathogenic microbes including multiresistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecium*. It was reported that the hBD3expression is upregulated upon treatment with transforming growth factor α?(TGFα),interferon γ?(IFNγ) and by bacterial supernatants. To further study the role of hBD3 ininnate immunity and its *in vivo* relevance, mouse models would be very useful. Therefore we decided to search for murine orthologs of hBD3 in gene databases. Sequence analysis revealed that murine β-defensin 14 (mBD14) is the ortholog of hBD3 with a homology of 68 %. In the present study we examined whether mBD14 is antimicrobial active and whether the expression may be inducible. We performed recombinant production of mBD14 in *E. coli* and tested its antimicrobial activity. Our results showed antibacterial activity of the recombinant protein at nanomolar concentrations against various bacteria and fungi including multiresistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecalis*. To examine the mechanisms of mBD14 gene regulation we stimulated primary mouse keratinocytes in culture. We found that mBD14 is induced by TGFα, IFNγ?and bacterial supernatants similar to hBD3. In summary, we performed successful cloning of murine β-defensin 14, found broad-spectrum antimicrobial activity and the induction of the gene expression upon treatment with TGFα, IFNγ?and bacterial supernatants. This indicates that mBD14 is not only a structural but also a functional ortholog to hBD3.

P172

Proinflammatory staphylococcal cell wall component lipoteichoic acid potently suppresses skin inflammation by direct inhibition of T cell activation

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Surface organs such as skin contact microbes continuously and microbial pathogen associated molecular pattern (PAMP) contribute to the signals that determine cutaneous responses. Skin infection with *Staphylococcus aureus* is a known trigger for inflammatory skin diseases, especially atopic dermatitis, whereas Staphylococcus epidermidis is part of the normal skin microflora. Both species release enormous amounts of the cell wall component lipoteichoic acid (LTA) and we aimed to discover the role of LTA for cutaneous immune responses. Therefore, murine fluoresce inisothiocyanate (FITC) contact hypersensitivity (CHS), which mimics atopic dermatitis with high IgE level and Th2 cells, was established and effects of LTA on FITC-CHS were studied. The presence of LTA during repeated contacts with FITC enhanced FITC-specific cutaneous inflammation and induced Th1-immunity demonstrating a role of LTA as type I PAMP. In contrast, limited application of LTA during initial contacts to FITC significantly suppressed lesional T cell cytokine expression such asIL-4 and IFN-ã. This LTA application also had functional consequences as T cells from draining lymph nodes demonstrated significantly suppressed T cell proliferation *ex vivo*. To understand the underlying mechanisms of this *in vivo* effect, CD4+ T cell activation was analyzed *in vitro* in the absence of other cell types by polyclon alactivation. Strikingly, whereas other TLR2 ligands acted as T cell costimulators, LTA significantly suppressed T cell proliferation. Taken together, we identified two opposing biological functions of LTA on cutaneous inflammation: 1) Enhancement of severe or ongoing inflammation through induction of type I immunity as clinically observed in atopic dermatitis and *Staphylococcus aureus* colonization. 2) Suppression of mild inflammation by direct inhibition of T cell responses. This second effect may be of great relevance as it indicates a possible strategy to stabilize the cutaneous immunological barrier by resident microflora such as *Staphylococcus epidermidis*.

P173

Regulation of the hyaluronan metabolism in human skin by topic aldexamethasone

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Hyaluronan, one of the most abundant components of the cutaneous extracellular matrix, is involved in tissue homeostatsis and repair processes. Since topical applied glucocorticoids are widely used in dermatology, this study aimed to investigate the regulation of hyaluronan metabolism in human skin under glucocorticoid therapy. Expression of the hyaluronan synthesizing enzymes HAS-2 and HAS-3 and the hyaluronan degrading enzymes HYAL-1, HYAL-2 and HYAL-3 was evaluated by qRT-PCR. HAS-2 was subject to a marked suppression by dexamethasone infibroblast and HaCat keratinocytes cell cultures, representative of the major cell types in dermis and epidermis, respectively. This was reflected by a decrease of HAS-2 expression in human skin biopsies after treatment with dexamethasone ointment for 3 days. Consistently, immunohistochemical staining for hyaluronan in human skin after dexamethasone treatment showed a decrease in epidermal and dermalhyaluronan. Together, these data demonstrate that possible explanations for the observed skin atrophy after long term glucocorticoid treatment.

P174

TNF-α binding capacity *in vitro* of alginate and silver containingalginate wound dressings

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Aim: TNF-α is an important pro-inflammatory immune modulator that leads to increased cytokine expression and elevated protease secretion. The overproduction of TNF-α in chronic wounds results in severe tissue damage and impairs healing. Therefore the reduction of this mediator seems to be a suitable way to improve the healing outcome. Within the present study we investigated the binding capacity of an alginate wound dressing for TNF-α. As well as the effect of two alginate wound dressings containing ionic silver and nanocrystalline silver respectively.

Methods: The wound dressing samples were cut into equal pieces. Each specimen was taken in a final volume of 1 ml of TNF-α solution (100 pg/ml). Samples were incubated up to 24 h at 37°C on a plate mixer. Supernatants were collected and stored at −20°C until testing. The concentration of unbound cytokine in the supernatants was determined by means of specific ELISA (Mabtech AB, Sweden).

Results: Alginate is able to bind TNF-α. Already after 1 h a highly significant decrease of the TNFα?concentration was observed. The wound dressings of alginate containing ionic or nanocrystalline silver were also able to reduce the level of TNF-α significantly. This binding seems to be irreversible as it was not possible to elute significant amounts of the cytokine from the wound dressing samples after incubation.

Conclusions: As our previous studies have shown, alginate possesses a high binding capacity for PMN elastase and inhibits the formation of free radicals. We have now been able to demonstrate that alginate and silver containing alginate are also able to bind significant amounts of TNF-α *in vitro*. The decrease of this pro-inflammatory cytokine should aid the establishment of a physiological wound milieu *in vivo*.

P175

Irreversible binding and activity reduction of elastase by native collagen type I *in vitro*

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Aim: Non-healing wounds represent a serious problem because degrading processes, like destruction of extracellular matrix and growth factors, prevent wound closure. It has been shown that chronic wounds in contrast to acute wounds contain elevated levels of neutrophil elastase which is responsible for most of the degradation of growth factors. Binding or inactivation of elastase by wound dressings could be a promising way to contribute to the treatment of chronic wounds. The aim of this study was to investigate the influence of bovine collagen type I (Suprasorb®C) on elastase activity and concentration *in vitro*.

Methods: Wound dressing samples were cut into equal pieces (0.5 cm^2). Each specimen was taken in a final volume of 1 ml of elastase solution (250 ng/ml forelastase binding or 0.1 U/ml for activity). Samples were incubated up to 24 h at 37°C on a plate mixer. Supernatants were collected and stored at −20°C until testing. Elastase concentration was determined by means of a specific ELISA (Milenia, Germany) and the activity with the EnzChek Elastase Assay Kit (MoBiTec, Germany).

Results: Suprasorb®?C reduced the activity of elastase already after an incubation of 1 h and the activity could not be recovered by elution of the wound material. Consistent with these results, the concentration of elastase was reduced after 8 and 24 h incubation with Suprasorb®?C and only a negligible amount of the enzyme was detected in the eluate.

Discussion: It was shown that Suprasorb® C is able to reduce the activity of the enzyme and to absorb considerable amounts of elastase *in vitro*. Elution of the wound dressing revealed a strong, possibly irreversible binding of elastase by Suprasorb®?C as reason for the diminished activity. This could contribute to maintenance of growth factors and thus support the healing process.

P176

Establishing *in vitro* models to determine atrophogenic potential

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Glucocorticoids (GCs) are the most widely used drugs for topical therapy of inflammatory skin diseases. Their long-term use, however, is limited due to their potential to induce undesired effects with atrophy being the most prominent one. Thus, determining the atrophogenic potential of novel compounds is of importance for drug discovery. Unfortunately, there are no predictive *in vitro* models available to test the skin atrophy potential of compounds. So far, novel compounds are tested in costly and time consuming animal models. The aim of our study is to establish a predictive atrophy *in vitro* test system. Such a test system will consist of two components, the assay itself and the read out parameter to be determined. A convenient marker (gene or protein) for GC-induced skin atrophy should be regulated *in vitro* and *in vivo* dose-dependently in a compound-specific manner. Rodent skin atrophy models as well as *in vitro* monolayer cells culture and 3 D *in vitro* skin model systems are used for identification and validation of potential surrogate markers. In a last step, evaluated surrogate marker have to be confirmed in rodent models. As a result, expression of matrix metalloproteinases (MMPs), such as MMP-1,MMP-2 and MMP-3, were repressed after 24 h GC-treatment of normal human epidermal keratinocytes (NHEKs). This GC-sensitive regulation was confirmed in a human full-thickness skin model (AST-2000, CellSystems®). Surprisingly, MMP-2mRNA expression was not found to be regulated in rodent atrophic skin biopsies after daily treatment with GCs. Moreover, it has been shown, that MMP activities do not only depend on gene expression, but also on post-translational activation processes.MMP-2 activity in the epidermis of ASTs analyzed in zymogram is shown to be reduced after GC treatment. Experiments to investigate whether other MMPs are affected similarly by GCs *in vitro* and *in vivo* are ongoing. So far, there are promising results obtained regarding the GC-regulation of MMPs indifferent *in vitro* and *in vivo* test systems to further evaluate (i) their impact on GCskin atrophy and (ii) their suitability to serve as surrogate marker to test the skin atrophy potential of new compounds.

P177

Detection of dimethylfumarate metabolites in humans

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Background: The treatment of psoriasis with Fumaderm®, a mixture of dimethylfumarate (DMF)and mono ehtyl fumarate (MEF), is a well established therapy but only little is known about their mode of action and pharmacokinetics. Meanwhile, DMF was found to be the essential component to treat psoriasis successfully. There is a great discrepancy between the ability of DMF to exert anti-inflammatory effects *in vitro* and the fact that DMF cannot be detected *in vivo*. In contrast, mono methyl fumarate (MMF), the hydrolysis product of DMF, can be detected *in vivo* but does not show potent effects *in vitro*. Glutathione conjugation is the formation of a thioether link between glutathione and a compound with an electrophilic centre. Once synthesized GSH conjugates could be converted into mercapturic acids and excreted via the urine. From the chemical point of view DMF is an electrophile that reacts with glutathione (GSH) by a Michael-type addition forming 2-(S-glutathionyl)-succinic acid dimethyl ester (GS-DMS). Therefore GS-DMS could be metabolized to its corresponding mercapturic acid2-(S-(N-Acetylcysteinyl))-succinic acid dimethyl ester (NAC-DMS).

Methods: In an *in vivo* study three psoriasis patients received two tablets Fumaderm®?underfasting conditions. Blood and urine samples were collected prior and after defined time intervals after drug intake and DMF, MMF, MEF were measured in plasma employing HPLC. The urine was screened for NAC-DMS using a HPLC-MS analytic.

Results: By using newly established HPLC-methods MMF and MEF, not, however DMF could be detected in plasma of psoriasis patients. In urine the DMF metabolite NAC-DMS could be detected employing the HPLC-coupled mass spectrometry method.

Conclusions: These data for the first time demonstrate that DMF enters the blood circulation after oral intake of Fumaderm®?and is finally excreted via the kidneys in the form of NAC-DMS. From these and other data published previously it can be assumed that DMF after uptake partly binds to GSH to form GS-DMS and partly is hydrolysed to MMF which is detectable *in vivo*. By these two competitive reactions free DMF is quickly and completely utilized, leading to no measurable concentrations of DMF in plasma. As DMF was found to be significantly more active as compared to MMF *in vitro* it can be assumed that most of the pharmacological activities can indeed be attributed to DMF.

P178

Proteasome inhibitors suppress angiogenesis by altering endothelial VEGFR-2 expression

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The ubiquitin-proteasome system is the major pathway for intracellular protein degradation in eukaryotic cells that controls a wide range of cellular regulatory proteins, including transcription factors and cell cycle regulatory proteins. Recent evidence established the importance of the proteasome also in tumour development, showing anti-tumour and anti-angiogenic actions by selective inhibitors *in vivo*. Assignalling via the VEGFR2 pathway is critical for angiogenic responses to occur, we explored whether anti-angiogenic effects via proteasome inhibition were mediated in part through diminished endothelial VEGFR2 expression. Our studies show that different proteasome inhibitors (MG132, ALLN and lactacystin) all blocked VEGFR2 expression in a time- and concentration-dependent manner, which was paralleled by respective inhibition of capillary like structure formation and endothelial cell migration. In contrast, tie-2 or VEGFR-1 expression was not significantly affected by proteasome inhibitor treatment. The suppressive effects on VEGFR2 expression were neither conveyed by increased shedding nor by shortened protein half-life, suggesting that transcriptional mechanisms accounted for the observed effects. Inline with this conclusion, proteasome inhibition significantly suppressed VEGFR2mRNA accumulation. In addition, inhibitor treatment considerably decreased transcriptional activity of 5'-deletional VEGFR2 promoter gene constructs. Proteasome inhibition-mediated repression was conveyed by a GC-rich region, harbouring one consensus Sp1 binding site. Subsequent EMSA analyses demonstrated diminished constitutive Sp1-dependent DNA binding in response to proteasome inhibition. Hence, VEGFR-2 expression may constitute a critical molecular target of proteasome inhibitors that may mediate their anti-angiogenic effects *in vivo*.

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Nanoscalic silver – a new silver composition with broad spectrum antimicrobialactivity and less toxicological side effects than silver sulfadiazine

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The rate of fungal infections in the general population has been increasing worldwide for several years. Even though there are several classes of antifungal substances available, resistances to these drugs rise constantly. Due to its well known broad spectrum antibacterial activities silver has been used successfully for decades and has become a standard treatment for burns and bacterial skin infections. Silver-containing creams, particularly silver sulfadiazine (AgSD), possess effective activities not only against bacteria but also against fungi, and resistances to these drugs are rarely reported. However, there is serious concern that silver ions applied to inflamed and/or denuded skin might be absorbed insignificant amounts, thus introducing the risk of silver deposition, potentially leading to internal organ injury. In view of these facts we compared the percutaneous absorption and the antimicrobial potency of AgSD with a new composition where nanoscalic silver is linked to an inorganic carrier material (NSAg) leading to a highly stable complex that is less likely to be absorbed via the skin. The backs of hairless mice (SKH-hr1) were treated on five consecutive days with AgSD 1%, NSAg 1%, and NSAg 0.1%, respectively. While both AgSD and NSAg showed strong antimicrobial activities against different bacterial strains, candida species, and dermatophytes, quantification of silver ions in organs, blood and faeces revealed significantly lower absorption rates in the NSAg-treated animals than in the AgSD group. These results suggest that NSAg-containing creams are well suited for the prevention and treatment of skin infections, even when applied to large surfaces.

P180

Glucocorticoid receptor activation effectively blocks sensitization and elicitation in hapten-induced murine contact hypersensitivity models

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T cell (TC) mediated immune reactions can be divided into a sensitization and anelicitation phase. Antigen-presentation by dendritic cells (DC) is of major importance during sensitization of the immune system for a given antigen, while primed T cells mediate largely the effector response after a second antigen contact. Both DC and TC are cellular targets for anti-inflammatory therapies. Glucocorticoids (GC) are well established for therapy of inflammatory skin diseases acting on T cells. Conflicting data exist whether GC target the sensitization phase of the immune response. Also, itis not known whether gene transactivation or gene transrepression activities by GCsare decisive in either phase. To address both questions mice were topically or systemically treated inhapten-induced contact hypersensitivity (CHS) models with mechanistically different Glucocorticoid receptor (GR) ligands: the classical GC prednisolone (triggering bothtransactivation and transrepression) and with non-steroidal, selective GC receptoragonists (SEGRA) (showing a preference for transrepression) during either the sensitization or the elicitation phase. These GR ligands were applied topically at the site of sensitization to focus oncutaneous DCs, or systemically, either during the sensitization or the elicitationphase. The CHS response was determined by ear swelling, weight and cellularity of draining lymph nodes (LN). In all settings, both the GC Prednisolone, and SEGRAs were able to suppress the elicitation. In addition, both GR ligands were comparably effective after topical application before sensitization indicating comparable effects oncutaneous DCs. After systemic application around sensitization, however, classical GC exhibited only weak inhibitory effects on CHS, whereas SEGRA compounds seemed to be more effective. This indicates that different GR ligands mediate inhibitory effects in both phases of CHS to a different extent, via partially different modes of action, with transrepression being the key molecular mechanisms. These findings may have impact for the design of new GR-ligands.

P181

Evaluation of non-invasive methods to assess the effect: side effect profile oftopically applied glucocorticoids

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Topical Glucocorticoids (GCs) are highly effective for the therapy of frequent forms of dermatitis, such as irritant contact dermatitis, allergic contact dermatitis and atopic dermatitis. The use of GCs, however, is limited by their potential to induce local and systemic side effects. It is therefore a strive of pharmaceutical companies to develop Glucocorticoid receptor-ligands with improved therapeutic indices, i.e. a better effect:side effect ratio. Animal models are in place to monitor effects and side effects, yet, due to practical reasons, the parameters determined in animal models are frequently different from those applied in clinical studies later. Use of the same, predictive read out parameters in rodent and man would contribute to more efficient drug development. This study therefore aims at evaluating non-invasive methods to measure anti-inflammatory effects and local side effects of topically applied GR-ligands in animal models suitable for rodents and man. Measurement of transepidermal water loss (TEWL), a well established method to assess barrier damage in humans, was now applied in hairless OFA rats. In addition, skin pH alterations were measured indicating skin homeostasis. Colorimetry was applied to monitor the anti-inflammatory efficacy as well as atrophogenic activity of GCs. Under non-inflammatory conditions, rat skin was treated with the strongly potent Clobetasol (Clb) or the moderately potent Prednicarbate (PC) over up to 26 days. TEWL was significantly enhanced in Clbtreated rats (>50% at 0.01%). This correlated well with reduced skin fold thickness. The skin pH increased from 5.4 to >6, while skin colour shifted towards blue. Under inflammatory conditions (DNFB-induced edema), Clb brought strongly increased TEWL values back close to normal at d3 of treatment. In parallel, skin pH improved from alkaline values to neutral, while skin redness was rendered normal. The observed effects were dose dependent and correlated with the clinical potency of the glucocorticoids used. Our data suggest that the methods evaluated here are accurately reflecting the clinical properties of established GCs and might therefore be suited for routine use in drug development.

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Hydroxychloroquine regulates metabolic activity, proliferation, and autophagiccell death of human dermal fibroblasts

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Hydroxychloroquine (HCQ) is a commonly used therapeutic agent in a variety of rheumatic diseases and inflammatory skin disorders. Anecdotal reports and clinical experience, moreover, indicate that HCQ may be useful in selected fibrosclerotic diseases. Therefore, we investigated the effects of HCQ in human dermal fibroblasts *in vitro*. HCQ significantly reduced metabolic activity as shown by XTT test and suppressed cell proliferation as demonstrated by reduced H3-thymidineincorporation. This effect was dose-dependent with an IC50 of 30 μM. The antiproliferative effect of HCQ was related to decreased phosphorylation of extracellular signal-regulated kinase-1 and -2, two key players of mitogenic signalling, cell proliferation and survival. Interestingly, the anti-proliferative effect of HCQ inhuman dermal fibroblasts was linked to induction of a distinct type of cell death asinvestigated by cell death and DNA fragmentation assays, annexin-V staining, lightmicroscopic and ultrastructural analysis. Although treatment of human dermal fibroblasts with HCQ resulted in dose-dependent annexin-V surface staining it failed to induce formation of mono- and oligonucleosomes and DNA fragmentation. These findings were in accordance with a lack of classical signs of apoptosis in HCQ-treated cells a shown by light and electron microscopy. In contrast, HCQ induced formationof autophagic vacuoles with double membrane structures and digested organellecontent. Induction of this autophagic cell death by HCQ was paralleled by increased RNA and protein levels of beclin-1, a newly defined cell death regulator of autophagy. Our findings support the concept that HCQ may affect fibrosclerotic diseases since one biological facet of the drug is induction of autophagic cell death infibroblasts.

P183

In graft versus host disease Extracorporeal Photopheresis increases numberand function of regulatory T cells

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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 celllymphoma 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 order to investigate whether ECP affects Treg function we analysed peripheral blood of patients with different T cell mediated diseases (GvHD after bone marrow transplantation, Sezary syndrome, cutaneous T cell lymphoma) during ECP-treatment. ECP treatment was performed on two consecutive days and blood samples were taken before and after each session. We observed an increase ofCD4+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 the functional properties of Treg in GvHD patients, we analysed the suppressive effect of isolated Treg before and after ECP using conventional suppression assays. As controls we used Treg from healthy volunteers. 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 (Pi) by two different ectonucleotidases, namely CD39 and CD73, which are also expressed on 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 of Treg isolated before and after ECP. After ECP, both activated as well as resting Treg released higher amounts of Pi as compared to those isolated before ECP, indicating that ECP enhances generation of immunosuppressive Adenosine. In conclusion ECP may exert its immunosuppressive functions by upregulating number and suppressive capacity of regulatory T cells and may thus be an appropriate therapy to prevent over-reactions of the immune system after stem cell transplantations.

P184 (V09)

Experimental extracorporeal photopheresis inhibits contact hypersensitivity by two mechanisms: generation of interleukin-10 and induction of regulatory T cells

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We have developed a murine model of extracorporeal photopheresis (ECP) which utilizes contact hypersensitivity (CHS) as a disease model. Leukocytes were obtained from dinitrofluorobencene (DNFB)-sensitized donors and exposed *in vitro* to8-methoxypsoralen and UVA (PUVA). Cells were injected i.v. into DNFB-sensitized mice. Ear challenge was significantly suppressed in the recipients, indicating that ECP does not only, as shown previously, inhibit the induction but also the elicitation of CHS. When splenocytes obtained from these recipients 3 days after injection of PUVA-treated leukocytes were injected i.v. into naïve mice, sensitization could not be induced. This indicated that ECP performed in sensitized mice induces regulatory T cells. Thus, we concluded that the inhibition of CHS was mediated by regulatory Tcells (ECP-Treg). We now report that purified ECP-Treg suppress CHS only in naive but not in sensitized mice, indicating the ECP-Treg like UVB-induced Treg suppressonly the induction but not the elicitation of CHS. This suggests that ECP may have atleast two mechanisms of action. One which inhibits the elicitation of CHS and one that drives the generation of Tregs which in turn can inhibit CHS induction. Interleukin (IL)-10 is known to inhibit both the induction and the elicitation of CHS. Therefore we analyzed serum obtained from mice after injection of either untreated or PUVA-exposed leukocytes. In mice, which had received PUVA-exposed leukocytes, significantly elevated IL-10 serum levels were found but not in mice which received untreated leukocytes. Thus, we propose that the inhibition of the elicitation phase of CHS upon infusion of PUVA-treated leukocytes is a process which does not require Treg but may be mediated via enhanced IL-10. In parallel, but independently, Treg are induced as demonstrated by the adoptive transfer experiments. Together, these data suggest that ECP inhibits both the induction and the elicitation phase of CHS and induces Treg, though most likely through different mechanisms.

P185

Optical detection of singlet oxygen produced by fatty acids and phospholipidsunder UVA irradiation

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UVA radiation has been known to generate reactive oxygen species such as single toxygen 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. Using highly sensitive IR-photomultiplier technology, we investigated the generation of singlet oxygen by fatty acids and lipids. In combination with their oxidized products, the fatty acids or lipids produced singlet oxygen under UVA radiation at 355 nm that is directly shown by luminescence detection. Linolenic or arachidonic acid showed the strongest luminescence signals followed by linoleic acid and docohexaenoic acid.The amount of singlet oxygen induced by lipids such as phosphatidylcholine was significantly higher as compared to the corresponding fatty acids within phospholipids. This result indicates a synergistic process of oxygen radicals and singlet oxygen during irradiation. UVA radiation initiates singlet oxygen generation, which subsequently oxidizes other fatty acids that in turn produces additionally singlet oxygen. This leads to anenhancement of UVA induced damage of fatty acids and lipids, which must have an impact regarding the oxidative damages in cells.

P186

Expression of MMP-1, Collagen-1, and 4 by keratinocytes upon low dose ofUV-B illumination

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Graft-versus-host disease (GvHD) is a cause of tissue damage in the gastrointest in altract, the liver and skin after allogeneic bone marrow transplantation. Acutecutaneous GvHD will develop in 20–80% of patients. The pathogenesis of GvHD involves the response of immunologic cells against host antigens and dysregulation of inflammatory cytokine cascades released by skin tissue. However, the precise pathophysiology of GvHD is not known. The beneficial effects of photochemotherapy with psoralens plus UVA irradiation (PUVA) have been described repeatedly; however, PUVA is limited by a wide range of unwanted effects. Recently, preliminary studies suggest that a novel form of UV-B phototherapy is useful as an adjuvant therapeutic modality in cutaneous GvHD. In our study, keratinocytes were illuminated with an UV-B light dose of 0.02–0.06 J/cm^2 corresponding to a minimal erythema dose of ~75% (MED) and expression of metallo-matrix proteinase (MMP-1), Collagen-1, and 4 was evaluated by quantitative RT-PCT analysis. MMPs are responsible for the degradation and/or inhibition of synthesis of collagenous extracellular matrix in connective tissues. Expression of MMP-1 showed a bi-phasic expression within 24 h upon light exposition. Immediately (2 h) after UVB exposition keratinocytes did show a threefold higher MMP-1 expression as compared to the dark control. During the next 12 h expression declined and after 24 h post illumination MMP-1 expression increased again. Surprisingly, Collagen-1 and Collagen-4 did equally show a twofold increase of m-RNA expression within 2 h after illumination as compared to the dark control. These results suggest that the induction of Collagen-1 and 4 may influence positively the immunophathogenesis of GvHD involved skin areas by UV-B illumination; whereas MMP-1 expression was increased. Additional comparative studies have to be performed to determine effects of UV-A and UV-B phototherapy in prevention and treatment of cutaneous GvHD.

P187

Infrared irradiation reduces UVB-induced apoptosis via reduction of DNAdamage and regulation of apoptosis-related proteins

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Infrared irradiation (IR) is increasingly used for cosmetic and wellness purposes. Inthis context it is often combined with artificial UV irradiations. Hence, the questionarises, which impact IR might have on photocarcinogenesis. Since UV-inducedapoptosis represents a protective mechanism preventing malignant transformation byeliminating UV-damaged cells we studied the impact of IR on UV-induced apoptosis. IR (IR-A, 760–1400 nm) 3 h before UVB irradiation significantly reduced theapoptotic rate of normal human as well as murine keratinocytes. Accordingly, thenumber of sunburn cells was remarkably reduced in murine skin upon IRpretreatment before UV exposure. Since UV-induced DNA damage is the majortrigger for the induction of apoptosis, the amounts of cyclobutane pyrimidine dimers(CPD) were measured by Southwestern dotblot analysis. Surprisingly, the amounts ofCPD were significantly reduced by pretreatment with IR. This was confirmed *in vivo* by immunohistochemistry, demonstrating a much weaker nuclear CPD staining in UV-exposed murine skin upon pretreatment with IR. In addition, pretreatment decreased the pro-apoptotic protein Bax and induced the anti-apoptotic protein Bcl-xL as demonstrated by intracellular FACS analysis. Furthermore, IR pretreatment restored UV-induced suppression of the expression of the anti-apoptotic protein FlipL. Taken together, this clearly indicates that IR reduces UV-induced apoptosis both *in vivo* and *in vitro*. This may be mediated by several pathways which may include reduction of DNA damage on the one hand and induction of anti-apoptotic proteins on the other hand. The anti-apoptotic effects of IR could support the survival of UV-damaged cells and thus support carcinogenesis. Since, however, IR via yetun known mechanisms reduces the formation of UV-induced DNA damage, the balance between these two effects may be important. Thus, *in vivo* carcinogenesis studies are required to define the role of IR and its interaction with UV inphoto carcinogenesis.

P188 (V14)

Cyclosporin A but not everolimus inhibits DNA repair in human fibroblasts and lymphoblasts

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Cyclosporin A (CsA) rather than other immunosuppressive drugs like ever olimusseems to enhance UV-induced skin carcinogenesis. Xeroderma pigmentosumpatients also exhibit an increased skin cancer risk due to reduced cellular nucleotide excision repair (NER) capacity. We measured post-UV survival of virus-immortalized normal human GM00637 fibroblasts and AG10107 lymphoblasts in the presence of increasing CsA oreverolimus concentrations by MTT. GM00637 and AG10107 cells showed a gradually decreased relative (excludes toxic effects) post-UV cell survival within creasing concentrations of CsA from 0.01 μM to 1.5 μM (e.g. AG10107: 24% reduction of survival at 0.1 μM CsA vs 0 μM CsA and 50 J/m2 UVC to cells; $P < 0.05$). In contrast, cells treated with increasing amounts of everolimus (0.25 nM–100 nM) showed unaltered relative post-UV cell survival. We also assessed the cellular NER capacity using host cell reactivation (HCR). AnUV-irradiated firefly luciferase (pcmvLUC) and a control untreated renilla luciferase (pcmvRL) reporter gene plasmid were cotransfected into GM00637 and AG10107cells. In both cell types incubation with increasing concentrations of CsA (0.1 μM–0.35 μM) resulted in a significantly decreased relative NER capacity in adose-dependent manner (i.e. AG10107: 15.2% repair without CsA, 14% with 0.1 μM CsA, and 7.9% with 0.35 μM CsA; GM00637: 13% repair without CsA, 10.6% with0.125 μM CsA, and 3 % with 0.2 μM CsA; $P < 0.05$). In contrast, incubation of the cells with increasing amounts of everolimus (0.5–1000 nM) did not impair relative NER (i.e. ~16% repair in AG10107 with and without everolimus). Furthermore we measured the removal of UV-induced DNA damage using an ELISA and specific antibodies against CPD or 6–4PP lesions. Incubation of GM00637 andAG10107 cells with CsA led to a reduced removal of DNA photoproducts after 24 h (e.g. 3% vs 32% CPD in AG10107 at 0.5 μM CsA vs 0 μM CsA and 5% vs 36%CPD in GM00637 at 0.1 μM vs 0 μM CsA; $P < 0.05$). As we used therapeutically relevant doses for CsA (0.08 μM–0.25 μM) and everolimus (3.1 nM–8.3 nM), our results suggest that CsA but not ever olimusimpairs cellular NER resulting in skin cancer proneness which is consistent with clinical observations.

P189

Endothelins and alpha-MSH are significantly increased in plasma of patients treated with UV light irradiation

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Introduction: There is growing evidence that UV light irradiation alone or in combination with photosensitizers, such as psoralens, exhibits systemic effects through induction of cytokine release. Several cytokines have been measured in plasma of UV light-irradiated patients but results are conflicting. Furthermore little is known about the kinetics of UV light-induced cytokine release.

Materials and methods: Plasma was collected from patients ($n = 10$) treated withUVA1 and PUVA before and 1 and 2 h following irradiation at day 0 and day 14 of therapy. Plasma endothelins (1–3), alpha-MSH, basic-FGF, HGF, SCF, and GM-CSF were assessed by ELISA.

Results: Significant elevation of both endothelins (potent melanocyte mitogens) and alpha-MSH (a melanocyte mitogen and immunomodulator) 2 h following irradiationon day 0 ($P < 0.05$) but not on day 14 was detected. Nevertheless, both endothelins and alpha-MSH values at day 14 remained higher than those at day 0. Interestingly, endothelins values were almost doubled under PUVA treatment than under UVA1therapy, whereas alpha-MSH values showed no clear difference between UVA1 and PUVA therapy. On the other hand, no significant changes in basic-FGF, HGF, SCF, and GMCSF plasma levels were detected after irradiation.

Conclusion: UV light irradiation, whether alone or in combination with psoralen, has a systemic effect through release of endothelins and alpha-MSH in the circulation. This may provide an explanation not only for the tanning but also the anti-inflammatory effects of UV light irradiation.

P190

UV-A irradiation leads to Warburg associated decrease of respiration leading to increase of progression markers in melanoma

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We showed that repetitive exposure to sublethal ultraviolet (UV)-A irradiation leads to Warburg effect-associated increase of lactate levels and transketolase-like-1 enzyme and mutations of mitochondrial (mt) DNA in melanoma in-vitro and in-vivo. However, the functional role of this in melanomagenesis has been unclear. In order to investigate the role of repetitive UV-A we employed an in-vitro system of melanomacell lines with different progression levels: WM 35 (radial growth phase) and WM 115 (vertical growth phase) were exposed to UV-A at 6 J/cm^2 for three weeks thrice daily. Mitochondrial function was assessed by Clark type electrode-based measurement of oxygen consumption and FACS measurement of mt mebrane potential. By RT-PCR and FACS we assessed the adhesion molecule L1 (CD171), involved in tumour migration as well as by RT-PCR and western blot proteinkinase B (Akt), a marker for melanoma progression. Repetitive UV-A exposure of melanoma cells declined respiration, reversible by addition of 75 μg/ml caffeine. In line with this, mitochondrial membrane potential was increased. RT-PCR and FACS revealed, that UV-A results in increased L1, suggesting increased invasiveness of melanoma cells after UV-A exposure. Moreover, RT-PCR and western blot analysis showed increased levels of Akt and pAkt, supporting a role of UV-A in melanoma progression. Annexin V and Insulin like growth factor binding protein (IGFBP)-3 indicated no alteration of apoptosis. Since it has been reported that Warburg-associated decrease of respiration increases activity of Akt, our results indicate a role for chronic UV-A not only ininitiation of melanoma but also in its progression, which is mediated by the Warburg effect.

P191 (V30)

ROS and UVA-1 lead to clustering and increased localisation of CSA and CSBin mitochondria of stressed cells

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Maintenance of mitochondrial DNA is a critical aspect 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 neurode generation. 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 deficient fibroblasts exhibit a higher level of UVA induced mitochondrial DNA mutations. In mitochondria of unstressed fibroblasts we could detect low amounts of CSA and CSB by western blot analysis and confocal microscopy. In order to further investigate the role of CSA and CSB in mitochondria we exposed fibroblasts to sublethal doses of chronic UV-A irradiation or non-toxic concentrations of H2O2. Western blot analysis, and confocal fluoroescent microspcopy showed that CSA and CSB are highly enriched inmitochondria upon oxidative stress, while vitamin E inhibited these effects. Immuno-gold labelling electron microscopy confirmed these results and showed clustering of CSA and CSB proteins in mitochondria, indicating localisation at distinct sites within mitochondria. We present evidence that NER-associated CSA and CSB proteins are present inmitochondria and increase in response to different types of oxidative stress. These results strengthen a possible role of CSA and CSB in protection from neurode generation and aging-associated processes.

P192

Persistent molecular changes after repetitive *in situ* uvexposures of human skin

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The incidence of melanoma has tripled in the past four decades, and epidemiological and laboratory data provide strong evidence that UV radiation is a major causative factor. We have recently shown that UV modulates production (by keratinocytes andfibroblasts *in vitro*) of growth factors regulating melanocyte functions. We now investigated long-term consequences of repetitive UV exposures of human skin *in situ*. We examined two sets of biopsies taken 1–4 years after repetitive UV exposures to 95% UVA/5% UVB (Source 1, six subjects) or with Solar Simulated Radiation (Source 2, four subjects). Melanin content was determined using Fontana-Masson staining. Changes in protein expression of melanogenic factors (tyrosinase, MART-1,MITF), growth factors and their receptors (SCF, c-kit, bFGF, FGFR-1, ET-1, ETBR,HGF, GM-CSF), adhesion molecules (β-catenin, E-, and N-cadherins), cell cycleproteins (PCNA, cyclins D1 and E2) as well as Bcl-2, DKK-1 and DKK-3 were analyzed by fluorescence immunohistochemistry. Changes in RNA levels (for ETBR) were detected by tissue *in situ* hybridization. Only 1/10 of the UV-treated samples showed an increased melanin content >1 year after UV exposure, and this was not associated with an increase in melanocyte number. However, 1/6 of Source 1 and 3/4 of Source 2-irradiated samples still showed a significantly increased expression of tyrosinase. Most of the molecular markers examined showed no detectable changes in their expression at >1 year after UV irradiation. While an increase in ETBR protein expression was detected in 3/10 UV-treated (2/6 Source 1 and 1/4 Source 2)subjects, ET-1 protein expression and ETBR mRNA expression showed no change. In summary, among 19 factors examined, only tyrosinase and ETBR protein expression, and only in some subjects, were elevated at >1 year post UV exposure. This suggests that persistent post-UV exposure changes are very minor and show a considerable subject-to-subject variation. A possibility that changes in the expression of the ETBR protein trigger downstream activation of abnormal melanocyte proliferation (and, hence, might potentially lead to melanoma) deserves further investigation.

P193 (V01)

Beta endorphin produced by melanoma promotes tumour growth and immune escape

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Beta endorphin is an agonist peptide for the mu opioid receptor (MOR); its major role is pain relief at proximal nerve endings. Interestingly, beta endorphin has also been found to be secreted in high amounts by several tumours of neuronal and non-neuronal origin where its role remains unclear so far. This project intended to investigate if beta endorphin (BE) secretion by tumour cells could play a role in cancer progression.

When analysing the expression of beta endorphin in 30 human melanoma biopsies obtained from patients, we found a correlation between beta endorphin expression and stage of the malignancy ($P < 0.05$). *In vitro* data demonstrated that BE acts anautocrine/paracrine growth factor for B16 melanoma cells as BE-blocking antibodies significantly reduced cell proliferation (60% inhibition at 72 H). The impact of BE on tumour immune escape was analyzed *in vivo* using mu opioid receptor deficient mice (MOR−/−) and their WT counterparts. As MOR−/− mice do not have the receptor for beta endorphin their immune system will not react to beta endorphin secreted by tumour cells. When injecting B16 melanoma cells s.c., a profoundly reduced tumour growth was observed in MOR−/− mice compared to WT animals (median volume 0.2 cm^3 vs 0.8 cm^3 at day 15 post injection; $P < 0.01$). This was paralleled by a significant higher infiltration of CD4+, CD8+, NK and dendritic cells at tumour site-determined by flow cytometry- in MOR−/− mice. This is at least in part due to inhibitory effects of BE on chemotaxis, as we were able to demonstrate reduced migration of human PBMCs in response to CXCL9, RANTES and Fractalkine; these chemokines are known to play a major role in leukocyte recruitment into tumours. Moreover, active attack of tumour cells is also modulated by BE as we observed by video microscopy with PBMCs isolated from mice once BE-blocking antibody was added to these cultures. These findings highlight new roles for endogenous opioids inperiphery and demonstrate that beta endorphin secretion by melanoma cells plays a role in tumour growth and immune escape. Blocking beta endorphin effects on effect or cells of the immune system could represent a new therapeutic strategy to limit cancer progression.

P194

High loss of heterozygosity rates on chromosome 9p in cutaneous squamouscell carcinoma

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Genetic alterations on chromosome 9p are frequent during carcinogenesis in a variety of tumours. Genomic mutations can be detected early by loss of heterozygosity (LOH) analysis. Incidence of cutaneous squamous cell carcinoma is much higher in immuno compromised than in immunocompetent patients . Our aim was to assess LOH at three micro satellite markers on chromosome 9p in the course from intraepidermal (actinic keratosis or Bowen's disease) to invasive squamous cell carcinoma in organ transplant recipients (OTR) ($n = 42$) and immunocompetent patients ($n = 43$). Thus, in 170 tissue samples equal numbers of tumour cells and internal control tissue cells were micro dissected. DNA was extracted, amplified by PCR at three micro satelliteloci (IFNA, D9S162 and D9S925) and then analyzed for LOH. In addition, correlation between LOH on chromosome 9p and protein p16 expression was assessed. We found high LOH rates at all three loci (61% at IFNA, 42% at D9S162 and 51% at D9S925). LOH at these loci was comparable between intraepidermal and invasiveforms of squamous cell carcinoma ($P = 0.59$, 0.66, 0.08 respectively). OTRs showed significantly more LOH than immunocompetent patients at D9S162 ($P = 0.04$) but not at IFNA or at D9S925 ($P = 0.88$, 0.92 respectively). P16 protein expression was similar in intraepidermal and invasive squamous cell carcinoma and similar between OTRs and immunocompetent patients. Correlation between LOH and p16immunoreactivity was poor (Spearman's rho ranging from 0.09 to 0.13). Our study revealed high genomic instability on chromosome 9p in cutaneous squamous cell carcinoma. Further efforts should be undertaken to identify new tumour suppressorgenes in those regions. Increased allelic imbalance around locus D9S162 in OTRs may be a genomic correlate for the clinically more aggressive behaviour of squamouscell carcinoma in these patients.

P195

Human Papillomavirus (HPV) 26 and HPV 88 replicate to extremely high levels in squamous cell carcinomas (SCC) of the nail unit in anHIV-immunosuppressed patient

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High-risk mucosal HPV have been implicated in the pathogenesis of a subset of digital SCC. A possible role in keratinocyte skin cancer at other locations is less clear. In an HIV-infected patient suffering from verrucous SCC of all fingertips, HPV26 and a new type, HPV88, were identified in all SCC. HPV26-specific real-time PCR determined high viral load (56–4.4*10^4Hari copies/cell) in all right hand tumours, whereas left hand SCC had copy numbers ranging from 0.4–1.2/cell. Conversely, the viral load of the left hand SCC had very high HPV88 copy numbers with up to 1.3*10^6/cell, whereas SCC of the right hand had low copy numbers (0.09–1.6/cell).The extremely high viral loads in these tumours suggested a major causative role for the respective dominant HPV type in SCC development. By *in situ* hybridization HPV26 E6 DNA was detectable in a majority of suprabasal tumour cells indicating productive viral infection. Tumour cells stained positive for p16INK4a, a marker of high-risk HPV-associated neoplasia. No missense or synonymous nucleotidic changes in the E6 ORF were detected, that could account for abnormal biologic properties of this HPV26 isolate as compared to HPV26 prototype. This indicated that productive infection with high-level amplification of the viral genome played a causal role in tumour progression. HPV88 was isolated from one of the SCC, comprising a genome of 7326 bp (61% similarity to its closest relative HPV60) and represents a new species of gamma papillomavirus. A survey of skin tumours and normal skin biopsies from immunocompetent (n = 362) and immunosuppressed (n = 26) patients detected HPV88 DNA in seven specimens. These showed 10-logarithms lower viral loads, implying that HPV88 infection is not generally associated with immunosuppression or SCC. HPV26 has been rarely detected in cervical cancer and in SCC of the nail unit in HIV-infected individuals. Here we identify HPV26 and HPV88 as the likely primary causes of digital SCC in an HIV-infected immunosuppressed patient.

P196

Lack of T cell receptor-stimulated CD95 ligand up-regulation protects cutaneous T cell lymphoma cells from Activation-induced cell death (AICD)

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Cutaneous T cell lymphoma (CTCL) is characterized by a monoclonal proliferation ofCD4+ lymphocytes. Restimulation of previously activated T cells via the T cellreceptor (TCR) leads to activation-induced cell death (AICD). CTCL cells (= CTCL tumour cell lines as well as primary CTCL tumour cells from CTCL patients) were resistant to TCR-induced AICD in contrast to sensitive controls (= lymphoma cell lines and normal T cells from healthy donors). AICD is dependent on the death receptor CD95 which can be blocked by the inhibitory protein cFLIP. Despite the fact that CTCL cells showed increased cFLIP expression they were sensitive to CD95 induced apoptosis. Furthermore, stable down-regulation of cFLIP by retroviral siRNA did not sensitize CTCL cells to TCR-induced AICD. Interestingly, TCR expression by CTCL cells was diminished when compared to controls; however, TCR signalling was reduced but not abrogated in respect to MAP-kinase phosphorylation. In the context of AICD TCR stimulation leads to expression of endogenous CD95 ligand (CD95L) which executes cell death via CD95. CTCL cells failed to up-regulate CD95L upon TCR stimulation compared to controls. This lack of TCR-stimulated CD95L induction precludes the sensitization of CTCL to AICD and could have future therapeutic implications to overcome apoptosis resistance in CTCL patients.

P197

Tumour depth influences vertical and horizontal expression of Cx26 in epidermis adjacent to malignant melanoma

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Gap Junctions are communicating cell-cell junctions which are formed by connexins. In human epidermis the main connexin is Cx43, while Cx26 and Cx30 are absent in healthy epidermis, but are induced by several pathogenic conditions. Before, we described the absence of Cx43, Cx26 and Cx30 in non-keratinocytic skin tumours(malignant melanoma, Merkel cell carcinoma) and an upregulation of Cx26 and Cx30as well as a downregulation of Cx43 in keratinocytic skin tumours (e.g. squamous cellcarcinoma, Morbus Bowen). Interestingly, we observed an induction of Cx26 andCx30 in epidermis adjacent to malignant melanoma and Merkel cell carcinoma, while there was no induction in epidermis adjacent to non-malignant tumours, e.g. melanocytic nevi. Here we wanted to examine whether the induction of Cx26 andCx30 correlates to tumour depth and other parameters, e.g. proliferation and ulcerformation. We investigated the localization of Cx26 and Cx30 in the epidermis of 42 cases of malignant melanoma with various Breslow-indices. We found a significant correlation of the dissemination of Cx26 protein expression to tumour depth vertically(Cx26 positive layers) as well as horizontally (horizontal area of Cx26 protein expression). There was no correlation to Cx30. We conclude that malignant melanoma influences Cx26 protein expression in adjacent epidermis in a size dependant manner.

P198

14-3-3 sigma, a novel tumour suppressor for malignant melanoma?

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The family of 14-3-3 proteins is centrally involved in intracellular signal transduction and cell cycle control, interfering with signalling molecules through binding to phosphoserine residues. Recently, we were able to demonstrate that 14-3-3 sigma is dramatically downregulated in melanoma metastases compared with primary melanomas. Based on current knowledge on cancer gene regulation, these findings were highly suggestive for gene silencing via epigenetic mechanisms. Indeed, by use of methylation-specific PCR it could be demonstrated that the 14-3-3 sigma genepromoter is highly methylated in melanoma metastases compared with primary melanomas. In line with this, treatment of melanoma cell lines with5-aza-2'-deoxycytidine (AZA), a potent inhibitor of cytosine methylation, resulted in enhanced gene and protein expression of 14-3-3 sigma. Addition of 4-phenylbutyricacid (PBA), a positive modifier of epigenetic gene regulation via inhibition of histonedeacetylation, further enhanced 14-3-3 sigma expression. These findings indicate that 14-3-3 sigma expression in melanoma cells is significantly regulated by epigenetic mechanisms such as gene methylation and histone deacetylation. Enhanced expression of 14-3-3 sigma after AZA/PBA treatment led to dramatically reduced proliferation rates of highly proliferative melanoma cells, as determined by flowcytometry. By transient transfection of melanoma cells with short interfering (si) RNAs against 14-3-3 sigma, the strong antiproliferative effect of enhanced 14-3-3sigma expression could be reversed. These findings were confirmed in a series of stably transduced melanoma cell clones, using lentiviral vectors carrying different short hairpin (sh) RNAs directed against 14-3-3 sigma. *In vivo* monitoring of melanoma cell growth in CD1 nude mice was performed by use of *in vivo*-imaging technology. Here subcutaneously injected 14-3-3 sigma high expressors, co-transduced with green fluorescence protein, showed significantly reduced growth, as compared with 14-3-3 sigma knockdown cells. Taken together, the presented data indicate that 14-3-3 sigma might act as a tumour suppressor in malignant melanoma. Its regulation via epigenetic mechanisms might open interesting therapeutic perspectives for metastatic disease.

P199

Regulatory T cell frequencies in patients with different melanoma stages and their correlation with T helper cell reactivity against recall antigens

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Naturally occurring CD4+CD25+ regulatory T cells (Tregs) are important contributors to the maintenance of immune tolerance in the periphery. Whereas Treg-deficiency is associated with several autoimmune diseases and type-I allergy, increased frequencies of Tregs are assumed to facilitate the development and progression of cancer. Unlike in the murine system, human Tregs cannot be distinguished from T helper cells easily by markers like CD25 or Foxp3 since both are also expressed by CD4+ T helper cells upon activation. We therefore used a panel of several Treg-associated markers to enumerated Tregs in the peripheral blood of stage II-IV melanomapatients. All marker combinations revealed increased ratios of Tregs in peripheral blood of melanoma patients compared to healthy volunteers. In addition, the relative ratio of Tregs increased with the progression of disease. Most important, accumulation of Tregs in progressed melanoma patients correlated with a general reduction of T cell responsiveness not only to different tumour-associated antigens but also to various recall antigens. Together, these observations suggest suppression of T cell reactivity through increased Treg ratios in patients with progressive melanoma and may explain the disappointing success of immunotherapies in these patients. However, for better understanding of Treg-associated mechanisms in cancer and autoimmunity, we generated novel antibodies by immunization of mice with human Tregs. Currently, promising candidates are integrated into the common marker combination for Treg quantification in patients with melanoma or type I allergy.

P200 (V06)

Gene patterns at the invasive front of melanoma metastases

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Malignant tumours and tumour metastases consist of heterogenous cell populations. In particular, cells at the invasive front of primary tumours show significant deregulation of genes involved in cellular proliferation, cell adhesion and extracellular matrix organisation. However, little is known about gene expression of tumour cells at the peripheral border (invasive front) of metastatic lesions. In the presented report, gene expression patterns in central and peripheral areas of melanoma metastases (n = 10)were analysed using DNA microarray technology. For this purpose, distinct centraland peripheral cell populations in kryosections of melanoma metastases were excised by laser-capture microdissection. Extracted RNA was pooled in each group, and labelled RNA targets were hybdridized to whole-genome DNA microarrays (54 000 probe sets). Overall, 248 differentially expressed genes were identified. Among these pro-invasive renal tumour antigen (RAGE), signal transducer and activator of transcription 1 (STAT1), and DNA repair gene O-6-methylguanine-DNAmethyltransferase (MGMT) showed significant upregulation at the invasive front. Downregulated genes in these areas included thrombospondin 1 (THBS1), angio-associated migratory cell protein (AAMP), and a larger series of genes with as yet undefined functions. Data were validated by real-time RT-PCR. The functional significance of these findings was further confirmed in *in vitro* migration assays. In these experiments, melanoma cell lines SK-Mel-103 and SK-Mel-147 were stably transduced with STAT1 targeting short hairpin (sh) RNAs, using lentiviral vector constructs. It could be shown that melanoma cell migration was significantly inhibted by STAT1 gene knockdown. Taken together, in the presented study gene expression patterns were identified, which might help to better understand invasive metastatic growth of melanoma cells. Inhibition of individual genes in signalling pathways, e.g. via RNA interference or small molecule inhibitors, might have beneficial effects on late metastatic spread of melanoma cells, which might open interesting therapeutic perspectives for the future.

P201

Functions of ADAM10 in human melanoma cells

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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. The interaction of HA and CD44on malignant and non-malignant cells has multiple impact on cell proliferation, cell motility or cell survival. Our group has shown that CD44-HA interactions promote cell proliferation of malignant melanoma (MM) cells [1]. 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*. Increased plasma levels of soluble CD44 have been observed in various malignancies. Therefore, the mechanism of CD44 shedding is of major interest for tumour biology and consequently putative shedding proteases are studied towards their ability to cleave CD44 from the surface of tumour cells. Among them MMP14, ADAM10 and ADAM17 are the most interesting candidates that are in the focus of the present study. We could detect ADAM10, ADAM17 and little amounts of MMP14 in tumour cells from MM by immunohistochemistry. Further we could demonstrate a co-localisation of ADAM10 and CD44 in the membranes of MM cells. ADAM10 is critically involved in the constitutive shedding of native CD44from human MM cell lines shown by inhibitor studies and siRNA techniques.ADAM10 specific inhibitors were able to block CD44 release from MM cells. All, ADAM10-, ADAM17- and MMP14-expression could be inhibited by siRNA techniques on mRNA and protein levels, but only ADAM-10 blocking was able to reduce the constitutive CD44 shedding from MM cell lines. ADAM10 silencing further more increases cell proliferation of MM cells suggesting that ADAM10 can influence MM cell proliferation by its implication in solCD44 shedding. Different from earlier studies using over expressed MMP14 and CD44 [2] we conclude, that ADAM-10 but not MMP14 is the native and essential protease forCD44 shedding from melanoma cells with functional impact on tumour cell proliferation.

1: Ahrens T et al. Oncogene 2001; **20**: 3399–3408. 2: Nakamura H et al. Cancer Res 2004; **64**: 876–882.

P202 (V08)

B16 melanoma infiltrating Treg are resistant to antibody depletion and help creating a microenvironment that prevents T cells from tumour infiltration

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Murine B16 melanoma is a suitable experimental animal model that resembles human malignant melanoma. To investigate the mechanism by which this tumour can escape immune surveillance, we screened tumours for infiltrating CD4+ cells and found that a subpopulation of CD4+ cells, namely CD4+CD25+FoxP3+ regulatory T cells (Treg), was accumulated in the B16 melanomas, comprising approx. 30% of all tumour infiltrating CD4+ cells. In other lymphoid organs as well as in blood, normal levels of Treg were detected in tumour bearing animals. When animals were injected with the Treg depleting anti-CD25 antibody PC61, substantial decrease of Treg in all secondary lymphoid organs was observed. In contrast, the number of Treg within the tumour remained unaffected. Moreover, using a combination therapy of Treg depletion and subsequent vaccination with tumour antigen loaded dendritic cells, no apparent effects on tumour growth were observed, indicating that the tumour residing Treg are able to protect melanomas from immune reaction(s). To identify the source of the tumour residing Treg, we isolated CD4+ cells or Treg from naïve or tumour-bearing mice, labelled them with PKH-PE or CFSE and injected them at different time points i.v. into B16-bearing mice. Several days later we took out lymph nodes, spleens and tumours and analysed the distribution of labelled cells in these organs by flow cytometry. We found that the injected cells in tumour-bearing mice home in comparable amounts to lymph nodes and spleen as in naïve mice. Surprisingly, we never found exogenous CD4+ or CD4+CD25+ in the tumour infiltrates. However, the endogenous CD4+ as well as CD4+CD25+Foxp3+ cells were still detectable. These results suggest that the tumour creates an immunosuppressive environment that makes it impossible for T cells to enter the tumour site and therefore mount an appropriate anti-tumour response.

P203

The Inhibitor-of-apoptosis (IAP) protein family member XIAP is an important downstream regulator of death receptor-mediated apoptosis

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Metastatic squamous cell carcinoma of the skin (SCC) has a severe prognosis due to its resistance to chemotherapeutic drugs. Because drug resistance of tumour cells strongly correlates with apoptosis resistance, inhibitory proteins could explain apoptosis resistance to current therapeutic agents in SCC. The inhibitor-of-apoptosisproteins (IAPs) such as X-linked IAP (XIAP) are known to block crucial steps of the apoptotic signalling pathways by interfering with active caspases downstream of mitochondria. To date, little is known about of the impact of XIAP for death receptor-mediated apoptosis in SCC. To analyze this aspect, we first characterized death receptor and XIAP expression in11 different SCC cell lines and compared their sensitivity to death ligands such as TRAIL or chemotherapeutic agents. We observed uniform surface expression ofTRAIL-R1, TRAIL-R2 and CD95, while all cell lines lacked TRAIL-R3/R4 expression. Of note, TRAIL resistance of several SCC lines correlated with XIAP expression. To test the function of XIAP for death receptor-mediated apoptosis in SCC more directly, we employed a retroviral stable knockdown (XIAP shRNA) against XIAP. Interestingly, specific downregulation of XIAP substantially sensitized SCC cells for TRAIL- or CD95L-mediated apoptosis, whereas the sensitivity to chemo therapeutic agents such as cisplatin was unchanged. Cell death was accompanied by increased caspase activation, indicative of the activation of apoptotic signalling pathways. In summary, we have identified XIAP as additional important regulator of death receptor-mediated apoptosis and show that downregulation of XIAP is sufficient to overcome death ligand resistance while not affecting the sensitivity to chemotherapeutic agents. Our data support the concept that XIAP may act as an important downstream regulator of death receptor-mediated apoptosis in SCC. In case of efficient activation of initiator caspases, XIAP might thus act as additional resistance factor avoiding elimination of SCC. Therefore combination therapies of death receptor agonists and XIAP antagonists may represent a novel approach for the treatment of metastatic SCC.

P204

Papular exanthema discloses AML: interphase FISH revealed deletion of p53and gain at 8q22/8q24/Tel8q without trisomy 8

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Leukemia cutis (LC) in acute myeloid leukemia (AML) is a rare event. We describe a 79-year-old patient who presented with fatigue, weight loss, and pancytopenia. Bone marrow biopsies resulted in a 'punctio sicca' suggesting idiopathic myelofibrosis. After 1 month the patient developed a papular exanthema. Skin histology displayed dermal infiltrations of monocytoid cells with the phenotype CD68+, CD15+, lysozyme+, CD7−, CD34−, myeloperoxidase-, and chloroacetate esterase-, consistent with the diagnosis of AML (FAB M5). The molecular mechanisms of extramedullary manifestations of AML are not completely disclosed. In our case CD4 and CD56expression, which are potential adhesion molecules, were positive. Numerical abnormalities of chromosome 8 (trisomy or tetrasomy) have been identified in association with LC. We performed FISH analysis on cutaneous tissue using directly labelled probes for various gene loci often involved in AML patients which showed deletion of p53 and excluded trisomy 8. However, application of probes for AML/ETO,MYC, and telomere 8q revealed a gain at 8q22/8q24/8q telomere in a significant number of infiltrating cells. We hypothesize that not trisomy of the whole chromosome eight but rather a partial gain at 8q exhibits an association with leukemiacutis in AML.

P205

The transcription factor c-Jun is regulated by loss of E-Cadherin 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 tumour progression. It forms homodimers or heterodimers with other members of the transcription factor superfamily AP-1, influencing the expression of a multitude of regulators of cell proliferation, migration and survival significantly involved in tumour development and metastasis. We could show by Western Blot analysis and immunhistochemical staining that c-Junprotein is upregulated in melanoma cells, whereas in melanocytes c-Jun protein is not expressed. Reporter gene assays revealed that inhibition of c-Jun by a dominant negative form of c-Jun (Tam67) leads to loss of the transcriptional activity of AP-1 inmelanoma 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 expression during melanoma development leads to proliferation and metastasis. Interestingly, there is a coincidence in the loss of E-Cadherin expression and upregulation in c-Jun protein in malignant melanoma. We, therefore, speculated whether E-Cadherin is a regulator of c-Jun. We examined three established model systems, which show a re-expression of E-Cadherin in melanoma cells: (1) viral transduction and vector based transfection of E-Cadherin, (2) as Snail cell clones and (3) HMB2-MIA negative cell clones. Our data could show that loss of E-Cadherin expression during melanoma development induces c-Jun activity, whereas in melanocytes active cell-cell-contacts viaE-cadherin have a negative impact on c-Jun, suggesting a direct link between E-Cadherin and c-Jun. Interestingly, c-Jun was not regulated on transcriptional level by E-Cadherin, since quantitative RT-PCR revealed equal levels of c-Jun mRNA in melanocytes and melanoma cells lines. Our data point to a regulation of c-Jun byE-Cadherin on posttranscriptional level. Further experiments show that c-Jun regulation by E-cadherin is cell-contactdependent. Treatment of primary melanocytes with an anti E-Cadherin antibodydestroying E-Cadherin dependent cell contacts between melanocytes, increased thec-Jun protein amounts in NHEM, which confirms the importance of cell-cell-contactsin regulating c-Jun.

P206

Immunostimulatory bcl2-specific 5#-triphosphate-siRNA: combining RIG-Iactivation and gene silencing activity for the therapy of melanoma

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Two hallmarks of melanoma development are increased cell survival and immuneescape. We hypothesize that treatment strategies which are able to simultaneous lypromote cell death and activate a cellular cytotoxic immune response can effectively counteract tumour growth. Here we designed short interfering RNA (siRNA) specific for the anti-apoptotic protein bcl2 which carry a 5# triphosphate (3p-siRNA).3p-bcl2-siRNA comprises two independent functional activities in one molecule: genesilencing of bcl-2 and immune activation via the cytosolic helicase RIG-I. Systemic treatment with 3p-bcl2-siRNA elicited strong anti-tumour activity against B16melanoma lung metastases. Like TLR agonists, RIG-I ligation by 3p-siRNA activate dinnate immune cells such as dendritic cells; unlike TLR agonists, activation of RIG-I directly induced a type I IFN response and apoptosis in B16 melanoma cells. Importantly, activation of RIG-I by 3p-bcl2-siRNA significantly enhanced B16melanoma cell apoptosis due to bcl-2 gene silencing. *In vivo*, these mechanismsacted in concert to provoke massive apoptosis of metastatic B16 melanoma cells in the lungs. The overall therapeutic activity of 3p-siRNA *in vivo* required NK cells as well as type I IFNs and was associated with downregulation of bcl-2 in metastaticB16 melanoma cells on a single cell level. Thus, 3p-siRNA represents a novel single molecule-based combinatorial approach in which RIG-I activation on both the immune- and the tumour cell level supports a cytotoxic immune response and in which gene silencing affects key molecular events that govern melanoma cell survival such as bcl2.

P207 (V11)

Construction of a new oncolytic adenoviral vector with doxycycline-inducibleexpression of CD95L: Efficient combination of selective oncolysis with apoptosis induction in melanoma cells

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No suitable therapy is available for metastasized melanoma thus characterized by high mortality rates. Apoptosis deficiency is a critical factor for therapy resistance. We have shown previously that TNF-related death ligands (CD95L/FasL and TRAIL) can induce apoptosis and enhance chemosensitivity of cultured melanoma cells. For preparing gene therapeutic strategies, we constructed a conditional replication-competent, oncolytic adenoviral vector (Ad5-FFE02), in which E1A and thus selective viral replication is driven by a tyrosinase promoter, and CD95Lexpression is triggered by a doxycycline-inducible promoter. Addressing further a safe application, a mutated variant of E1A was used (E1AΔpRb), which allows replication preferentially in tumour cells. Here, we analyzed efficiency and selectivity of gene expression as well as of induction of oncolysis and of apoptosis by Ad5-FFE02in melanoma cell lines (SK-Mel-19, Mel-HO and Mel-2a, A-375, MeWo), as compared to non-melanoma cell lines (MCF-7, HeLa, PFSK-1). After infection with Ad5-FFE02and induction with doxycycline (24 h), efficient induction of apoptosis as monitored by a DNA fragmentation ELISA, was found in tyrosinase-positive and CD95L-sensitivemelanoma cells (SK-Mel-19, Mel-HO and Mel-2a) but not in tyrosinase-negativeA-375, in CD95L-resistant MeWo and particularly not in the non-melanoma cell lines. Protein analyses revealed highly selective expression of E1A and selective induction of CD95L in tyrosinase-positive melanoma cells. Four days after infection withAd5-FFE02, oncolysis as monitored by crystal violet staining, was restricted totyrosinase-positive melanoma cells SK-Mel-19, Mel-HO, Mel-2a and MeWo. Non-melanoma cell lines showed no response. These data prove the high selectivity of tyrosinase promoter-driven adenoviral vectors in pigmented melanoma cells as well as the high efficacy of proapoptotic genes. Selective replication-competent adenoviral vectors may develop as novel therapeutic strategies and may significantly increase the efficacy of gene therapeutic approaches in melanoma.

P208

Relevance of beta-Catenin signalling for melanoma cell proliferation and survival

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For several years it is known that beta-catenin expression and cellular distribution is altered in melanoma cells. During melanoma progression there is an increase in beta-catenin expression and commonly a translocation of beta-catenin into the nucleus. Nevertheless, the involvement of the beta-catenin pathway in the progression of malignant melanoma is a matter of debate. Mutations in the beta-catenin gene are rarely found in melanoma cells. Until now unknown mechanisms enforce melanoma cells to redistribute beta-catenin into the nucleus and to increase its expression thereby potentiating the beta-catenin signalling and transcriptional activity. In order to elucidate a) the molecular mechanisms by which beta-catenin affects melanoma progression and b) the mechanisms governing the expression and activity of beta-catenin during melanoma progression we analyzed the biological effects of an inhibition of beta-catenin in invasive and metastatic melanoma cells. Interestingly, normal human melanocytes were not affected by beta-catenin downregulation. However, after treatment of several invasive growing melanoma cell lines with a beta-catenin specific inhibitor and shRNAs against beta-catenin we found a strong reduction of melanoma cell growth and survival and induction of apoptosis. Further more, several genes known to be involved in proliferation, migration and invasion of tumour cells were downregulated by beta-catenin targeting. These dataindicate that beta-catenin is an important player in melanoma cell proliferation and survival opening a door for new therapeutical strategies for malignant melanoma.

P209

Treatment of established RMA-Tag tumours via DC directed targeting of tumour antigens is further improved by depletion of regulatory T cells

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Tumour antigens, chemically coupled to the antiDEC205 antibody, can target dendritic cells *in vivo* and were successfully applied in murine preventive tumour vaccination studies. In this study our aim was to test whether antiDEC205-protein conjugates have the potential to reduce the tumour growth of already established tumours. For this end we coupled the tumour antigen Tag to antiDEC205 and treated RMA-Tag tumour-bearing mice with these conjugates. When the tumours had reached an average diameter of 5 mm the mice were injected with antiDEC205-Tag conjugate or Tag protein, plus adjuvands in 5-day intervals. Although tumour growth was initially slowed down after injection of the anti-DEC205-Tag conjugates as compared to Tag-treated mice, it resumed two to three weeks after the first vaccination. Analysis of the immune response revealed initially a strong induction of tumour specific CD8+ cells inanti DEC205-Tag treated mice. However, when tumour growth revived increased numbers of CD4+CD25+FoxP3+ regulatory T cells (Tregs) in the tumour draining lymph node were noticeable. Thus, to assess the suppressive effects of Tregs during tumour therapy, anti-CD25 antibodies were used to deplete Tregs from tumour bearing animals during anti-tumour treatment. By concomitant injection of antiDEC205-Tag conjugates we could cure up to 40% of the tumour bearing mice and tumours were rejected. Furthermore, the survival of tumour bearing mice of the autochthonous tumour model RIP-Tag5 could be prolonged for up to ten weeks after treatment with anti-DEC205-Tag conjugates compared to untreated, or Tag-treated mice respectively. In aggregate our data indicate that depletion of CD25+ Treg during cancer therapy bolsters the effectiveness of DC based anti-tumour therapies and thatantiDEC205 targeted vaccination also improves treatment of autochthonous tumours.

P210

Deposition of hyaluronic acid in malignant melanomas is controlled bytumour-stroma-interactions

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Tumour-Stroma-Interactions are thought to be important for tumour growth and metastasis. Previously we have shown that fibroblasts (FB) in the tumour stroma surrounding malignant melanomas (MM) support MM progression by synthesizing growth factors and matrix-degrading enzymes [Anderegg et al.]. Histochemical studies suggest that these fibroblasts further deposit large amounts of hyaluronic acid (HA) in the tumour stroma, thus supporting MM-cell proliferation and motility. In the present paper we analysed the gene expression pattern of cultured FB and MM-cells concerning HA synthetases and HA degrading hyaluronidases by RT-qPCR. The resulting amount of deposited HA was measured by HA-ELISA. We demonstrate that HA-Synthetase-2 (HAS2) is the main synthesizing enzyme and is expressed at highest levels in FB. MM-cells synthesize only little amounts of HA-synthetases and consequently MM-cell supernatants contain very low concentrations of HA. FB express HAS2 and HAS3 synthetases and secrete 500–1000 times more HA than MM-cell lines do. Medium transfer experiments showed that MM-cells secrete soluble mediators which induce HAS2 expression and HA-secretion by FB. Interleukin-1β?and TGFβ?found in MM-cell culture supernatant scan induce HAS2-expression and HA deposition by FB thus being candidate mediators of this tumour-stroma-interaction. This model demonstrates that stroma cells produce HA found deposited in MM under the control of MM-derived mediators. These experiments not only establish a model to study tumour promotingtumour-stroma-interactions but may also identify novel targets for anti-proliferative orantimetastatic therapies in malignant melanoma.

Anderegg, U et al. Exp Dermatol 2005;**14**:709–718.

P211 (V15)

Skin tumour development induced by carcinogens is controlled by IL-10 and mast cells

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Chronic inflammation can result in skin tumour development. Also, proinflammatorycytokines such as TNF, IL-1, and IL-6 have been shown to contribute to cutaneous carcinogenesis. In contrast, anti-inflammatory signals and mechanisms are thought to be involved in the prevention of skin tumour development. However, there is little evidence from *in vivo* studies to support this hypothesis. Here, we subjected mice deficient for IL-10, a prototype anti-inflammatory cytokine, to chemical multi-step skin carcinogenesis. We found that IL-10−/− mice exhibit a dramatic increase in the rate of tumour bearing mice and the number of tumours per treatment site was significantly elevated as compared to normal C57BL/6 mice (e.g. 90% vs 30% tumour bearing mice and 1.3 ± 0.2 vs 0.1 ± 0.1 tumours per cm^2 skin at week 18 after tumour induction). As skin mast cells (MCs) are a major source of IL-10 and because MC-derived IL-10 has been shown to downregulate skin inflammation in various settings, we assessed carcinogen-induced tumour development in MC-deficient KitW/KitW-v mice. Interestingly, KitW/KitW-v mice exhibited similar, albeit less pronounced, responses as IL-10−/− mice, i.e. the induction of skin tumours was significantly increased as compared to normal mice. Also, KitW/KitW-v mice that had been repaired for their deficiency in skin MCs showed normal cutaneous carcinogenesis. Taken together, these observations suggest that MCs control skintumour development and that this protective effect of MCs may be due, at least in part, by their release of IL-10. These findings are in contrast with a recent report showing that IL-10 promotes skin tumour development following UV irradiation, which is driven by immunosuppression. This suggests that the protective effects of IL-10 and MCs may be specific for tumours induced by contact with carcinogens, which is associated with prominent and chronic inflammation. In support of this hypothesis we and others found that mice deficient for TNF, a signature proinflammatory cytokine, exhibit dramatically reduced skin tumour development in response to carcinogen treatment. In summary, we present, for the first time, evidence that IL-10 and MCs contribute to the control of carcinogen-induced skin tumour development.

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The cell-type specific S100/RAGE axis drives tumour development by sustaining inflammation

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Chronic inflammation plays a key role in promoting tumour development which is highlighted by a broad range of experimental and clinical evidence. However, the molecular mechanism converting a transient inflammatory reaction into a tumour-promoting microenvironment remains largely elusive. We show that mice deficient for the receptor for advanced glycation end-products(RAGE) are resistant to DMBA/TPA-induced skin carcinogenesis, characterized by a severe defect in sustaining inflammation during the promotion phase, thereby providing direct genetic evidence for a novel role for RAGE in linking chronic inflammation and cancer. Accordingly, upon single and repeated TPA treatment Rage−/− skin exhibits reduced epithelial cell proliferation, stromal inflammation and impaired up-regulation of COX-2 and MIPs representing novel targets of RAGE signalling *in vivo* in a bone marrow cell dependent manner. Finally, RAGE-dependent upregulation of its potential ligands S100A8 and S100A9 supports the existence of a cell-type specific S100/RAGE-driven feed-forward-loop in chronic inflammation and tumour promotion. Novel *in vivo* data will be presented on cell-type specific RAGE deficient mice in order to provide the genetic evidence that cell-type specific S100/RAGE signalling drives the strength and maintenance of an inflammatory reaction during tumour-promotion and thereby critically contributes to tumour development demonstrating that sustained inflammation is a key determinant of carcinogenesis.

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Identification and functional characterization of Polo-like kinase 1, a novel gene responsible for progression of cutaneous melanoma

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Melanoma is a very rapidly growing and lethal cancer. Disruption of the cell cycle regulation has been implicated in the development and progression of malignant melanoma albeit the exact underlying factors and mechanisms are not characterized yet. We used cDNA microarray technique to evaluate gene expression of 125 samples including normal skin, melanocytic nevi, primary melanomas, melanoma metastases and human melanoma cell lines. By combining pathway enrichment analysis with top 50 genes expressed in primary melanomas and melanoma metastases as compared to nevi we could identify cell cycle pathway and its member polo-like kinase 1 (plk1, a mitotic serine/threonine kinase) but not plk2, 3 or 4 to be significantly over expressed in primary melanomas and melanoma metastases. This finding could be confirmed using real-time RT-PCR analysis on an independent set of specimens. In *in vitro* analysis of human melanoma cell lines we observed the peak expression of the plk1 to be at the G2/M phase of the cell cycle. To explore the role of plk1 in human melanoma cell biology, melanoma cell lines were transfected with pDNA vector transcribing plk1 siRNA. This strategy as compared to control pDNAvector led to: (i) significant reduction of plk1 mRNA and protein, (ii) reduced cell proliferation, (iii) G1 phase arrest with increased number of octoploid cells, (iv) cell death with apoptotic features and, (v) induction of chemosensitization to campthotecin. This study shows that: (i) plk1 expression is dynamically regulated during the cell cycle of human melanoma and, (ii) knock down of plk1 can lead to inhibition of human melanoma cell proliferation, survival and induction of chemosensitization. Our data suggest plk1 to represent a potentially attractive target in melanoma therapy.

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The farnesyl transferase inhibitor lonafarnib combined with the RAF inhibitor sorafenib potently inhibits growth and induces apoptosis of metastatic melanoma cells

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Farnesyl transferase inhibitors (FTIs) inhibit the post-translational farnesylation of a number of target proteins, including RAS, preventing its signalling function. RAS signals to the RAF-MEK-ERK (MAPK) and PI3K-AKT-mTOR (AKT) signalling pathways which are constitutively activated in melanoma and appear to play a major role in tumour progression and chemoresistance. There is evidence that FTIs may synergize with other anticancer agents and could prove useful at overcoming chemoresistance. Using a panel of melanoma cell lines we evaluated the effects of the FTI lonafarnibalone and in combination with chemotherapeutic agents or pharmacological MAPK orAKT pathway inhibitors on melanoma cell growth and survival. Lonafarnib inhibited growth of melanoma cells isolated from primary melanomas in a dose-dependent manner. However, lonafarnib did not significantly inhibit growth of melanoma cells isolated from metastases. Monochemotherapy of melanoma cells with temozolomideor cisplatin produced diverse effects on growth of the different melanoma cell lines. In general, melanoma cells isolated from metastases were more resistant to chemotherapy than melanoma cells isolated from primary tumours. Co-treatment of melanoma cells with lonafarnib and chemotherapy did not enhance the inhibitory effect on growth of most metastatic melanoma cell lines tested. Also, most combinations of the FTI lonafarnib with MAPK or AKT inhibitors lacked significant enhancement of growth inhibition compared to monotherapy. In contrast, lonafarnib combined with the RAF inhibitor sorafenib potently inhibited growth and inducedapoptosis in most metastatic melanoma cell lines. These data suggest that lonafarnib combined with sorafenib may be a promising strategy for treatment of metastatic melanoma and merits in-depth investigation.

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Proteinase-activated receptors and melanoma cell invasiveness

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The proteinase-activated receptors (PARs) belong to a novel G-protein-coupled receptor subfamily, which can be activated by specific proteolytic cleavage of the extracellular N-terminus. PAR-1 can be stimulated by thrombin and matrixmetalloproteinase-1 (MMP-1), whereas PAR-2 can be activated by proteinases with trypsin-like specificity and certain kallikreins. Recent findings suggest a major role of PARs in the progression of various tumours. Both PAR-1 and PAR-2 expression is upregulated in melanomas. In contrast, primary melanocytes show no immunoreactivity for PAR-2, and only moderate immunostaining of PAR-1. Hence, only stimulation with PAR-1 agonists resulted in an intracellular calcium mobilization response in these cells. To further analyze signal transduction pathways turned on byPAR-1 and PAR-2 in malignant melanoma, we investigated the melanoma cell lineWM9 *in vitro*. WM9 cells demonstrated an overexpression of PAR-1 and PAR-2, and activation of both receptors resulted in a calcium mobilization response. In addition, quantitative PCR analysis revealed an upregulation of the pro-invasive metalloproteinase MT1-MMP after stimulation with PAR-1 and PAR-2 agonists. Furthermore, we observed a fast activation of ERK1/2 MAP kinases, whereas the MAP kinase p38 showed no phosphorylation. In contrast, both the protein kinase B(PKB)/Akt and PKC alpha/beta II were permanently activated in WM9 cells. We also found a PAR-1 and PAR-2 dependent phosphorylation of the protein kinase D(PKD/PKCμ) in melanoma cells. Recent data showed an important role for PKD in the motility and invasion of different tumour cells by the recruitment of integrins to focal contacts. Knockdown of PKD transcript in WM9 cells using a gene silencer plasmid for PKD revealed a significant decrease in proliferation and motility compared to normal WM9 cells. In conclusion, both PAR-1 and PAR-2 contribute to melanoma cell invasion and metastasis, which is at least partly dependent on the expression and activation of PKD.

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Role of erythropoietin receptor expression in malignant melanoma

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Erythropoietin (Epo) is known to promote proliferation and differentiation of erythroid precursors and to prevent apoptosis. Epo receptor (EpoR) belongs to the cytokinereceptor superfamily and exerts its effects via receptor homodimerization, subsequent phosphorylation of Janus kinase 2 (Jak2) and activation of signal transducers and activators of transcription (STAT)-3 and -5. Epo is also able to induce the activation of the Ras-Raf-MEK-ERK pathway in different types of cells. Recombinant human erythropoietin (rHuEpo) can prevent anemia in tumour patients; recent evidence has suggested that rHuEpo may adversely affect the survival of selected cancer patients by promoting tumour growth. In this study, we have investigated the expression of EpoR in malignant melanoma specimens and analyzed the effect of rHuEpo on proliferation and apoptosis of melanoma cells *in vitro*. We observed a transcriptional upregulation of EpoR in 50% of malignant melanoma metastases in comparison to benign nevi and primary melanoma using quantitative real-time RT-PCR. EpoR expression was weak in normal human melanocytes (NHM)and different melanoma cell lines (e.g. A375 and BLM cells), but was high in MV3cells and melanoma metastasis specimens as assessed by Western blot analysis. Furthermore, rHuEpo treatment also induced the phosphorylation of JAK-2, STAT-3and activated the Ras-Raf-ERK pathway as shown by Western blot experiments. In addition, rHuEpo seemed to prevent the cisplatin-induced apoptosis in certain melanoma cell lines. In summary, we demonstrate functional EpoR expression in stage IV melanoma specimens, thus cautioning the use of rHuEpo for the treatment of anemia in these patients.

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Imiquimod and its analogues induce apoptosis in melanoma and breast cancer cell lines independent of MAP kinase signalling

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Background: Imiquimod is a topical immune response modifier (imidazoquinoline)with antiviral and antitumour properties, which is effective against skin and breast cancer. Besides stimulating the production of pro-inflammatory cytokines through Toll-like receptors, novel apoptotic mechanisms have been identified. In this study, we examined the molecular mechanism through which imiquimod and its analogues induce cell death in melanoma and breast cancer cell lines.

Material and methods: Cell viability assay (MTT), H&E staining, Western blot, flowcytometry analysis using annexin V/propidium iodide.

Results: The treatment of different melanoma and breast cancer cell lines with imiquimod or its analogues resulted in the reduction of cell viability between 10 and 60% at 24 h as assessed by MTT assays. Data obtained from H&E stained cytospins and flow cytometry analysis using Annexin V/PI demonstrated ongoing apoptosis invarious melanoma (A375, BLM) and breast cancer cell lines (BT20, MDA-MB-468) that was confirmed by Western blot analysis using anti-PARP antibodies as a marker of late apoptosis. To show whether MAP kinase signalling was involved in the regulation of imiquimod-induced apoptosis, the cells were pre-treated with specific inhibitors of c-jun-N-terminal kinase (JNK), p38 and extracellular signal regulated kinase (ERK). Data obtained from MTT assays demonstrated that the three MAP kinase pathways were not involved in the modulation of imiquimod-inducedapoptosis. The examination of pro- and anti-apoptotic proteins including bax, bak,bcl-2 or Mcl-1 at the expression level demonstrated no regulation in response to treatment with either imiquimod and its analogues in contrast to mouse embryonic cells (Wild type, Bax−/−, Bak−/−knock out cells). However, our data suggest significanttranslocation of Bax to the endoplasmatic reticulum to cause intrinsic apoptosis inresponse to imiquimod and its analogues as demonstrated by confocal laserscanning microscopy.

Conclusion: Our data demonstrate the ability of imiquimod and its analogues to killmelanoma and breast cancer cells by an apoptotic mechanism without the involvement of MAP kinase signalling. The role of endoplasmatic reticulum in apoptosis caused by imidazoquinolines merits further investigation.

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Identification of a distinctive multi-gene signature in cutaneous melanomausing qRT-PCR-based microfluidic cards

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Melanoma is a complex genetic disease, the management of which will require anin-depth understanding of the biology underlying its initiation and progression. Recently, oligonucleotide microarray-based comparison of gene expression profiles of a series of nevi, primary cutaneous melanomas and cutaneous melanoma metastases have yielded several differentially regulated genes that are potential determinants in melanomagenesis and progression (Nambiar et al. 2007). However, high-thoroughput qRT-PCR-based validation and generation of multigene prediction models in melanoma have received limited attention. Therefore, a selection of 62genes previously found to be differentially expressed in melanoma progression was used in the novel qRT-PCR platform called TaqMan®?low density array (LDA) ormicrofluidic card; 30 well-characterized clinical specimens (Five benign, five dysplastic nevi, 10 primary melanomas and 10 cutaneous melanoma metastases) were analyzed. These genes were then ranked in the order of their discriminatory potential between nevi and primary melanomas using multiclass and two-class significance analysis of microarrays (SAM) and validated by analyzing an additional 42 samples. Consequently, a 4-gene signature of ASK/Dbf4 and Tpr in combination with the established markers melanoma cell adhesion molecule (MCAM/MUC18) and hepatocyte growth factor receptor (c-MET) was generated that could distinguish benign and atypical nevi from malignant melanomas. In summary, our study reports aqRT-PCR-validated novel 4-gene signature that differentiates nevi from melanoma and may complement histopathological findings.

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Taxol-induced mitochondrial stress in melanoma cells is mediated by activation of c-Jun N-terminal kinase (JNK) and p38 pathways via uncoupling protein 2

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Taxol (paclitaxel) is a new antineoplastic drug that has shown promise in the treatment of different tumour types. However, the molecular mechanisms governing taxol-induced apoptosis are poorly understood. Activation of mitogen-activated protein (MAP) kinases is induced by a wide variety of external stress signals and may lead to apoptosis. Therefore, we challenged the human melanoma cell lines A375and BLM with taxol and characterized the molecular mechanisms regulating taxol-induced apoptosis. Taxol resulted in the activation of apoptosis signal regulated kinase (ASK)1, c-jun NH2-terminal kinase (JNK), p38MAPK and extracellular-regulated kinase (ERK) together with the downregulation of uncoupling protein 2 (UCP2). In addition, reactive oxygen species (ROS) were induced andDNA-binding activity of the transcription factors AP-1, ATF-2 and ELK-1 was enhanced. Ultimately, cytochrome c was released, and caspases-9 and -3 as well as PARP were cleaved. Pretreatment of melanoma cells with the JNK inhibitor(SP600125) or the p38 inhibitor (SB203580) blocked taxol-induced UCP2downregulation, ROS generation and apoptosis, whereas the ERK inhibitor(PD98059) had no such effect. Our data provide evidence that taxol-induced mitochondrial stress occurs through the activation of both JNK and p38 pathways, and suggest a novel role for UCP2 in the modulation of taxol-induced apoptosis of melanoma cells.

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Sézary syndrome is a unique cutaneous T-cell lymphoma as identified by an expanded gene signature including diagnostic marker molecules CDO1 and DNM3

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Sézary syndrome (SS) is a rare, aggressive CD4+ CTCL; molecular traits differentiating SS from non-leukemic mycosis fungoides (MF) and from inflammatory skin diseases (ID) are not sufficiently characterized. PBMC of 10 SS patients and 10healthy donors (HD) were screened by Affymetrix U133Plus2.0 chips for differential gene expression. Ten candidate genes were confirmed by qRT-PCR to be significantly over-expressed in CD4+ T-cells of SS versus HD/ID. For easier clinical use, these genes were re-analyzed in PBMC; qRT-PCR confirmed five novel (DNM3,IGFL2, CDO1, NEDD4L, KLHDC5) and two known genes (PLS3, TNFSF11) to be significantly overexpressed in SS. Multiple logistic regresssion analysis revealed thatCDO1 and DNM3 had the highest discriminative power in combination. Upon comparison of PBMC and skin samples of SS versus MF, CDO1 and DNM3 were found up-regulated only in SS. Using anti-CDO1 antisera, differential expression of CDO1protein was confirmed in SS CD4+T-cells. Interestingly, DNM3 and CDO1 are known to be regulated by SS-associated transcription factors TWIST1 and c-myb, respectively. Furthermore, CDO1 catalyzes taurine synthesis and taurine inhibits apoptosis and promotes chemo-protection. In summary, CDO1 and DNM3 may improve the diagnosis of SS and open novel clues to its pathogenesis.

P221 (V20)

Novel therapeutic strategies significantly reduce lymph node and pulmonary metastases in a spontaneously metastasizing mouse melanoma xenograft model

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We have recently established a spontaneously metastasizing mouse melanoma model using human M24met melanoma cells to demonstrate that dimethylfumarate(DMF) reduces melanoma growth and lymph node metastasis. We have now used this model to compare therapeutic effects of DMF, a VEGF-A blocking antibody and dacarbazine on melanoma growth and metastasis. Single as well as combination therapies have been used. Volumes of primary tumours could be reduced significantly with all different treatmentconditions. Most importantly, lymph node and pulmonary metastasis as analyzed byimmunohistochemistry, was most effectively reduced by a combination of DMF and dacarbazine or a combination of DMF and VEGF-A blockade. Pulmonary metastasis was inhibited by all of the therapies used without any significant differences between different treatment groups. These results correlated with reduced lymph vessel angiogenesis in primary and metastatic tumour sites. In summary, DMF might be an appropriate adjuvant in the treatment of advanced stage melanoma in humans.

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CD147 impacts angiogenesis and metastasis formation

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CD147 (EMMPRIN/Extracellular Matrix Metalloproteinases Inducer) is highly expressed on many tumour cells; its role for tumour invasiveness and metastasis has been deduced from its capacity to induce MMPs i.e. MMP-1, -2, -3 and -9. However, as previously demonstrated in the murine B16 melanoma model, MMP-2/-9expression occurs independent of CD147. To scrutinize the impact of CD147 on metastasis formation and angiogenesis in this model, CD147 was stably knocked down in B16 cells. This silencing of CD147 expression resulted in a reduced capability of the tumour cells to metastasize to the draining lymph nodes. Notably, theCD147 knock down caused a decreased VEGF expression *in vivo* accompanied by reduced blood vessel formation. Thus, in the B16 melanoma model, CD147 promotesmetastasis formation by induction of angiogenesis in a MMP independent manner.

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Proliferation arrest in B-Raf mutant melanoma cell lines upon MAPK pathway activation

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In benign tumours the activation of oncogenes primarily induces senescence, associated with cessation of cellular proliferation; e.g. in melanocytic nevi expressing mutant B-Raf. These putative control mechanisms include the RB and/or the p53pathway. The current model of melanomagenesis postulates that progression toimmortal melanoma cells requires inactivating aberrations in signalling cascades controlling senescence. Thus, melanoma cells carrying mutant B-Raf should be resistant to mitogen-activated protein kinase (MAPK) pathway-induced senescence. Here, we demonstrate that hyper-activation of the MAPK pathway following activation of an inducible form of oncogenic C-Raf induces a senescence-like proliferation arrest in three B-Raf mutant melanoma cells. This Raf-induced senescence is initially strictly dependent on MEK signalling, but seems to be independent of MAPK signalling after prolonged continuance. It is associated with reduced levels of RBphosphorylation and an increase in p21 expression, but is independent of p16Ink4aand p53. These data argue against the existence of fundamental changes in melanoma cells completely precluding senescence.

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The iron-containing nucleoside analogue N69 efficiently induces apoptosis inhuman melanoma cells by activating new pathways enclosing lysosomalactivation

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Treatment of malignant melanoma is impeded by its pronounced chemotherapy resistance related to defects in apoptotic signalling. As present chemotherapeutics are virtually ineffective for melanoma therapy, new cytostatic drugs are urgently required to overcome apoptosis resistance. Nucleoside analogues have a high pharmacological potential for anti-cancer treatment, and metal carbonyl complexes, such as the recently described nucleoside analogue N69, offer new opportunities for drug development. As important prerequisites for efficient drugs, these complexes are stable in solution and also reactive in the cell. Our studies focused on the effects of N69 as well as on its mode of action in melanoma cell lines (A-375, Mel-HO,SK-Mel-13 and Mel-2a). Apoptosis was determined by DNA fragmentation, TUNEL and DAPI positivity as well as by counting sub-G1 cells. Cytotoxicity was quantified by a lactate dehydrogenase release assay. Changes in the lysosomal pH weredetermined by fluorescence microscopy after acridine orange staining. For analysis of caspase and cathepsin activities, cells were treated with irreversible, selective peptide protease inhibitors. N69 (20 μM) strongly induced apoptosis in A-375 and Mel-HO, whereas SK-Mel-13and Mel-2a responded to higher concentrations. At the used concentrations, cytotoxicity was neglectable in these cells. Cytochrome c was released from mitochondria. However there was no indication of any caspase activation in melanoma cells, and also selective caspase inhibitiors could not block apoptosis byN69. Interestingly, there was a strong effect of N69 treatment on the lysosomal pH indicating a permeabilization of lysosomes, but also selective cathepsin inhibitors were not able to reduce the apoptosis rate. Thus, N69 represents a novel drug with particular efficacy in melanoma cells, and it triggers proapoptotic pathways, which have not been described in melanoma cells before. Their further elucidation may expose new ways for targeting this highly therapy-refractory tumour.

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Vitamin D status in melanoma patients

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Sun exposure, particularly intensive short-time and recreational sun exposure, isconsidered to be the major etiologic factor for melanoma. But on the other hand 90%of all requisite vitamin D has to be formed in the skin through the action of the sun – a serious problem due to the fact that new scientific findings convincingly demonstrate vitamin D deficiency to be associated with a variety of severe diseases including various types of cancer (e.g. colon, prostate and breast cancer). According to recent reports sun exposure is associated with a relatively favourable prognosis and increased survival rate in various malignancies, including malignant melanoma. It has been speculated that these findings were related to UV exposure-induced relatively high serum levels of vitamin D which may lead to a more favourable course of melanoma. To prove this hypothesis serum levels of 25-hydroxyvitamin D (25(OH)D)were analyzed among 205 patients with histologically proven melanomas of different stages and compared with a control group ($n = 141$). The 25-hydroxyvitamin D serum levels of melanoma patients in large did not significantly differ from those in the control group. But on the other hand stage IV melanoma patients had significantly reduced 25(OH)D serum levels in comparison with stage I melanoma patients ($P = 0.006$). Furthermore we demonstrated that patients with low 25(OH)D serum levels (<10 ng/ml) had primary cutaneous melanomas with greater tumour thickness as compared to melanoma patients with 25(OH)D serum levels >20 ng/ml (0.078).Tumour thickness at time of diagnosis is strongly associated with prognosis of melanoma and considered to be the best predictor for melanoma progression. Although we did not find statistical significant differences comparing time periods until progress in melanoma patients with low (<10 ng/ml) and normal 25(OH)D serum levels we observed seasonal variation with more rapid progression of melanomas when diagnosed in spring and autumn compared to those diagnosed in winter and summer.

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Down regulation of proapoptotic Bcl-2 proteins with tumour progression, as determined by immunohistological analysis of corresponding melanoma primary tumours and metastases

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The proteins of the Bcl-2 super family play crucial roles in regulation of the intrinsic proapoptotic pathway. The family consists of proapoptotic proteins, such as Bax, Bak, PUMA and NOXA as well as antiapoptotic proteins as Bcl-2 and Mcl-1.According to the presence of Bcl-2 homology domains (BH 1–4), proapoptotic-proteins further subdivide into multi-domain proteins (Bax, Bak) and BH3-onlyproteins (PUMA, NOXA). For control of apoptosis, the expression of these proteins is balanced, thus a dys balance may result in apoptosis deficiency and may promote tumour progression and therapy resistance. Their expression depends on diverse signalling cascades frequently dysregulated in cancer. Especially melanoma metastases are characterized by pronounced therapy resistance associated with a block of proapoptotic pathways. Recently, we reported an association between loss of Bax and Bak in primary melanomas with poor prognosis (metastasis). Here, we have investigated the expression levels of Bcl-2 proteins in melanoma metastases and compared these with the corresponding primary tumours of the same patient. Twenty primary melanomas (nodular/lentigo-maligna/acral lentiginous, unclassified and superficial-spreading melanomas: $n = 1/1/1/3/14$) and the corresponding metastases (mainly from lymph nodes) were examined by immunohistology (APAAP). Primary antibodies were used for human Bcl-2, Mcl-1, Bax, Bak, PUMA and NOXA, and their specificity was proven in Western blot analyses. The microscopical evaluation of the slides interestingly revealed a tendency for a downregulation of Bcl-2 in metastases, whereas Mcl-1 expression was more continuous. Also multidomain proapoptotic Bcl-2 proteins, especially Bax, showed weaker expression in metastases, thus paralleling the previous findings in primary tumours. Most pronounced was a downregulation of PUMA in 61% of the melanoma metastases, whereas Noxa was weakly expressed both in primary tumours and metastases. Our results indicate a critical role of proapoptotic Bcl-2 proteins in course of melanoma progression. These data may be further helpful for identification of molecular markers as well as of therapeutic targets in melanoma.

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Mitogen-activated protein kinases (MAPK) activation in human keratinocytes:p38 plays a crucial role for TRAIL-mediated target gene induction

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Because of its ability to specifically induce apoptosis in transformed keratinocytes the TNF-related apoptosis inducing ligand (TRAIL) may represent a hopeful novel therapeutic option for skin tumour treatment. TRAIL binding to its death receptors 1 (TRAIL-R1) and TRAIL-R2 promotes recruitment of procaspase-8 at the death inducing signalling complex (DISC) that further induces the apoptotic process. TRAIL is also involved in activation of non-apoptotic signalling pathways such as the transcription factor NF-κB. Much less, however, is known about the role of TRAIL for MAPK activation. We thus characterized posttranslational MAPK modifications following TRAIL stimulation in HaCaT cells. Interestingly, TRAIL highly induced ERK 1/2, p38 and JNK activation with markedly different kinetics. While ERK was activated in a caspase-independent manner, p38 and JNK activation occurred caspase-dependently. To block caspases more physiologically, HaCaT cells were stably transduced with the intracellular caspase-8 inhibitor cFLIPL. Interestingly, cFLIPL was unable to interfere with TRAIL-mediated ERK activation, while delayed JNK or p38 activation was fully blocked. To assess the physiological relevance of these TRAIL-activated signalling path ways, we investigated TRAIL-mediated induction of the known target gene interleukin-8 (IL-8) in the presence of different MAPK inhibitors. While MEK inhibitor U0126efficiently blocked TRAIL-induced ERK activation, it did not interfere with IL-8secretion. In contrast, p38 inhibitor SB203580 partially repressed TRAIL-mediatedIL-8 secretion, indicating a crucial role of p38 activation for target gene induction by TRAIL. Taken together, our data indicate that TRAIL-induced MAPK activation might initiate apoptosis-independent signals including skin differentiation, proliferation and inflammation. We speculate that TRAIL, under apoptosis-resistant conditions, could exert protumoural activity via activation of different MAPK in a tissue-specific manner.

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Stage-specific effects of Hepatocyte growth factor on snail, slug, twist and Src in melanoma

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Hepatocyte growth factor/Scatter Factor (HGF/SF) is a known mitogen, motogen, and morphogen for many epithelial cells. It is physiologically secreted by cells of mesenchymal origin and is supposed to be crucial for melanomagenesis. Previous work has shown that HGF down-regulates E-cadherin in melanoma cell lines. Mechanisms of HGF induced down-regulation of cadherins in melanoma are unknown. Based on recent reports, which showed that the Snail super family of zinc-finger transcription factors represses E-cadherin and up-regulates N-cadherin, we hypothesized that HGF down-regulation of E-cadherin may be mediated through these transcription factors, Twist or Src. Melanocytes and six melanoma cell lines were probed for changes in protein levels by immunoblotting of Snail, Slug, Twist and Src after exposure to recombinant HGF for 8 h at 10 or 50 ng/ml. After nuclear extraction of exposed and unexposed cells the subcellular location of these proteins was determined. Cell extracts from cell lines, derived from superficial melanomas (WM35, WM164 and WM793) and metastatic melanomas (WM9, WM278 and 1205 Lu) were used. In all cell lines transcription factors Slug and Snail were mainly localized in the nuclear fraction. Phosphorylated Src was predominantly found in the cytoplasmic fraction. Melanoma cell lines expressed Src, Snail and Slug in a cell type specific manner. Exposure to HGF modulated phosphorylated Src in the nucleus, Snail and Twist in melanocytic cells at different levels and indicated that Snail is not the only target of HGF. From this survey it can be concluded that in superficial melanoma cell lines the transcription factor Slug is mainly affected by HGF, whereas in metastatic cell lines Twist is mostly induced. Therefore we suggest that HGF mediated effects on these proteins are stage-dependent and may have important implications for the biology of HGF and the E- to N-cadherin switch in melanoma.

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Effect of hepatocyte growth factor on the expression of Wnts and Wnt regulators in melanoma

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Hepatocyte growth factor/scatter factor (HGF/SF) plays an important role in development and progression of melanoma. Wnts activate pathways involved in differentiation, proliferation and migration and are supposed to be crucial for all stages of melanoma development. Emx2 and Six3 have been described as repressors of Wnt1 expression in mammalian forebrain development. In a previous study transforming growth factor-β?(TGF-β) induced Wnt7a expression in human mesenchymal progenitor cells. We investigated whether HGF may cause similar effects on Wnt expression in human melanocytic cell lines and may lead to alterations in Emx2 and Six3 transcription. Melanocytes (FOM101) and melanoma cell lines of different stages (superficial melanoma: SBcl2, WM35, WM793; metastatic melanoma: WM9, 451Lu) were exposed to 50 ng/ml recombinant HGF. Unexposed and exposed cells were harvested at different time points, varying from 1 to 24 h. RNA was reverse transcribed and semi quantitative PCR was performed with primers for WNT1,WNT3a, WNT5a, WNT7a, WNT7b, WNT10b, WNT11, EMX2 and SIX3. Wnt3a, -5a, -10b and -11 were expressed constitutively in all cell lines, Emx2 andSix3 in a couple of them, independently of exposure to HGF. Differential expression of Wnt1, -7a and -7b was observed after HGF treatment in some cell lines. Further, we found a stage dependent expression of Emx2 with decreasing levels of transcripts in the course of progression. Since no information about the role of Emx2 in melanoma is currently available we will study its impact for melanomagenesis in more detail.

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Expression of CD44v6 in Melanocytic Lesions and Influence of the hepatocyte growth factor on the expression of CD44v6 in Melanocytes and Melanoma cell lines

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CD44 molecules, a family of multifunctional, transmembrane glycoproteins have been implicated in diverse normal and pathological cellular functions. Different isoforms ofCD44 are mainly generated by alternative splicing of 10 variant (v1 -v10) exons. Hepatocyte Growth Factor (HGF) is a known mitogen, motogen and morphogen formany epithelical cells and acts through c-Met, a transmembrane associated tyrosinekinase receptor. CD44v6 forms a complex with both c-Met and HGF and is required for HGF-induced c-Met activation. Because expression levels of CD44v6 in the progression of melanoma and the influence of HGF on CD44v6 are unknown, immunohistochemistry of melanocytic lesions and the dependence of c-Met mediated signalling on the co-expression ofCD44v6 in melanocytes and melanoma cell lines from different stages (primary melanoma: WM35, WM793, WM278, SBcl2; metastatic melanoma: WM9, WM164,1205Lu, 451Lu) after HGF-stimulation were studied. Changes in c-Met and CD44v6expression were analysed at different time points after exposure to 50 ng/ml recombinant human HGF at the RNA level (c-Met, CD44v6, real-time PCR) and on the protein level (CD44v6, immunoblotting). Immunohistochemistry showed that CD44v6 is mainly represented in primary melanomas, cutaneous and lymph-node metastases, whereas positive staining in nevi and visceral metastases of melanomas was rare and weak. The exposure of melanocytes and melanoma cells to recombinant human HGF leads to an increase ofCD44v6 expression in melanocytes at the RNA and protein level. Based on these results we want to abrogate the effect of HGF with a neutralizing antibody, down regulate CD44v6 by using siRNA and investigate on the involved transcription factors and functional effects after HGF stimulation.

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Role of the immune system in the pathogenesis of malignant melanoma in anew genetic mouse model

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Our group has established a new mouse model for melanoma (HGFxCDK4R24Cmice) where primary autochthonous melanomas in the skin develop spontaneously and can be induced by neonatal UV radiation or DMBA treatment. Melanomas spontaneously metastasize into the draining lymph nodes and the lungs. Here we investigated the role of cellular immune responses in the pathogenesis of melanoma. HGFxCDK4R24C mice were treated neonatally with 20 μg DMBA or were left untreated. Mice with progressively growing melanomas were sacrificed and tumour tissue as well as draining lymph nodes were harvested for further analyses. The immune infiltrate in primary melanomas of the skin and the composition of immune cells in metastatic lymph nodes was characterized by immunohistochemistry and by flow cytometry. DMBA induced primary cutaneous melanoma did not show significant immune infiltrates of lymphocytes or dendritic cells. In the draining lymph nodes melanoma cells were found to penetrate into the T- and B-cell areas with a significant decrease of T-cells, particularly CD8+ T-cells and DC. Interestingly, alltumour-bearing HGFxCDK4R24C mice developed a significant splenomegaly with a diffuse increase of cells in the red pulp. When primary melanomas were transplanted subcutaneously to tumour free mice this phenomenon could also be seen. Therefore splenomegaly appears to be tumour-induced. These data extend our previous observations showing that autochthonous melanoma in HGFxCDK4R24C mice appear to effectively evade the cellular arm of the immune system. We are currently investigating the *in vivo* function of dendritic cells and cytotoxic T-cells in tumour-bearing mice using adoptive T-cell transfer experiments.

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Anti-tumour effects of solubilized triterpene acids from Viscum album *L. onmurine* and human skin cell lines *in vitro*

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Mistletoe (Viscum album L.) contains triterpene acids such as oleanolic acid and betulinic acid in relatively high amounts. However, due to their poor solubility these interesting compounds have not been largely used in the clinical setting. For both of these triterpene acids anti-tumourigenic, anti-inflammatory and anti-microbial effects are reported. Here, we have taken a new approach to generate water soluble formulations of triterpene acids from mistletoe. Water solubility was achieved by complexation of triterpene acids with cyclodextrinsas carrier molecules. We tested the effects of different neutral and alkaline aqueoustriterpene-cyclodextrin solutions on cell viability and apoptosis of human (HaCaT andCal-39) and murine (B16.F10) skin-derived cell lines and compared them to triterpene acids dissolved in dimethyl sulfoxide (DMSO). We show here that these solubilized triterpene acids dose-dependently inhibited cell proliferation and induced apoptosis of HaCaT (immortalized keratinocytes), Cal-39(squamous cell carcinoma) and B16.F10 (melanoma) cells *in vitro*. Toxic side effects were observed when DMSO was used as solvent, but not when triterpene acids were solubilized with cyclodextrins. In summary, we have demonstrated the use of solubilized triterpene acids for induction of anti-tumour effects *in vitro*. Further experiments will reveal whether these solubilized triterpene acids are potent in the induction of tumour cell apoptosis *in vivo*.

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Macroscopic and microscopic characterization of spontaneous and UV-induced melanomas in the skin of HGF × CDK4R24C mice

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C57BL/6 mice which carry a mutant cyclin-dependent kinase 4 (CDK4R24C) andoverexpress hepatocyte growth factor (HGF) spontaneously develop cutaneousmelanoma at an age of about one year. A single neonatal dose of erythemal UVB (6kJ/m^2) radiation significantly increases the penetrance and decreases the latency of primary melanomas in the skin. Here we macroscopically and microscopically characterized spontaneous and UV-induced melanomas in HGFxCDK4R24C mice and investigated whether primary melanomas show an infiltration with immune cells. Macroscopically, spontaneous melanomas appear as nodular lesions in the skin whereas UV-induced melanomas showed both nodular as well as superficially spreading growth. Both types of tumours disseminated to regional lymph nodes and lungs. Histopathologically, both primary as well as metastatic melanomas were composed of two types of tumour cells. Epitheloid melanoma cells were arranged in files and nests and were separated by thick bundles of much less pigmented, spindle-shaped melanoma cells. Melanoma cell proliferation involved the dermo-epidermal junction and occasionally showed intraepidermal spread, particularly in UV-induced melanomas. Immunohistological stainings did not reveal significant infiltration of primary tumours with immune cells. Thus, UV-induced melanomas are morphologically similar to spontaneous melanomas inHGFxCDK4R24C mice but show a horizontal growth pattern in the skin more frequently. Future investigations will have to address the molecular and cellular mechanisms how UV-irradiation promotes melanomagenesis in this genetic melanoma model.

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Resistance of cutaneous T-cell lymphoma cell lines to TNF-ligand-mediated apoptosis correlates to overexpression of cFLIP and loss of caspase-10 and Bid

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Tumour necrosis factor (TNF-α), CD95L/FasL and TNF related apoptosis-inducing ligand (TRAIL), critically contribute to lymphocyte homeostasis due to their induction of apoptosis, and they may further represent safeguard mechanisms to prevently mphoma development. Thus there is a high selective pressure for lymphomas for acquiring resistance to death ligands. This may be different for the various entities of T-cell lymphomas dependent on their origin and the pathway of tumour development.

We investigated eight cutaneous T-cell lymphoma cell lines (CTCL) with regard to their sensitivity to the three death ligands and compared them to four T-cell acutely mphoblastic leukemia (T-ALL) cell lines. Four CTCL cell lines derived from CD30(-) mycosis fungoides or Sézary syndrome, whereas four other cutaneous cell lines derived from CD30(+) cutaneous anaplastic large cell lymphoma (cALCL). Whereas all T-ALL cell lines revealed general sensitivity to the death ligands, CTCL cell lines revealed pronounced resistance to TNF-α?and TRAIL, and one of theCD30(-) cell lines was in addition resistant to agonistic CD95 stimulation. In resistant CTCL cell lines, caspase activation was completely blocked. In response to CD95 stimulation caspase-10 was cleaved selectively in CTCL cells but not in T-ALL cells. Resistance to CD95 stimulation in one CTCL cell line and resistance to TNF-alpha in several correlated with loss of the respective death receptors (CD95 and TNF-R1, respectively). Several changes of other apoptosis regulator proteins of the families ofBcl-2 proteins as Bid and Bcl-2 were identified. Significant overexpression of cFLIP (cellular FLICE-inhibitory protein) was found in 8/8 CTCL cell lines whereas in T-ALL cell lines cFLIP was not detectable. Parallels with regard to expression of apoptosis regulators were seen in PBMCs and biopsies of CTCL patients. Thus, the *in vitro* study underlines the significance of apoptosis control for cutaneous lymphoma, furthermore suitable markers can be identified, which may become targets for therapeutic approaches.

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Use of *in vivo* confocal laser scanning microscopy for monitoring of superficial basal cell carcinoma after cryotherapy

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The confocal scanning laser microscopy (CLSM) enables the examination of skin tissue *in vivo* at nearly histologic resolution. CLSM features of basal cell carcinomas (BCCs) have already been described in the literature and correlate well with histology. Typical findings of superficial BCCs in CLSM are elongated nuclei of tumour cells oriented along the same axis, increase of tortuous vessels and presence of prominent collagen bundles within the surrounding stroma. We included five consecutive patients with a total number of 10 histological proven superficial BCCs of the trunk and monitored tissue changes 5 and 24 h after standardized cryotherapy by means of CLSM. All BCCs showed characteristic findings in CLSM before cryotherapy. Five hours after therapy, all BCCs showed numerous necrotic cells indicated by bright pycnotic nuclei within basal epidermal layers and incipient blistering. Seven BCCs showed also tissue damaging within upperdermal layers and displayed necrotic cells in next proximity to collagen bundles. After removal of necrotic tissue 24 h after cryotherapy, connective tissue with pycnoticcells beneath collagen bundles was displayed in all cases. Our preliminary results suggest that the presence of pycnotic cells within basal epidermal layers and upperdermal structures with blistering are followed by ablation of tumour tissue after cryotherapy. A second cryotherapy should be considered if tissue damaging through upper dermal layers indicated by pycnotic cells next to collagen bundles can not bedetected 24 h after therapy. The predictive value of the method has to be proven by long-term follow-upexaminations, but in our opinion CLSM holds promise in the diagnosis and monitoring of effective cryotherapy of superficial BCCs.

P236 (V34)

Platelets support vascular integrity in inflammation and tumour angiogenesis

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Platelets are required for plug formation upon traumatic vessel injury. They have also been reported to support vessel stability - the precise mechanism still being a matter of current research. This study investigated the role of platelets for vessel stability during inflammation and angiogenesis in the microcirculation of the skin. We made the surprising discovery that, during thrombocytopenia, inflammation (Irritant contact dermatitis; reverse Arthus reaction) and tumour angiogenesis (melanoma) led to severe skin hemorrhage. By imaging the cutaneous Arthus reaction and melanoma growth through a skin window, we observe in real-time the loss of vascular integrity and the kinetics of skin hemorrhage in thrombocytopenic mice. Bleeding – mostly observed from small venules - occurred as early as 20 min upon challenge, pointing to a fundamental continuous need for platelets to maintain vascular integrityin inflamed and angiogenic microcirculation. Importantly, thrombocytopenic mice didnot bleed in uninflamed tissue. Platelet transfusion experiments showed that 10% ofplatelet count is sufficient to prevent inflammatory hemorrhage. Geneticallyengineered mice revealed that maintenance of vessel integrity is independent of the major platelet adhesion receptors, indicating that this platelet function completely differs from that in primary hemostasis. Our studies reveal that platelets play a crucial role for the maintenance of vascular integrity during inflammation and tumour angiogenesis. Targeting platelets and thus modifying vascular integrity may be an interesting strategy for inhibition of tumour survival and inflammation.

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Defects in dna mismatch repair do not account for early onsetbasal cell carcinoma

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Genetic factors contribute considerably to the risk of developing basal cell carcinoma(BCC), particularly in Gorlin syndrome. However, a small cohort of patients aged 40or less do not have clinical symptoms of this syndrome, but still develop basal cellcarcinoma at a young age. Defects in DNA mismatch repair have been associated with development of hereditary as well as sporadic forms of skin cancer. Also, microsatellite instability has been found in sporadic BCCs . We therefore hypothesized that inherited mismatch repair defects, manifesting as instability of microsatellites, might contribute to the development of BCC at a young age. We examined twenty-two patients with multiple BCCs before the age of 40 for evidence of microsatellite instability using DNA isolated from sun-exposed and non-exposed skin and peripheral blood leucocytes. Exposure to known environmental risk factors was excluded by means of a questionnaire. We screened the PTCH1 gene for pathogenic mutations by direct sequencing of all exons and intron-exon boundaries. Microsatellite-instability status was established in all DNA sources by amplifying mononucleotide repeats BAT-26, BAT-25, NR-21, NR-22 and NR-24. We found no pathogenic mutations in PTCH1 in any of the patients. We noted no size difference in mononucleotide repeats in any patient sample. Thus, microsatellite instability was not detected in tissue samples or in germ line DNA. We conclude that our study does not support a causative role for defects in mismatch repair in the development of multiple early onset BCCs.

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Paired basic amino-acid-cleaving enzyme 4 in human melanoma – a novel player in tumour cell invasiveness?

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Basic amino-acid-cleaving enzyme 4 (PACE4) is a member of the prohormoneconvertase (PC) family that consists of at least eight different serine endoproteases with overlapping substrate specificities. Due to their well established enzymatic activity on prohormones such as pro-opiomelanocortin, PCs - especially PC1/3 and PC2 – have been investigated in cutaneous biology mainly with regard to pigmentation and generation of pro-opiomelanocortin-derived peptides. Here we investigated the role of PCs, especially PACE4, in melanoma cell biology. Expression analysis of a large panel of human melanoma cell lines revealed dramatically increased levels of PACE4 in the majority of transformed melanocytes as compared to normal melanocytes. Immunohistochemical analysis confirmed PACE4 immunoreactivity *in situ* in 89% of47 tumour specimens. To precisely investigate the impact of increased expression ofPACE4 in melanoma cells we ectopically expressed ratPACE4 in A375 melanomacells. Aberrant PACE4 expression did not have any effect on cellular proliferation but interestingly increased pigmentation as measured by melanin assay. Most importantly, ectopic expression of ratPACE4 led to increased matrix metalloprotease(MMP)1 and 2 expression and enhanced gelatinase activity as shown byzymography. Invasion assays *in vitro* revealed a significantly increased invasionactivity of the PACE transfectants as compared to vector-alone transfected cells re-emphasizing the role of MMP2 in tumour cell invasion. Our data unravel members of the PC family such as PACE4 as novel players in melanoma biology and suggest a role for them in tumour cell invasion. In light of the recent pharmacological development of specific PC inhibitors these findings may also have therapeutic value for the treatment of patients with melanoma.

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Characterization of a mouse model to investigate lymphocyte homing to human melanoma

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We have previously shown that blood vessels in human metastatic melanoma do not, in general, express leukocyte adhesion receptors, effectively blocking entry of cytotoxic lymphocytes and restricting the anti-tumour immune response. Based on these results, we hypothesize that modulation of tumour vasculature to express adhesion receptors will enhance immune responses to melanoma, resulting in more effective vaccination protocols. To study vascular adhesion receptor expression and lymphocyte extravasation within melanoma, we developed a humanized mouse model of melanoma progression. As tumour angiogenesis is proposed to occur from surrounding tissues we implanted human A375 melanoma cells into human skin xenografts 3 weeks after transfer onto RAG−/−JAK−/− mice in order to study development of melanoma nodules within a human tissue microenvironment. Three weeks after tumour cell injection, tumour nodules formed inside the skin grafts and presented morphology similar to fresh human melanoma metastases. Immunohistochemical examination of tumour vasculature using a species specific CD31 antibody showed human and mouse vessels throughout the tumours. A time course study indicated that mouse vessel penetration of human skin xenografts started between day 5 and 10 after engraftment. In the same time frame, preexisting human vessels gained continuity with the mouse circulation as identified by *in vivo* staining of human endothelium with FITC labelled Lectin specific for human endothelia. Over 10 weeks, increasing numbers of mouse vessels penetrated the graft while the number of human vessels was continuously reduced. Adoptive transfer of human lymphocytes to grafted mice resulted in infiltration of human skin xenografts. We conclude that the xenograft model can be used for human lymphocyte homing studies that focus on lymphocyte extravasation in individual vessels labelled for species specificity. Analysis of tumour infiltrates in this model needs to consider whether infiltration occurs through human or mouse vessels. Studies should be restricted to a time frame of 10–20 days after graft implantation based on the time dependent decline of human vessels inside the grafts. Previously published studies using human skin xenografts to study lymphocyte homing need to be interpreted with regard to these findings.

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Proteins of the Bcl-2 family are involved in Bcl-xAK-mediated apoptosis

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Bcl-2 proteins are crucial in the control of apoptosis via the mitochondrial pathwayand exert both proapoptotic and antiapoptotic functions. Most antiapoptotic proteins contain four Bcl-2 homology domains (BH 1–4), whereas the proapoptotic proteins may lack one or several BH domains. All the so far described proapoptotic Bcl-2proteins carry BH3. The bcl-x gene gives rise to several alternative splice products resulting in proteins with distinct functions, e.g. the antiapoptotic Bcl-xL with all four BH domains and the proapoptotic Bcl-xS (BH3 and BH4). In a previous study, we reported the identification of Bcl-xAK, a novel Bcl-2 protein with a unique BH domain structure (BH2 and BH4), and we showed that its overexpression triggered apoptosisin human melanoma cells. Here, we investigated the mechanism of apoptosis induction by Bcl-xAK. A replication-deficient, tetracycline-regulatable (Tet-Off) adenoviral vector was constructed for its overexpression. With the strong expression of Bcl-xAK after infection and tetracycline withdrawal, apoptosis was efficiently induced in human melanoma cell lines demonstrated by DNA fragmentation analysisand identification of sub-G1 cells. Also efficient activation of the caspase cascade(caspase-3, -6, -7 and 8) was found, unlike to the situation previously found after stable and transient plasmid transfection. By examining the involvement of otherBcl-2 proteins, we observed that both Bax and Bak activity contributed toBcl-xAK-mediated induction of apoptosis, whereas overexpression of Bcl-2 largely blocked apoptosis by Bcl-xAK. These results elucidate molecular mechanisms of Bcl-xAK-induced apoptosis. Future studies will further clarify the proapoptotic pathways which are of particular interest for melanoma as they are not related to theBH3 domain, unlike to other proapoptotic Bcl-2 proteins.

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SOX10 but not BRN2 is required for nestin expression in human melanoma cells

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Nestin is an intermediate filament protein and a marker of neuroectodermal stemcells indicating multi-potentiality and regenerative capability. In melanoma tissues, nestin re-expression was demonstrated and correlated with tumour progression.Activation of the nestin neural enhancer was shown to be dependent on the binding of class III POU transcription factors, among which Brain-2 (BRN2, POU3F2) was suggested to play a key role. BRN2 expression was demonstrated in melanocyte precursors and correlated inversely with differentiation. BRN2 was also found in melanoma and implicated in control of proliferation. However, the effector genes ofBRN2 remain unknown and it is not clear if BRN2 regulates nestin expression in melanoma. We found nestin and BRN2 mRNA in almost all of 13 analyzed melanoma cell lines of different progression stages, but expression levels of nestin did not correlate with expression levels of BRN2. Protein expression of nestin was detected in 11/13 and BRN2 in 7/13 melanoma cell lines independent of progression stage. Downregulation of BRN2 by small interfering (si) RNA did not alter nestin expression in melanoma cells. However, inhibition of BRN2 led to a decreased migratory ability of the melanoma cells, which was also observed after siRNA-mediated downregulation of nestin. POU proteins, such as BRN2, commonly cooperate with SOX proteins by binding toa nearby DNA site for their action. Since SOX10 has been shown to interact withBRN2, SOX10 was inhibited in melanoma cells. This led to decreased levels of BRN2and of nestin. Thus, SOX10 but not BRN2 seems to be required for nestin expression in human melanoma cells.

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Firm adhesion of melanoma cells to endothelial cell derived ultra large von Willebrand factor fibres under shear flow conditions

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Tumour endothelial crosstalk is the basis of efficient tumour cell extravasation, a pivotalstep in hematogenous metastasis. Melanoma cell supernatant induced PAR1activation is - at least partially - mediated by tumour secreted MMP-1. Endothelial cell activation leads to a prompt luminal secretion of ultra large von Willebrand factor (VWF) fibres, P-selectin and IL-8 using a microfluidic device mimicking *in vivo* blood flow. Our data indicate that this haemostatic active VWF released by endothelial cells(EC) and immobilized at the vessel wall occurs within seconds upon melanoma cell exposure. Surprisingly, high molecular weight multimers of VWF were found to for melongated fibre-like structures (>200 μm in length) on the EC cell surface. After application of platelet-poor plasma during a 5 min stimulation time cell surface attached platelets were found to be almost exclusively sticking to VWF fibres. Furthermore, these VWF fibres are capable to anchor melanoma cells under flow conditions. Therefore, luminal immobilized VWF network plays a pivotal role in firm adhesion of melanoma cells to the vessel wall under shear flow conditions. This report provides evidence for a new concept of efficient and instantaneous tumour-endothelial communication via a functional MMP-1/PAR1 axis. Melanoma cell derived MMP-1 canonically cleaves endothelial expressed PAR1 and apparently paves the metastatic path generating a prothrombotic, proinflammatory and cella dhesive endothelial surface. Moreover, we show that von Willebrand factor firmly binds melanoma cells under high shear flow conditions and therefore may play apivotal role in tumour progression.

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Tumour immune escape by the loss of homeostatic chemokine expression

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The novel keratinocyte-specific chemokine CCL27 plays a critical role in the organization of skin-associated immune responses by regulating T cell-homing under homeostatic and inflammatory conditions. Here we demonstrate that human keratinocyte-derived skin tumours may evade T cell-mediated anti-tumour immune responses by down-regulating the expression of CCL27 through the activation ofEGFR-Ras-MAPK-signalling pathways. Compared to healthy skin, CCL27 mRNA and protein expression was progressively lost in transformed keratinocytes of actinickeratoses, basal and squamous cell carcinomas. *In vivo*, precancerous skin lesions as well as cutaneous carcinomas showed significantly elevated levels of phosphorylated ERK compared to normal skin suggesting the activation of EGFR-Ras signalling pathways in keratinocyte-derived malignancies. *In vitro*, exogenous stimulation of the EGFR-Ras signalling pathway through EGF or transfection of the dominant active form of the Ras oncogene (H-RasV12) suppressed while an EGFR-tyrosine kinase inhibitor increased CCL27 mRNA and protein production in keratinocytes. In mice, neutralization of CCL27 led to decreased leukocyte recruitment to cutaneous tumour sites and significantly enhanced primary tumour growth. Collectively, our data identify a novel mechanism of skin tumours to evade host anti-tumour immune responses.

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Contribution of Rac1 to the formation of malignant sweat gland tumours in mice

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The small GTP binding protein Rac1 can regulate the proliferative potential as well asadhesion and migration of epidermal keratinocytes. It plays an important role inepidermal wound healing and the morphogenesis of hair follicles. Over- expression and constitutive activation of Rac1 have been described in squamous cell carcinomas of the skin and mucosa. We have asked whether activation of Rac1 was sufficient to cause progression of benign skin lesions to invasive tumours. Mice with epidermis specific expression of delta n LEF, a truncated version of the transcription factor LEF (K14ΔnLEF transgenic mice), develop sebaceous adenomas of the skin. We have activated Rac1 signalling in these benign skin lesions by crossing mice expressing a mutated version of Rac1, L61Rac1, as a transgene under the control ofthe keratin 14 promoter (K14Rac1L61 mice) to K14ΔnLEF transgenic mice. ResultingK14ΔnLEF/K14Rac1L61 double transgenic mice developed, in addition to sebaceous adenomas, fast growing, poorly differentiated, invasive skin tumours at low frequency. These tumours stained positive for keratin 14 but only focally expressed markers of squamous epithelial differentiation. Instead, expression of markers of sebaceous differentiation could be demonstrated. These tumours did not develop in K14ΔnLEF single transgenic mice or in K14Rac1L61 mice. We conclude that constitutive activation of Rac1 contributes to the progression of sebaceous adenomas to invasive sebaceous tumours of the skin.

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ADAM-9 mediates heterotypic interactions of melanoma cells andfibroblasts

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Invasion and metastasis of human tumours are multistep processes requiring cell-cell and cell-matrix interactions with cellular and structural constituents of the host stroma. During these processes, tumour cells modify the extracellular environment either directly, or indirectly through the interaction with stromal cells, and produce a microenvironment suitable for their growth and migration. In the recent years the family of ADAMs proteases (A Disintegrin And Metalloproteinase) has acquired an important role in tumour invasion and their multifunctionality with proteolytic and adhesive functions has suggested a pivotal role for these proteins in a variety of physiological and pathological conditions. We have previously shown that ADAM-9 is strongly expressed in human melanoma at the tumour-stroma border where direct or indirect interactions between tumour cells and fibroblasts occur. To analyse the role of ADAM-9 in the interaction of melanoma cells and stromal fibroblasts, we have produced in eukaryotic system there combinant disintegrin-cysteine domain. Melanoma cells of various invasive abilities and human primary fibroblasts from different donors adhered to immobilized recombinant protein in an Mn2+-dependent fashion suggesting that integrins mediate this process. Inhibition studies showed that adhesion was mediated by beta-1integrin receptors and independent of the RGD recognition motif. Interestingly, stimulation of high but not of low invasive melanoma cells with soluble recombinant disintegrin-cysteine domain resulted in an enhanced de novo synthesis of MMP-2. To investigate the role of ADAM-9 in mediating cell-cell interactions we used the soluble DisCys-His domain to interfere with melanoma cells adhesion to fibroblasts. In this system we obtained a 40% inhibition of cell-cell adhesion. In addition, downregulation of ADAM-9 protein in melanoma cells by siRNA to 50% was associated with a 30% decrease of adhesion to fibroblasts. Moreover, ADAM-9 depletion infibroblast and its silencing in melanoma cells, resulted in a reduction of 70% of total heterotypic cell adhesion. In summary, these results suggest that up-regulation of ADAM-9 expression inhuman melanoma might play an important role in mediating cell-cell contacts and contributes to proteolytic activities in the tumour microenvironment.

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IL-31 receptor alpha expression in epidermal keratinocytes is modulated by cell differentiation

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Recently, interleukin (IL)-31 has been identified as a 4-helix bundle cytokine that is expressed by activated T cells preferentially by T-cells skewed toward a TH2-typecytokine profile. While an enhanced expression of IL-31Rα?has been described in inflammatory skin diseases, the influence of cell differentiation on the expression ofthe IL-31 receptor in skin cells was largely unknown. Therefore we analyzed the effects of the cellular differentiation state on epidermal mRNA and protein levels ofIL-31Rα?in culture conditions when epidermal cultures exhibit predominantly basal, spinous, and granular cell phenotypes. Interestingly, IL-31Rα?mRNA completely disappeared during the process of differentiation and already at the transition from basal to spinous phenotype (d5) hardly any mRNA was detectable by quantitative RT-PCR. Expression of OSMRβ?mRNA was decreased on day 5 but not significantly altered during the late phases of keratinocyte differentiation. Using an IL-31Rαspecific antibody we confirmed a strong down-regulation of the receptor at the protein level when differentiation of NHEK was induced by culturing the cells in growth medium containing 1.4 mM Ca2+ for more than 3 days. Protein expression was not traceable in terminal differentiated keratinocytes (day 15) resembling a granular cell phenotype. Additionally, we found no IL-31Rα?expression in dermal fibroblasts or primary human melanocytes. Monocytes stimulated with 1000 U/ml IFNγ?for 48 h revealed a strong upregulation of IL-31Rα?expression. Loss of receptor expression in late-stage keratinocytes consequently led to a loss of cytokine responsiveness: while proliferating keratinocytes respond to IL-31 treatment with increased tyrosine phosphorylation of STAT3, we could not detect any STAT activation in differentiated keratinocytes. In contrast, stimulation with OSM resulted in STAT3 tyrosine phosphorylation irrespective of the differentiation state which indicates that differentiated keratinocytes do respond to cytokine treatment in general. Therefore *in vitro* studies analyzing the effects of the novel cytokine IL-31 on epidermal keratinocytes should consider the variation of IL-31Rα?expression which depends on the status of cellular differentiation.

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Expression of matrix metalloproteinases, cytokines and connexins in diabetic and non-diabetic human keratinocytes before and after transplantation

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Wound healing is known to require a well-organized balance of numerous factors, e.g. cytokines, matrix metalloproteinases (MMPs) and their inhibitors, as well as direct cell-cell communication (connexins, Cx). Disruption of this balance may lead to the formation of chronic wounds such as diabetic foot ulcers. The transplantation ofautologous keratinocytes is a promising therapy for diabetic foot ulcers, however little is known about what happens on molecular level. Therefore, we intended to characterize keratinocytes from diabetic and non-diabetic origin before and after transplantation. We isolated human keratinocytes from diabetic and non-diabeticorigin and transplanted them into an ex-vivo wound healing model. To characterize the keratinocytes we investigated mRNA expression of MMP1, 2 and 9, TIMP1 and 2,IL-1ß, TNFa, Cx26 and Cx43 by using quantitative PCR and, for Cx, localisation by using immunofluorescence microscopy. We found no significantly increased expression of the molecules investigated in cultured keratinocytes from diabetic compared to non-diabetic origin, even though there were significant differences forMMP-2, IL1-ß and TNFa in skin biopsies. Expression of IL-1ß was significantly lower in keratinocytes from diabetic origin. Interestingly in the course of wound healing, differences in the dynamics of expression of MMP-1, IL-1ß and Cx43 were observed. Our results suggest that keratinocytes from diabetic origin are as capable for transplantation into chronic wounds as keratinocytes from healthy origin at the starting point of therapy. However, differences in expression dynamics later on might reflect the systemic influence of diabetes resulting in a memory of the transplanted keratinocytes.

P248 (V03)

Cancer-retina antigens as paraneoplastic antigens in melanoma-associated retinopathy

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Melanoma-associated retinopathy (MAR) is a rare paraneoplastic neurological syndrome characterized by retinal degeneration in melanoma patients. The main photoreceptor proteins have previously been found to be expressed as cancer-retina antigens in melanoma. Here, we present evidence that cancer-retina antigens can function as paraneoplastic antigens in MAR. Expression of these antigens and auto-antibodies against arrestin and transducin were detected in MAR patients. To investigate this phenomenon comprehensively, we used ret transgenic mice which develop spontaneously skin melanoma as a murine model for MAR. Retinal degeneration in tumour-bearing mice was frequently detected by electroretinogram and eye histology. We observed degeneration of photoreceptor cells, bipolar cells and pigment epithelium as well as retinal detachment. In most cases these defects were combined. Cancer-retina antigens were expressed in tumours of these mice and autoantibodies against arrestin were revealed in some sera. Adoptive transfer of splenocytes and sera from tumour-bearing, but not from healthy mice, into wild-type mice led to the induction of retinopathy in 25% of the animals. We suggest that MAR can be mediated by humoral and/or cellular immune responses against a number of cancer-retina antigens, and may function as paraneoplastic antigens in MAR.

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Isolation and characterisation of human melanocytes for clinical use

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Despite significant progresses in tissue engineering and cell biology there is still an unmet need for treating patients suffering from vitiligo. The disease is characterized by depletion of melanocytes in more or less extended areas of the skin. In correlation to the extent and localisation of the depigmented areas patients ask for medical treatment. Today the treatment regimen includes dermabrasio followed by transplantation of a mixture of keratinocytes and melanocytes taken from biopsies ofnonaffected areas. The transplantation is supported by treatment with an excimer laser. However, this modern treatment is restricted to few centres, <5 atleast in Germany. The hair follicle bulge area is an abundant easily accessible source of actively growing pluripotent adult stem cells. These cells can be differentiated into various cell lineages, e.g. keratinocytes and melanocytes amongst others. Euroderm GmbHuses ORS (outer root sheath) technology and possesses the technological know-how for generating autologous keratinocytes. The strategic concept of this translational project is to isolate and propagate melanocytes and keratinocytes, preferentially from ORS cells, or alternatively from skin biopsies, in order to achieve sufficient quantities. A mixture of these cells will then be administered to the affected skin of a patient. The technical challenge lies in the definition of optimal culture conditions to foster the growth of melanocyte precursors. Additionally the culture conditions have to meet GMP requirements. The results of these preclinical studies will then be the prerequisite for starting clinical trial (Phase I).

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Activation pattern of MAPK and NF-kappaB pathways in skin cancer and inflammation

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The transcription factor NF-kappaB, most often composed of p50 and p65/RelA, is a crucial mediator of inflammatory processes. Inflammatory stimuli that are capable of activating the catalytic IkappaB kinase 2 (IKK2) result in serine-phosphorylation, ubiquitination and subsequent degradation of IkappaBalpha which allows translocation of NF-kappaB to the nucleus. In addition to degradation of IkappaBalpha, phosphorylation of the transactivating subunit RelA is an essential regulatory step for its transcriptional activity. The three typical elements of a classic MAPK cascade are a MAPK, a kinase that activates the MAP2K and the kinase that activates the MAP2K. The extracellular signal-regulated kinase (ERK), the Jun-Nterminal kinase (JNK) and p38 MAPK are the most common cascades. Up to the level of the MAPKs, the cascades resemble a funnel, as each MAPK can be activated by two MAP2K which in turn are activated by multitudinous MAP3Ks. Both NF-kappaB and the MAPK cascades are involved in inflammatory processes as well as carcinogenesis. In mice lacking skin-specifically NF-kappaB/RelA, we observed a significantly retarded initiation of skin cancer development following treatment with DMBA and TPA, pointing to a key role of RelA in skin carcinogenesis. Staining of sections from human spinocellular carcinoma demonstrated weak phosphorylation of RelA at Ser536, accompanied by weak IKK activation. In contrast phosphorylation atSer276 was strong, accompanied by a respective activation of MSK1 in MPM2positive cells. c-Jun phosphorylation was also clearly visible with a respective phospho-specific antibody, associated with respective JNK1/2 activation. We compared the reaction pattern observed in spinocellular carcinomas with lichenruber, atopic eczema and psoriasis. In contrast to spinocellular carcinomas we detected a strong phosphorylation at Ser536 and its kinase IKK, whereas Ser276was barely visible, despite MSK1 activation. This points to a different function of NF-kappaB/RelA depending on the respective phosphorylated site. Whereas c-Jun was activated in Lichen ruber, this was not the case in atopic eczema and psoriasis. This difference in signal transduction may be a reason why lichen ruber cantransform.

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Viability and proliferation of fibroblasts, keratinocytes and hacat-cells influenced by polihexanide

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Aim: Wound dressings combined with antimicrobial agents are increasingly utilized in the treatment of critical colonized or infected chronic wounds. Povidene-iodine andoctinedine are considered more or less equivalent for disinfecting of acute wounds, whereas polihexanide is regarded first choice for 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. Therefore we investigated the influence of polihexanide on viability and proliferation of three skin connected cell lines.

Methods: Viability and proliferation of human fibroblasts, keratinocytes and HaCaT-cells were investigated via microscopic evaluation of live and dead cells (analySIS®?3.1 Soft Imaging System GmbH, Germany) and by means of the ATPLite™-M kit (Packard Bioscience BV, The Netherlands). To distinguish between live and dead the cells were stained with SYTO-13 and Ethidiumhomodimer-2(Molecular Probes, U.S.). The luminometric ATP assay is based on the detection of light generated by the ATP dependent enzymatic conversion of D-luciferin by luciferase.

Results: A significant increase of the proliferation of human keratinocytes and HaCaT-cells by polihexanide was found (significant versus control: 0.2–2 μg/ml). In these concentrations a slightly positive effect on viability of all cell types could be noted. In higher concentrations a decrease of viability and proliferation was observed dose-dependently.

Conclusions: These *in vitro* results (obtained with two different methods) demonstrate an impressive positive influence of polihexanide on the proliferation of different cell types. The observations are in accordance with *in vivo* studies recently published (Kramer et al. 2004). Therefore 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.

P252

Human dermis-derived cells demonstrate strong features of controlled mesodermal differentiation

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Multiple tissue niches in the human body are now recognized to harbour adult stem cells. Here, we have examined a panel of putative stem cell/progenitor markers on progenitor cells derived from mechanically and enzymatically processed adult human scalp skin, which may serve as a convenient and abundant source of adult human progenitor cell populations. After removal of the epidermis, adherent growing cells ofa purposely heterogenous mixture of intradermal cells (including sweat glands, fibroblasts, and hair follicle-derived cells), were propagated up to 17 passages in serum free or serum-supplemented medium as well as in specific minimal media with defined supplements ('B27 supplement', BIT 9500), cocktails of selected mitogens/peptides, as well as defined growth supports (fibronectin, laminin, orotherwise coated flasks and glass coverslips). The diverse cell populations generated in this manner were then tested for spontaneous differentiation markers by immunocytochemistry and RT-PCR. Interestingly, human skin-derived stem/progenitor cells obtained in this manner exhibit a strong differentiation pattern of mesodermal lineage (e.g. SMA+). This intriguingly differentiation potential of progenitor cells contained in normal, aging human scalp skin (mean age: 50 ± 5 years) warrants systemic follow-up, since it raises the possibility that adult human skin-derived progenitor cells can serve multiple cell-based therapy purposes for regenerative medicine.

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The transcriptional response to distinct growth factors is impaired in Werner syndrome 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 aging 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 inepigenetic regulation.

The transcriptional response of RNA polymerase II to growth factors as determined by microarray analysis is substantially altered in Werner Syndrome cells with a profound lack of stimulation of growth related genes. This together with alterations in Pol I transcription provide a novel mechanism underlying at least in part the severe growth retardation and premature aging in Werner Syndrome patients.

P254

Truncated Cockayne syndrome B protein represses transcription by RNA polymerase I

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Cockayne syndrome represents a premature aging syndrome with atrophy and photosensitivity of the skin, cataract formation and neurode generation. Cockayne Syndrome group B (CSB) protein plays a role both in transcription-coupled DNA repair and transcriptional regulation of RNA polymerase I and II. A truncating mutation of CSB results in Cockayne Syndrome, while complete absence of CSB in UV-sensitivity syndrome does not. Here we addressed the role of the truncated CSB protein and the complete CSB absence on the molecular mechanisms underlying PolI transcription and ribosomal biogenesis impaired in CSB patients. We show that recombinant CSB specifically reconstitutes RNA polymerase I *in vitro* transcription in extracts from CSB mutant cells. CSB stimulates a transcriptional step after initiation complex formation and enhances elongation by RNA polymerase I. We were able to isolate promoter and gene-internal sequences of the rDNA with CSB antibodies in ChIP experiments suggesting a function of CSB in initiation and elongation of Pol Itranscription. Analysis of rDNA transcription in cells from UV-sensitivity syndrome with a null mutation in CSB showed that CSB is not essential for rDNA transcription. In contrast, cells with a truncating mutation of CSB revealed a substantially repressed Pol I transcription. Truncated CSB represses also RNA polymerase I *in vitro* transcription. Here we identify for the first time that only truncated CSB but not the lack of CSB interferes with Pol I transcription with subsequent impairment of ribosomal biosynthesis, which is most likely causal for premature aging and degeneration in CSB patients. These data may also have relevance for mechanisms underlying intrinsic aging.

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Cytokine-induced antimicrobial peptide secretion from keratinocytes is not regulated by intracellular glutathione

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Keratinocytes are able to produce antimicrobial peptides(AMP) constitutively or after appropriate stimulation. It has been shown previously that a stimulation with interleukin 22 (IL-22) and interferon-gamma (IFNγ) are most efficacious in inducing AMP. In psoriasis patients skin infections are prevented by an overexpression of AMP in scales and living epidermal cell layers. Oxidative stress which is present in inflamed tissues is able to profoundly modulate cellular redox systems, mostimportantly the glutathione (GSH) system. A shift in the balance of oxidized and reduced GSH is able to regulate important cellular activation systems such as the NFκB-pathway. The aim of the present study was to investigate whether changes of intracellular GSHmay modulate the production and secretion of AMP in keratinocytes. Normal human keratinocytes or HaCaT-cells were pre-treated with but hionine sulfoximine (BSO) to deplete or with dimethyl fumarate (DMF) to increase intracellular GSH. Thereafter cells were stimulated with IL-22 and IFNγ. The AMP psoriasin (S100A7), human beta-defensin 2 (hBD2) and RNAse 7 were measured in the supernatants by using specific ELISA. Cells without any treatment served as negative, cells stimulated with IL-22/IFNγ?as positive control.

The data shows a strong down-regulation of GSH after BSO- and an up-regulation after DMF-pretreatment. Stimulation with IL-22/IFNγ?upregulated psoriasin and hBD2 but not RNAse 7 protein secretion. Decreasing GSH by BSO- or increasing GSH through DMF-pre-treatment did not lead to a significant modulation of AMP secretion. The results of our study provide evidence that modulation of intracellular GSH which occurs in response to oxidative stress does not lead to changes in cytokine(IL-22/IFNγ)-induced AMP upregulation in keratinocytes.

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Investigation of the vitamin D endocrine system in human sebocytes *in vitro*

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Vitamin D metabolites has been shown to be of high importance for regulation of growth, differentiation and other cellular functions in various tissues. In this study, we have investigated whether sebocytes are target cells for biologically active vitamin Dmetabolites, and whether they possess the enzymatic machinery for the local synthesis and metabolism of the biologically active vitamin D metabolite1,25(OH)2D3. Using real time PCR we detected VDR expression in SZ95 sebocytes. Incubation with 1,25(OH)2D3 resulted in a dose-dependent regulation of cell proliferation (crystal violet dye uptake and MUH techniques), whereas inhibition ofSZ95 sebocyte proliferation (up to 30%) occurred under serum-supplemented and stimulation under serum-free conditions. Moreover, modulation of cell cycle regulation and of apoptosis was detected by flow cytometry. In addition, minorchanges on SZ95 sebocyte lipids (nile red fluorescence assay) but marked inhibition of IL6 and IL8 secretion (ELISA) resulted after incubation of SZ95 sebocytes with1,25(OH)2D3. RNA for vitamin D-25-hydroxylase, 25-hydroxyvitaminD-1a-hydroxylase (1aOHase) and 1,25-dihydroxyvitamin D-24-hydroxylase(24OHase) was detected in SZ95 sebocytes by real time PCR. Expression of VDR and 24OHase was upregulated along with vitamin D analog treatment. Although several other splice variants of 1aOHase were detected by nested touchdown PCR, our findings indicate that the full length product represents the major 1aOHase gene product in SZ95 sebocytes. In conclusion, the vitamin D endocrine system is of high importance for sebocyte function and physiology. SZ95 sebocytes express the VDR and the enzymatic machinery to synthesize and metabolize biologically active vitamin D analogs and represent target cells for biologically active metabolites. Our findings indicate that sebaceous glands represent potential targets for therapy with vitamin Danalogs or for pharmacological modulation of 1,25(OH)2D3 synthesis/metabolism.

P257

Epidermal transplants made from outer-root-sheath keratinocytes retain stem cell properties superior to human epidermis

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To overcome limitations of split-thickness skin grafts, alternative transplantation concepts are based on the use of precursor cells, either from the outer root sheath (ORS, keratinocyte sheets) (EpiDex) or the basal membrane (BM, keratinocyte suspensions). However, their stem cell properties have not yet been characterized. We investigated serial cultures from human keratinocytes derived from the ORS and BM, respectively, for their clonogenic potential and compared expression of keratinocyte precursor cell markers to those of human skin. Multilayered sheets were reconstructed from ORS-derived keratinocytes, isolated and expanded from human anagen follicles. By immunohistologic staining expression of CD34, CD29, CK15, follistatin, CD71, nestin, and p63 as markers for stem (SC) and transient amplifying cells (TAC), respectively, was detected, and was compared to the expression in human skin obtained from reconstructive surgery of age-matched patients. From serial cultures of ORS- as well as BM- derived keratinocytes cytospins were prepared and percent expression of precursor cell as well as differentiation markers was determined. To assess the proliferative capacity and to classify theclonal type clonality assays were performed from parallel cultures. Multilayered epidermal transplants made from ORS-derived keratinocytes showed an expression pattern of SC and TAC markers comparable to that of human epidermis. Whereas expression of CD29, CK15, and follistatin was confined to the basal membrane, CD71 and p63 were also expressed suprabasally. Percent expression ofCK15, follistatin, and CD29 was higher in ORS-derived compared to BM-derived keratinocytes. Whereas truncated hair bulbs led to lower CK15 and higher involucrin expressivity, CK15 was almost completely lost during serial culture. Percent amount of holoclones (SC-clones) was higher and of paraclones lower in ORS-compared to BM-derived keratinocytes. Based on these findings ORS-derived keratinocytes seem to be a favourable source for reconstruction of epidermal transplants delivering precursor keratinocytes with highly proliferative capacity. For this, integrity of the hair follicle seems to be essential. To enhance the yield of transplantable cells, factors should be defined to preserve precursor cells under prolonged culture conditions.

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

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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 as well as in the establishment of cell polarity. Therefore we investigated expression and localization of TJ associated proteins, including the cell polarity complex aPKC/Par3/Par6 in developing and mature epidermis as well as in primary keratinocytes of wild type(WT) and CD44 knock-out mice. In addition, we investigated TJ functionality and upstream regulators of TJ assembly, e.g. Tiam1. We observed a downregulation of TJ-transmembrane protein Claudin 4 on protein and RNA level and less intense cell–cell junction staining in CD44 knock-out compared to WT mice in several embryonal stages. In addition, we observed a downregulation of Par3 at the cellborders. Transepithelial resistance, a measure for TJ functionality, was altered in cultured keratinocytes of CD44 knock-out mice compared to WT mice. This study strongly suggests an influence of the transmembrane glycoprotein CD44 on TJ assembly and function.

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Kindlin-1 Regulates proliferation, polarity and motility of epidermal keratinocytes through Integrin and EGF mediated signalling pathways

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Kindlin-1 is a focal adhesion phosphoprotein mainly expressed in skin keratinocytes. Mutations in the kindlin-1 gene, KIND1, cause the Kindler syndrome (KS), a human disorder characterized by skin blistering, progressive poikiloderma and mild photosensitivity. We showed previously *in vivo* and *in vitro*, that kindlin-1 deficient keratinocytes exhibited reduced proliferation and adhesion, as well as impaired directed migration and defective polarization. Here we demonstrated that KS keratinocytes displayed altered distribution of the actin cytoskeleton and focal adhesions, corresponding to their multipolar shape described before. Furthermore, to investigate the role of kindlin-1 in proliferation, adhesion, migration and polarization, we screened pathways involved in these cellular processes using primary kindlin-1deficient and normal keratinocytes. Kindlin-1 deficiency led to reduction of RhoA and Cdc 42 pools, and to a 2.2 fold increase of Rac-1 activity. Accordingly, the phosphorylation of the downstream mediator of Rac-1 cofilin was 40 % reduced, reflecting higher cofilin activity. Phosphorylation of MAPK Erk1 and Erk2 was 60 %decreased, while phosphorylated JNK and the total amount of paxillin were increased. Since EGF plays an important role in these pathways, we treated normal human keratinocytes with 10 ng/ml EGF and observed a strong induction of kindlin-1phosphorylation, suggesting that EGF activates kindlin-1 through phosphorylation.Kindlin-1 interaction partners were identified by co-immunoprecipitation experiments:migfillin, alpha-actinin, focal adhesion and beta1 integrin co-precipitated withkindlin-1. Taken together, our results place kindlin-1 at the crossroad between beta1integrin and EGF signalling pathways, involved in the regulation of the cell shape, migration and proliferation by modulation of RhoGTPases and MAP kinases.

P260 (V17)

Complex ageing phenotype in mice with conditional deficiency form itochondrial manganese superoxide dismutase in the connective tissue

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Connective tissue consisting of resident mesenchyme-derived cells (e.g. fibroblasts)and extracellular matrix proteins is essential for organ function and homoeostasis. The free radical theory of ageing postulates that the production of mitochondrial reactive oxygen species is the major determinant of ageing and life span. Its role in ageing of the connective tissue has not yet been established, even though the incidence of ageing-related disorders, like arteriosclerosis, wrinkle formation and impaired wound-healing of the skin, osteoarthrosis and osteoporosis is high and mainly occurs in connective tissue rich organs. We have now addressed this question experimentally by creating mice with conditional deficiency of the mitochondrialmanganese superoxide dismutase in fibroblasts and other mesenchyme-derived cells of connective tissues throughout all organs. Here we show that homozygous deficiency for the manganese superoxide dismutase with lack of superoxide aniondetoxification results in structural changes of mitochondria and a diminished capacityof homozygous MnSOD deficient fibroblasts to maintain and organise the surrounding extracellular matrix. These connective tissue specific alterations are associated with reduced lifespan and premature onset of ageing-related phenotypessuch as weight loss, reduced subcutaneous fat, tissue kyphosis (curvature of the spine), osteoporosis, motodegeneration and reduced fertility. Our results provide a causative link between the lack of manganese superoxide dismutase in residentmesenchyme-derived cells of connective tissues and severe ageing phenotypes of many organs in mammals.

P261

Alpha-tocopherol derivatives of amino acids protect human keratinocytes against hyperosmotic stress

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Epidermal keratinocytes evolved mechanisms to adapt to changing osmotic conditions. It is known that osmotically perturbed keratinocytes express specific transporter systems for the uptake of compatible organic osmolytes mediating cellular protection and cell volume homeostasis. In this study we show that amino acids are able to restore the decrease in cell proliferation of hyperosmotically stressed HaCaT keratinocytes functioning as compatible osmolytes. However, because of their polar characteristics amino acidsare not able to penetrate easily into the deeper viable skin layers after topical application. Therefore we developed a prodrug concept coupling lipophilic moieties to various amino acids by esterification of the polar carboxylic acid function in order to enhance skin penetration. Amongst others we chose alpha-tocopherol (vitamin E) asa lipophilic moiety due to its known beneficial effects as one of the most important lipid soluble antioxidants. The alpha-tocopherol derivatives DL-alpha-tocopheryl-(mono)-glycinate and DL-alpha-tocopheryl-(mono)-prolinate showed similar osmoprotective effects under salt stress than glycine and proline, respectively. Our *in vitro* data indicate that lipophilic derivatives of amino acids may improve the hydration state especially of dried skin. The lipophilic moiety acts as a carrier for the enhancement of skin penetration after topical application. This concept may be useful for the treatment of many clinical and cosmetic indications associated with dry skin such as atopic or aged skin.

P262

Ontogeny of langerhans cells in prenatal human skin

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Despite a considerable number of studies, the precise phenotype of the epidermal Langerhans cell (LC) precursor has not yet been determined in humans. This elusive LC precursor is believed to migrate into the embryonic skin and to show a primitive surface marker pattern that subsequently develops into the profile of adult LCs. Thus, the study of the LC ontogeny could provide valuable hints about the origin and the development of these cells. Using confocal laser scanning microscopy and flowcytometry, we evaluated the acquisition of the LC marker profile in cryostat sections and single cell suspensions of embryonic and fetal skin. At an estimated gestation alage (EGA) of 9 weeks, some CD45+HLA-DR+ cells in the epidermis and dermis expressed CD1c but not LC-specific molecules, such as CD207 and CD1a. By 11 weeks EGA, a minority of CD45+HLA-DR+CD1c+ skin cells began to expressCD207, which in skin sections was exclusively found in the epidermis.CD45+HLA-DR+CD207+ cells started to acquire CD1a at 12 weeks EGA.CD45+HLA-DR+CD207-CD1a+ cells, representing a subset of dermal dendritic cells, were not found in the skin before midgestation. The phenotypic characteristics of LCs are strikingly influenced by cytokines. In this respect TGF-β?plays an important role in the development and the differentiation of LCs. To test, whether the acquisition of CD207 and CD1a on LC precursors correlates with changes in the production of TGF-β, we immunohistochemically stained embryonic and fetal skin sections. Notably, we found no TGF-β?reactivity at 8 weeks EGA. However, one week later the periderm and the intermediate epidermal cell layer showed a faint TGF-β?expression. With advancing gestational age, the staining intensity of TGF-β?in all epidermal cell layers increased with the exception of the basal layer, which remained negative at all time points. Collectively, these data suggest that CD45+HLA-DR+CD1c+CD207-CD1a- LC precursors sequentially acquire CD207 and CD1a in the epidermis, probably as a consequence of the higher TGF-β?concentration in the epidermal microenvironment.

P263

MAPK independent impairment of T cell responses by Sorafenib

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Sorafenib, originally developed as Raf1 inhibitor, is currently widely tested for thetreatment of different cancers either alone or in combination therapy. To investigatepossible side effects of Sorafenib on immune responses we analyzed its effect on Tcells *in vitro*. To this end, we demonstrate by flow cytometry analyses that the presence of Sorafenib concentrations comparable to the observed plasmaconcentrations in treated cancer patients impairs the activation of T cells. Accordingly, Sorafenib impairs specific immune responses; ELISPOT data of PBLs obtained from vaccinated melanoma patients demonstrate markedly diminished survivin specific immune responses in the presence of Sorafenib. Surprisingly, the observed inhibition of T cell activation was not associated with reduced ERK phosphorylation. In fact, phospho-ERK levels and phospho-MEK were transiently elevated in the presence of Sorafenib indicating that the suppression of T cellfunction was not mediated by effecting RAF. In conclusion, our data demonstrate that T cell function is sensitive towards Sorafenibin an MAPK independent fashion. This observation has important implications for the use of Sorafenib as therapy for immunogenic cancers.

P264 (V26)

Pathogenesis of spongiosis: suprabasal keratinocyte-apoptosis and protection of basal keratinocytes by cFLIP

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In healthy skin epidermal keratinocytes display low levels of Fas receptor (CD95) and are highly resistant to CD95-mediated killing. However, during the elicitation phase of eczematous dermatitis, dermis- and epidermis-infiltrating, IFN-γ?secreting activatedCD45RO+ T-cells pave the way to keratinocytes-apoptosis, in part by promotion ofCD95 expression on keratinocytes. CD95-induced apoptosis of keratinocytes mediated by T-cells either through direct CD95L/CD95 interactions or paracrine secretion of soluble CD95L has been proposed as a major mechanism for spongiosis formation, the histopathological hallmark of eczematous dermatitis. Caspase-8 and/or caspase-10 are critically required for CD95-mediated apoptosis in keratinocytes, and these initiator caspases are inhibited by the catalytically inactivecaspase-8/-10 homologue cFLIP long (cFLIPL). In order to test the impact of inhibition of upstream caspase activation in our *in vitro* eczema-model, we used the keratinocyte cell line HaCaT that was retrovirally transduced with cFLIPL. These cells were then cocultured for 48 h with anti-CD2/-CD3/-CD28 mAb activated, purified human CD4+ T-cells from peripheral venous blood. While vector carrying keratinocytes efficiently died by apoptosis in HaCaT/CD4+ T-cell cocultures, cFLIPL-expressing keratinocytes were largely protected against T-cell mediated apoptosis. Moreover, *in situ* correlation of cFLIP protein expression and keratinocyte apoptosis in eczematous dermatitis revealed a highly restricted expression of cFLIP in basal keratinocytes, whereas active caspase-3 was mainly detected in suprabasal epidermal layers. Therefore, this pattern of cFLIP expression in human epidermis may explain the spatial localisation of spongiosis in eczematous dermatitis insuprabasal layers. Taken together, the apoptosis inhibitor cFLIP may attribute to the rapid regression of spongiosis in an uncomplicated eczema – where no secondary changes or new attacks occur – by ensuring the survival of basal keratinocytes.

P265 (V27)

Keratinocyte-specific knockout of the Tfam protein elucidates an essential role of the mitochondrial respiratory chain for skin differentiation *in vivo*

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Mitochondrial DNA (mtDNA) encodes 13 subunits of the electron transport chain(ETC). A lack of mtDNA in the epidermis, leading to a non-functional ETC, could lead to a reduction of oxidative stress, possibly influencing physiological processes. To better understand the functional significance of the ETC in these processes, we have selectively deleted ETC activity in the epidermis, as previously reported, by crossing mice expressing Cre-recombinase, driven by the human Keratin 14 promoter, with mice having the Tfam gene flanked by loxP sites. Nuclear encoded Tfam controls transcription and replication of mtDNA. Surprisingly, despite loss of ETC activity, interfollicular epidermis appears to undergo normal differentiation, and is even hyperkeratotic, since the number of proliferating (Ki67+) epidermal keratinocytes in k.o. epidermis is significantly higher compared to wildtype (wt) mice. However, the dermis and, most pronouncedly, the subcutis of k.o. mice displays massive atrophy.At the same stages of development, k.o. mouse subcutis is significantly thinner thanthat of wt mice. Furthermore, k.o. mice show major growth and pigmentation defects in skin appendages. Hair follicle morphogenesis of k.o. animals is incomplete and shows premature catagen induction. The total number of hair follicles is decreased in Tfam k.o. mice and, as a sign of premature involution, follicular dermal papillae are condensed, and mast cells accumulate. Instead of normal melanin banding patterns of the hair shaft and the usual intraepithelial melanin distribution, prominent ectopicintrafollicular melanin granule location and melanin incontinence are observed. Furthermore, tongue epithelium of k.o. animals is disordered and tongue papillae show progressive postnatal degeneration. The resulting tongue ulcerations may prevent proper feeding, which could be the main cause of premature mutant death by day 9. These results point out an unexpectedly complex role of the ETC in skin biology *in vivo*, since adequate ETC activity is required for normal skin architecture and function, including keratinocyte proliferation, hair follicle morphogenesis and pigmentation.

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Purification and characterization of vaspin in human skin – a new epidermal serine protease inhibitor

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Proteolytic degradation of extracellular proteins plays a crucial role in the physiological fdetachment of corneocytes from human stratum corneum. The presence of protease inhibitors regulates their proteolytic activity and contributes to the integrity and protective function of the skin. Their importance in diseases has been revealed impressively in principal in Netherton's disease, where the Kazal-type protease inhibitor LEKTI is absent. We speculate that decreased expression ofprotease inhibitors in human skin are involved in inflammatory skin diseases likeeczema and that enhanced expression leads to thickening of the cornified envelopeas seen in plantar callus. Therefore we analyzed human callus for the presence of protease inhibitors, which might contribute to the stratum corneum architecture. After purification by high performance liquid chromatography (HPLC) we identified by Nano-Electrospray-Ionisation Mass Spectrometry (ESI-MS) and MS/MS-sequenceanalyses the serine protease inhibitor (SERPIN) A12, also named vaspin, in the extracts of human callus. Vaspin was previously only described as a visceral adiposetissue-derived serine protease inhibitor with the function of a insulin-sensitizing adipocytokine in obesity. Primary cultured keratinocytes exhibited vaspin mRNA-expression by RT-PCR after a four-day-long pretreatment with 1.7 mM calcium to induce differentiation of keratinocytes. Immunohistochemical analysis of paraffin-embedded skin samples revealed vaspin expression at the apical part of the epidermis and the stratum corneum. The expression of vaspin was observed atvarious localizations of healthy human skin including face, trunk as well as palmarand plantar localizations. Confocal Microscopy revealed vaspin expression in close localization to the serine protease kallikrein 5. In conclusion we identify the protease inhibitor vaspin in human skin. Our finding that keratinocytes express vaspin suggests a new physiological role for this protease inhibitor than the previously described function in adipose tissue. We speculate that vaspin contributes to the epidermal barrier function of healthy human skin.

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Overexpression of the putative ubiquitin c-terminal hydrolase UCH478 results in senescence in human dermal fibroblasts

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Premature ageing of the skin is a prominent side effect of psoralen plus UVA (PUVA)photochemotherapy used for inflammatory skin disorders. Following PUVA-treatment fibroblasts reveal long-term growth arrest, increased expression of interstitial collagenase and senescence associated ?-galactosidase reminiscent of replicative senescence. Since the molecular basis of the functional and morphological changesis unknown, we applied subtractive hybridization to isolate differentially expressed genes. We isolated a full length cDNA of a transcript, named UCH478, with sixfold induction after PUVA by 5#- and 3#-RACE (Rapid Amplification of cDNA Ends)resembling the sequence of the putative ubiquitin specific protease USP53. Expression of UCH478 is increased on mRNA and protein level in PUVA-treated and in replicative senescent fibroblasts. Stable overexpression in human dermal fibroblasts resulted in a premature senescent phenotype with enlarged cell size, growth arrest, expression of senescence associated β-galactosidase and of matrixmetalloproteinase-1, and continuously increased levels of reactive oxygen species. To get insight into the role of UCH478 in cellular senescence and to identify putative UCH478 dependent pathways, a Yeast two-Hybrid analysis was performed. The screen identified tumour necrosis factor receptor associated factor-2 (TRAF2) andTBC1D20, a gene with sequence homology to the tre-2 oncogene, mitosis regulators BUB and cdc16, as binding partners of UCH478. In silico analysis revealed that onlyTBC1D20 is evolutionary conserved. Since TRAF2 is an adapter protein and signal transducer involved in the age-dependently and redox-regulated NFκB signalling pathway we focused on the analysis of TRAF2. As deubiquitination enzymes are involved in growth and differentiation, cell cycle progression, and signal transduction, the analysis of UCH478 and its signalling pathways may promise useful in sights into the role of deubiquitination in cellular senescence and degenerative disorders.

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Expression of metabolic systems in a newly developed full-thickness skin equivalent

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Reconstructed human skin equivalents gain importance as animal-free alternative to assess toxicological effects and the efficacy of raw materials, cosmetics and pharmaceuticals. We developed a multilayered human full-thickness skin equivalent consisting of a fully stratified epidermis and a dermis rich in extracellular matrix (ECM). In order to show resemblance to native skin we analyzed the expression of two separate enzyme systems: the ECM-degrading matrix metalloproteinases (MMPs) and the xenobiotic phase I and II enzymes. In human skin both dermal collagen fibres and the elastic fibers are subjected to degradation and rearrangement, eventually leading to skin ageing, especially under stress conditions. Responsible for those reactions are the matrix metalloproteinases. We detected MMPs 1-3 and 9 at a basal level in the cell culture medium of the skin equivalents, either in their inactive or active form. The secretion of all 4 MMP smarkedly increased after UVA irradiation indicating that these enzymes are induciblein our *in vitro*-system comparable to native human skin. The MMPs could also be detected immunohistologically in the cells of the skin equivalent. The xenobiotic metabolism in the skin is essential for the assessment of toxicological effects because many potentially genotoxic substances are biotransformed into the ultimate mutagenic form by endogenous enzymes, e.g. CYP450s. In order to elucidate the xenometabolic properties we analyzed the expression of several xenobiotically relevant enzymes in the skin equivalent and an epidermal model. Gene expression studies of phase I and II enzymes indicated that most of the genes were expressed at a basal level in both models. In addition upregulation of CYP1A1 and1B1 after treatment with beta-naphthoflavone occured similarly to native skin. Further approaches comprise the measuring of phase I and II enzyme activities. Our results demonstrate that the full-thickness skin model expresses two sets of enzymes. The presence of MMPs and several phase I and II enzymes corroborate the high grade of similarity between the bioartificial skin equivalent and the native human skin. Therefore our model represents a promising tool to study questions of toxicology and efficacy related to skin *in vitro*.

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Downregulation of Cx43 results in accelerated wound healing, enhanced keratinocyte proliferation and fibroblast migration in higher mammals

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Connexins are transmembrane proteins that form Gap Junctions (GJ), communicating channels that allow the exchange of small molecules, e.g. metabolites and second messengers, between adjacent cells. GJ 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 downregulated during early wound healing at the wound margin and in regenerating epidermis. The fact that no downregulation takes place at the margins of chronic wounds implicates that the downregulation of Cx43 is important for an effective wound closure. To further elucidate the role of Cx43 we investigated the influence of Cx43 mimeticpeptides (gp27) which result in a disruption of gap junctional intercellular communication in porcine *ex vivo* wound healing models and cultured human keratinocytes and fibroblasts. Treatment of wound healing models with gp27 resulted in a significantly accelerated wound healing compared to the controls. The treated models also showed a higher number of proliferative keratinocytes in there generating epidermis and at the wound margin. Confluent fibroblast cultures that were treated with gp27 prior to a scrape wound assay showed an enhanced migration that resulted in a faster wound closure. These data suggest that, by reducing the gap junctional communication by applying Cx-mimetic peptides, the natural downregulation of Cx43 can be enhanced, resulting in accelerated wound healing, enhanced keratinocyte proliferation and fibroblast migration.

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Alpha-melanocyte-stimulating is a powerful agent in the bleomycin model of collagen synthesis and fibrosis

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Identification of novel treatments for the prevention and treatment of fibrosclerotic diseases is a major therapeutic challenge in medicine. Using the well established bleomycin (BLM) model we investigated the *in vitro* and *in vivo* effects of the neuropeptide alpha-melanocyte-stimulating hormone (alpha-MSH) on collagen synthesis and fibrosis. Alpha-MSH *in vitro* significantly suppressed collagen type Iand III synthesis in human dermal fibroblasts exposed to BLM. A modulatory effect on BLM-induced collagen expression was likewise observed with adrenocorticotropin, with the superpotent MSH analogue NDP-alpha-MSH, and for skolin suggesting that the cAMP pathway is crucially involved in the observed effect of alpha-MSH. When looking further at the signal transduction events neither BLM nor alpha-MSH affected the Smad pathway, a key signalling pathway orchestrating collagen expression induced by the profibrotic cytokine transforming growth factor-beta1. However, N-acetylcysteine and MnCpx3 significantly suppressed BLM-induced collagen expression indicating that reactive oxygen species (ROS) play a central role in the action of BLM. Alpha-MSH strongly suppressed BLM-induced generation of ROS and significantly upregulated the expression of heme oxygenase-1 (HO-1) andsuperoxid-dismutase-2 (SOD2), two redox-sensitive enzymes involved in suppression of fibrosis and ROS detoxification. To assess the *in vivo* relevance of these findings we evaluated the effect of alpha-MSH in a mouse model of scleroderma induced by injections with BLM for 21 days. Alpha-MSH reduced cutaneous collagen mRNA and protein levels as well as tissue fibrosis and this salutary effect was paralleled by increased *in situ* expression of HO-1 and SOD2. Our data suggest that alpha-MSH and related peptides may become novel therapeutic agents for the treatment of human fibrotic/sclerotic disorders. At the mechanistic level alpha-MSH appears to exert these modulatory effects by reducing oxidativestress.

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Regulation of Nrf1/2 expression in skin cells – a novel functional facet of alpha-melanocyte-stimulating hormone

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Human skin is constantly exposed to ultraviolet (UV) light and other proinflammatory stressors which can lead to increased amounts of intracellular reactive oxidative species (ROS). The transcription factors Nrf are members of the Cap' n' collar family and play a crucial role in regulation of protective genes e.g. phase II detoxifyingenzymes and therefore in the cellular stress response. In this study we wished to precisely identify and localize expression of Nrf1 and 2 in human skin cells *in vitro* and situ. In addition, we wanted to explore if alpha-melanocyte-stimulating hormone (alpha-MSH) could affect expression of Nrf1/2 and subsequently, phase II detoxifyingenzymes. Expression analysis revealed that both Nrf1 and 2 expression is broadly expressed in human skin cells *in vitro* and *in situ* as demonstrated by real-time RT-PCR, Western immunoblotting and immunohistochemistry. In particular normal human melanocytes (NHM) *in vitro* displayed the highest RNA and protein expression levels of Nrf1 and 2. Irradiation of NHM and normal human keratinocytes (HNK) with UVB (10 mJ/cm^2) *in vitro* resulted in a robust decline in the mRNA expression levels of Nrf1/2 as well as heme oxygenase-1 (HO-1),gamma-glutamylcysteine-synthetase and glutathione-S-transferase. This effect of UVB could be confirmed in human skin organ cultures irradiated with UVB and subsequent expression analysis by real-time PCR and semiquantitative immunohistochemistry. Interestingly, in NHM but not in HNK alpha-MSH *in vitro* attenuated the UVB-induced decline in Nrf1/2 and HO-1 expression. Our data add a novel functional facet to the list of biological effects of alpha-MSH in skin cells, i. e. compensation of UVB-induced suppression of Nrf1/2 and detoxifying enzymes in melanocytes. Further studies are underway to identify the mechanism how alpha-MSH upregulates Nrf1/2 expression in human melanocytes.

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Glycine receptors in human epidermis

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The inhibitory glycine receptor (GlyR) is a member of the nicotinicoid receptor superfamily. This ligand-gated heteropentameric Cl - channel is composed of different α?(1–4) and a β-subunit and mediates fast synaptic transmission in the central nervous system. Recently, members of this superfamily, the nicotinicacetylcholine receptors, have been found to be involved in the regulation of various biologic functions of human skin. Since glycine, the natural ligand of GlyR has been found to enhance epidermal barrier recovery, we wanted to know, whether GlyR are indeed found in human skin and aimed at characterizing their function in skin physiology. We examined expression of GlyR in cultured keratinocytes, fibroblasts and human skin on mRNA level using PCR. In addition we performed immunofluorescence analysis of GlyR expression in normal human skin, eczema and psoriasis samples. In order to determine functional effects of GlyR stimulation and inhibition we performed proliferation analysis on keratinocyte and fibroblast monolayers and cultured keratinocytes organotypically in the presence of glycine orthe nAChR/GlyR antagonist strychnine. In human skin we found the mRNA of theα2-, α3- and β-subunits while the α1-subunit was not detectable. In cultured keratinocytes and fibroblasts the α1- α3- and the β-subunit mRNA could be amplified. In keratinocytes, glycine specifically induced a 50% increase in cell number after 3 days in culture, while fibroblasts proliferation was not influenced. Organotypically cultured epidermis-equivalents were significantly thicker than control if cultured in the presence of glycine. In human skin, GlyR immunoreactivity (IR) was detected in the upper epidermal layers and in the cuticle of the inner root sheath of anagen hairfollicles, while all other adnexal structures remained negative. Interestingly, in specimens of eczema and psoriasis, GlyR IR was dramatically reduced in areas with prominent parakeratosis suggesting a role of GlyR in terminal differentiation and epidermal barrier control. Further studies are needed to exactly elucidate the role ofthe hitherto neglected ion chloride in cutaneous physiology and pathophysiology and its regulation through GlyR.

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Keratinocyte stem cells in different compartments of human skin-defining conditions for serial culture

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Limited availability of donor skin and mechanical instability of split-thickness skin grafts raise the need for alternative transplantation concepts. Adult stem cells play acentral role in tissue homeostasis and wound repair based on their high proliferative capacity and unlimited life-span. In order to generate autologous grafts based on keratinocyte stem cells we characterized the expression of precursor cell markers inhuman scalp and analyzed the stem cell behaviour of keratinocytes from different compartments during serial culture. For immunohistologic double-staining of human scalp CD34, CD29, CK15, CK19, follistatin, CD71, nestin, and p63 were used as markers for stem (SC) and transient amplifying cells (TAC), respectively. Keratinocyte precursor cells were isolated from biopsies of human scalp and hairless skin, respectively, using different protocols, and expansion was studied in dependence on the use of different media, serum and feeder cells. From serial cultures of follicular- as well as basal membrane- derived keratinocytes cytospins were prepared for immunohistologic staining and percent expression of precursor cell as well as differentiation markers was determined. To assess the proliferative capacity and to classify the clonal type clonality assays were performed from parallel cultures. In human scalp we could identify CK15, follistatin, and CD200 as markers forbulge-stem cells of the hair follicle, CD71 and p63 as markers for supra-basally located precursor cells. Follicular precursor cells showed higher proliferative activity as well as formation of holoclones superior to epidermal precursor cells. Whereas in both compartments CK15 got almost lost during primary cultures, CD29, CD71, and p63 are largely expressed after isolation and give rise to high levels during serial culture. Whereas addition of serum and feeder cells temporarily lead to enhanced expression of CK15, proliferative capacity and clonality, especially of holoclones, were diminished and differentiation was accelerated in serial culture. Follicular keratinocyte precursor cells seem to be a promising source for developing alternative tissue engineered concepts for reconstruction of epidermal defects. However, culture conditions must be precisely defined to preserve stem cell properties.

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Dual regulation of placenta growth factor activity by genetic and proteolytic mechanisms

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Placenta Growth Factor (PlGF) belongs to the family of VEGF proteins and is a multifunctional cytokine that is a key regulator in angiogenesis. By differential mRNA splicing, the human PlGF gene gives raise to protein isoforms PlGF-1 and -2, which differ primarily in the absence or presence of a carboxyl-terminal domain of highly basic 21-amino acids, respectively. Unlike for VEGF-A protein isoforms, little is known about the functional differences of PlGF splice variants. We synthesize dre combinant human PlGF proteins and performed structural and functional analysis to identify different functions of PlGF isoforms. *In vitro* chemotaxis analysis revealed striking differences between the two PlGF forms. Whereas PlGF-2 induced a robustchemotactic response on porcine endothelial cells (PAE) stably transfected with the cell surface glycoprotein Neuropilin-1 (Nrp-1), PlGF-1 showed no effect. The chemotactic activity of PlGF-2 was abolished in control cells non transfected withNrp-1, indicating that PlGF-2 binds to Nrp-1 and mediates the chemotactic response. Furthermore, unlike for PlGF-1 (KD 83×10^{-6} M), BIAcore analysis demonstrated a high affinity of PlGF-2 (KD 49×10^{-9}M) to heparan-sulfate. Interestingly, analysis of protease sensitivity revealed that PlGF-2 is a target of the serine protease plasmin. Western-blot analysis and MALDI-TOF-mass-spectrometry of PlGF-2 fragments identified a plasmin cleavage site resulting in loss of the carboxyl-terminal domain comprising the heparin binding domain (HBD). Importantly, plasmin cleavage ofPlGF-2 resulted in loss of its chemotactic activity on Nrp-1 transfected PAEs, supporting the functional relevance of the HBD for PlGF biology. Our results revealed novel functions of the carboxyl-terminal HBD of PlGF-2. Furthermore, these results indicate that removal of the carboxyl-terminal domain of PlGF, whether it is due to alternative splicing of mRNA or to proteolysis, is associated with a significant loss in bioactivity. Overall the results point to the idea that plasmin-mediated proteolysis is a major switch to control the activity of VEGF protein family members and might serve as an important mechanism during processes of angiogenic remodeling such as in tumour formation or tissue repair.

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Phospholipase stimulates lipogenesis in SZ95 sebocytes

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Introduction: With progressing ageing human sebocytes reduce lipid production. However, the association between the ageing events and sebaceous lipid synthesis as well as ways to influence the latter is not fully identified. Lipids act as ligands of nuclear receptors such as PPAR. Phospholipase (PLA2) catalyzes the hydrolysis ofthe sn-2 fatty acyl bond of phospholipids to yield free fatty acid and lysophospholipid. It has been hypothesized that PPAR are activated by eicosanoids obtained throughPLA2 activity.

Materials and methods: A method to stimulate sebaceous lipid synthesis by Bothropsmoojeni gel filtration fractions (Botmo GF) was studied on SZ95 sebocytes *in vitro*. Botmo GF fractions were partly purified, and a fraction with PLA2 activity (BotmoGF11-117) and a fraction without enzymatic activity (Botmo GF11-101) were identified and additionally tested.

Results: Botmo GF fractions increased lipogenesis in SZ95 sebocytes without inducing apparent toxic or apoptotic effects. Botmo GF11-101 (1 μg/ml) enhancedneutral lipid synthesis by up to 170% and polar lipid synthesis by up to 120%. The enzymatically active PLA2 Botmo GF11-117 (1 μg/ml) increased synthesis of neutral lipids by up to 200%, and polar lipids by up to 120% compared to untreated SZ95sebocytes.

Conclusion: PLA2 activation or suppression could be important for human sebaceous lipogenesis. PLA2 modifiers may be attractive for skin lipid research and pharmacological/cosmetic products.

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Specific interaction between chitosan and matrix metalloprotease 2

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The crucial event in metastasis is tumour invasion which in the case of melanoma cellsis dependent on matrix metalloprotease 2 (MMP2). Moreover, MMPs play a pivotal in impaired wound healing. Chitosan (MW ca. 5×10^5 g/mol, degree of acetylation ca.30%) is supposed to comprise anti-tumour properties and to improve wound healing of chronic ulcera. Here we show that chitosan attenuated the invasive activity of melanoma cells in a cell-based invasion assay and reduced MMP2 activity in the supernatant of melanoma cells. While the expression level of MMP2 was not affected, the amount of MMP2 in the cell supernatant was reduced, indicating apost transcriptional effect of chitosan on MMP2. Atomic force microscopy revealed a direct molecular interaction between MMP2 and chitosan forming a complex with a diameter of 349.0 ± 69.06 nm and a height of 26.5 ± 11.50 nm. Affinity chromatography revealed a high binding-specificity of MMP2 to chitosan, and a colorimetric MMP2 activity assay suggests a non-competitive inhibition of MMP2 bychitosan. We conclude that the biopolymer chitosan binds MMP2 followed by a decrease of MMP2 activity and tumour invasion. Therefore, chitosan may represent a new type of MMP2 inhibitor. Beside their role in tumour cell invasion, MMPs are also involved in wound healing where pathological MMP regulation results in chronic ulcerations. Since chitosan is known to improve wound healing, we speculate that the MMP binding capacity of chitosan is at least partly responsible for the improved wound healing mediated by chitosan-based wound dressings.

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Light activated curcumin induces growth arrest in human melanocytes and human melanoma cells at low concentrations

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Curcumin is a pharmacological active component from the rhizome of the plant Curcuma longa with well known anti-tumour activities. Unfortunately, the use of curcumin is hampered by a low absorption rate *in vivo*. Recently we could show that curcumin combined with UVA or visible light induces cell growth arrest and apoptosis in human keratinocytes at very low doses (Dujic et al., 2007). Based on these results we investigated the effect of curcumin combined with light on human melanocytes and melanoma cells. Both cell species were treated with curcumin (0.2–1 μg/ml) and irradiated with UVA (1 J/cm^2) or visible light (5.500 × 5 min). After 24 h BrdU incorporation and cell membrane damage (LDH release) were measured. Light activated curcumin inhibited the cell proliferation rate of the melanocytes down to 2–18% compared with untreated controls. Similar results were observed in melanoma cells IPC-298, G361, A375 and SKMEL-13. These effects were not obtained when cells were incubated with 0.2–1 μg/ml curcumin alone in the absence of light. We did not find any sign of toxic membrane damage after incubation with curcumin alone or in combination with UVAor visible light. These findings demonstrate the growth inhibitory effect of curcumun in combination with light in different pigmented cell species.

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Crucial role for fibroblast integrin-linked kinase in murine wound healing

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The extracellular matrix is a key regulator of cell functions, e.g. proliferation, differentiation and migration. Binding of matrix macromolecules to integrins initiates the assembly of an intracellular multiprotein complex, the focal adhesion, of which integrin-linked kinase (ILK) is a central component. ILK binds to the intracellular tail ofβ1 integrins and recruits adaptor proteins, thus connecting the outside environment to the actin cytoskeleton. Here, we report the generation of an inducible fibroblast-specific ILK-deficient mouse.ILK-deficient skin fibroblasts show impaired cell spreading, focal adhesion assemblyand stress fiber formation on collagen and fibronectin substrates. This wasassociated with reduced mRNA levels of the collagen-binding integrins α1, α2 andα11, as well as decreased cell surface expression of β1 integrin. Motility of ILK-deficient fibroblasts was enhanced. Transduction of mechanical forces was reduced, as ILK-null fibroblasts show a greatly impaired capacity to contract collagenlattices. In line with altered mechanical properties, the expression ofα-smooth muscle actin was diminished, indicating that myofibroblast differentiation was hampered. Since all of the before mentioned processes are pivotal during cutaneous wound healing, we wounded fibroblast-specific ILK-deficient and control animals .Fibroblast-specific ILK-deficient animals show strongly reduced wound contraction and greatly disturbed granulation tissue formation. Fewer myofibroblasts were present in the granulation tissue, most likely due to reduced proliferation of the ILK-deficient fibroblasts. In conclusion, ILK plays an important role in fibroblasts in sensing environmental cues and thereby maintaining dermal homeostasis.

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Role of MT1-MMP in cell migration

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In wound healing or pathological processes like tumour progression, migration of cells on components of the extracellular matrix (ECM) plays a crucial role. During these processes, expression and activation of Matrix Metalloproteinases (MMPs) is required for degradation or cleavage of ECM with release of bioactive fragments that modulate migration. In particular, membrane-type 1 matrix metalloproteinase (MT1-MMP) has been involved in migration of endothelial cells, keratinocytes andfibroblasts on various substrates including collagen type I and fibronectin. To further analyze the role of MT1-MMP in migratory processes we have used cells derived animals with complete inactivation of this enzyme. By adhesion assays we have first analyzed whether ablation of this protease interferes with adhesive capacity of fibroblasts to different substrates. Indeed, MT1-MMP deficiency resulted in a significant reduction in cell adhesion to collagen type I but not to collagen type IV, fibronectin and laminin. To investigate whetherMT1-MMP ablation effects also migration of cells, we utilized primary murine fibroblasts derived from MT1-MMP deficient mice and their wild-type littermates fortime lapse video microscopy in a random migration assay. Migration on collagen type I and fibronectin, was significantly impaired in knock-out fibroblasts as compared to wild-type controls. In addition, motility of wild-type fibroblasts could be reduced to levels comparable to those observed with MT1-deficient cells by adding recombinant mouse TIMP-2, an endogenous inhibitor of MT1-MMP activity. Morphological analysis of fibroblasts adhered to collagen type I or fibronectin revealed enhanced filopodia formation in MT1-MMP deficient cells plated on these substrates. In summary, we could show that ablation of MT1-MMP results in reduced adhesion primary murine fibroblasts to collagen type I whereas cell migration was reduced on both, collagen and fibronectin *in vitro*. These data suggest that altered adhesion to collagen type I might be responsible for altered migration, while cell migration onto fibronectin requires an additional unknown event, e.g. proteolyticcleavage of this substrate and release of stimulatory fragments.

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Hazardous polycyclic aromatic hydrocarbons are present in tattoo inks that additionally generate singlet oxygen under UVA irradiation

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About 10 million people in Germany have a tattoo at least and most of the tattoos show a black colour. Modern black tattoos are made with ink that not only contain multiple impurities but also are originally produced for the staining of consumer goods. Since black inks are usually produced by imperfect combustion, we analyzed eight commercially available tattoo inks for polycyclic aromatic hydrocarbons (PAH). Beside normal carbon, we found in 500 mg dry substance PAH like phenanthrene (12–129 μg), anthracene (9–73 μg), fluoranthene (12–135 μg), pyrene (8–93 μg)that is in total 32–342 μg of PAH. We also detected 0.02–3.6 mg phenol. Since tattoos are frequently exposed to solar light, these PAH can act as photosensitizer in the skin and generate reactive oxygen species such as single toxygen. Therefore, we excited the polycyclic aromatic hydrocarbons in solution with UVA light and determined the quantum efficiency of singlet oxygen generation by measuring its luminescence at 1270 nm. All polycyclic aromatic hydrocarbons showed a strong singlet oxygen luminescence and their quantum efficiency is higher as compared to porphyrins used in photodynamic therapy. Our results show high amounts of toxic or carcinogenic PAH in black inks that are tattooed in skin. Moreover, the PAH generate the highly reactive singlet oxygen molecule that could affect skin integrity.

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Mechanisms of growth modulatory effects of caffeine and testosterone in female and male human hair follicles

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Caffeine has been reported to counteract the growth suppressive effects of testosterone in the androgenetic-alopecia-analogue male human hair follicle organ culture model. The present study investigated the differential mechanisms contributing to the growth modulatory effects of testosterone and caffeine in both female and male human hair follicles *in vitro*. Whole human female hair follicles from face-lift operations and male hair follicles from electively taken biopsies from men affected with androgenetic alopecia (AGA) after informed consent were cultured over120 h and incubated with either a) testosterone alone or b) with testosterone in combination with caffeine or c) with normal growth medium as control. Hair shaftelongation was measured every 24 h. At the end of the culture period, follicles were frozen and cryosections for immunofluorescence performed. Histomorphometric hair cycling analysis revealed an anagen rate reduction induced by testosterone (5 μg/ml) that was counteracted to an almost normal (non-testosterone-suppressed) anagenrate by caffeine (0.0005%, 0.001%). Hair shaft elongation was significantly suppressed in testosterone-treated hair follicles compared to controls ($P < 0.001$) that was counteracted by caffeine at the concentration of 0.0005% in female hair follicles ($P < 0.05$) and at the concentration of 0.001% in male AGA hair follicles. The catageninducer TGF-beta2 was partly up-regulated in testosterone-treated hair follicles and down-regulated by caffeine as shown by quantitative immunohistomorphometry. Proliferation as indicated by Ki67-staining was reduced by testosterone and again enhanced by caffeine, whereas apoptosis of hair matrix keratinocytes was up-regulated in testosterone-treated hair follicles and treatment with caffeine reduced matrix keratinocyte apoptosis. Thus, caffeine counteracts the growth suppressing effects of testosterone by enhancing human hair growth via pro-proliferative and anti-apoptotic effects, leading to increased hair shaft elongation and late catagen entry that seem to be mediated by attenuation of TGF-beta2 activation. Moreover, female hair follicles seem to react to lower concentrations of caffeine than male AGA hair follicles giving evidence for possible differences in response to topical caffeine treatment *in vivo*.

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Die Transplantation multipotenter hämatopoetischer Progenitorzellenverbessert die dermale Wundheilung über parakrine Mechanismen

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Studien in den letzten Jahren haben gezeigt, dass Stamm- oder Progenitorzellenpotent in die Geweberegeneration eingreifen können. Unserer Arbeitsgruppe ist eskürzlich gelungen, durch die retrovirale Überexpression eines β-catenin-Konstruktesin lineage-negative Knochenmarkszellen von C57Bl6 Mäusen eine signifikante exvivo Expansion und Immortalisierung von murinen hämatopoetischen Stammzellenzu etablieren. Ziel dieser Studie war es, den Einfluss dieser neu generiertenmultipotenten hämatopoetischen Progenitorzelllinie und deren Zellkulturüberstandauf die dermale Wundheilung zu untersuchen. Nach Induktion der Wunde mittels 5 mm Hautstanzen wurden die Tiere in 3Versuchsgruppen randomisiert und entweder mit $1 \times 10E7$ Progenitorzellen, demkonzentrierten Kulturüberstand von $1 \times 10E7$ Progenitorzellen oder mitKontrollmedium behandelt. Die Wundheilung war in den mit Progenitorzellen- bzw.deren Kulturüberstand behandelten Gruppen zwischen Tag 3 und Tag 10 signifikantschneller im Vergleich zur Kontrollgruppe. Dabei zeigte sich kein Unterschiedzwischen der Progenitorzelllinie und dem Überstand in den behandelten Gruppen.Des Weiteren konnte auch ein konzentrationsabhängiger Effekt desKulturüberstandes auf die Angiogenese in einem Matrigel-Assay beobachtet werden.Mittels Protein-Array-Analyse des Zellkulturüberstandes konnte eine Vielzahlinflammatorischer und proangiogenetischer Zykotine und Wachstumsfaktoren (u.a.IL-6, MCP1, M-CSF) detektiert werden. Diese Ergebnisse indizieren, dass die Transplantation von multipotentenhämatopoetischen Progenitorzellen die dermale Wundheilung über parakrineMechanismen beschleunigt. Durch die Generierung der multipotentenhämatopoetischen Progenitorzelllinie können *in vitro* für die dermale Wundheilungwichtige Faktoren unbegrenzt hergestellt werden.

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Evidence that thyroid hormones directly affect human hair follicles: prolongation of anagen, stimulation of hair matrix proliferation, pigmentation and energy metabolism

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Both insufficient and excess levels of thyroid hormones (T3, T4) can result in altered hair/skin structure and function (e.g. effluvium). However, it is still unclear whetherT3/T4 exert any direct effects on human hair follicles (HFs). Therefore, we have investigated the effect of thyroid hormone receptor stimulation on microdissected, organ-cultured human scalp HFs. Here, we show that both T3 and T4 up-regulate the proliferation of hair matrix keratinocytes and down-regulate their apoptosis. T3 andT4 also prolong anagen duration *in vitro*. Intrafollicular immunoreactivity (IR) for the thyroid hormone-responsive keratins CK6 and CK14 is modulated by T3 and T4 (CK6: enhanced, CK14: down-regulated). Both T3 and T4 significantly stimulate human HF melanin synthesis, with supraphysiological concentrations of T4 showing the strongest stimulation of intrafollicular melanogenesis. Since thyroid hormones reportedly can stimulate erythropoietin expression and since we had previously identified human scalp HFs as an extrarenal erythropoietin source, we also assessed whether thyroid hormones increase intrafollicular erythropoietin IR and mRNA expression. Both is indeed the case. T3 and T4 also upregulate cytochrome- coxidase subunit I (COX-I) IR in the outer root sheath, a key respiratory chain enzyme. The response of TGF2 to thyroid hormone stimulation is currently being assessed. These data clearly demonstrate that human HFs are a direct target tissue for thyroid hormones.

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Differential expression of influx and efflux transport proteins in human antigen presenting cells

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The multidrug resistance related protein (MRP, ATP binding cassette C transporters, ABCC) family and the solute carrier organic anion transporting (SLCO) family function as efflux and influx transporters, respectively, of a variety of large organic anions or their conjugates in hepatic detoxification, drug distribution and drug resistance of tumour cells. Thus, these transport proteins are part of the metabolism and elimination of drugs, xenobiotics and certain endogenous molecules and are involved in active vectorial transport of inflammatory mediators. Further we demonstrated that normal human keratinocytes express a specific profile of influxtransporters, support the concept that the uptake of large organic cations like drugs is an active transport process mediated by members of the SLCO family. Human macrophages (M$\emptyset$) express cytochrome P450 enzymes verifying their capacity to metabolize a variety of endogenous and exogenous substances. Here we analyzed the mRNA and protein expression of transport proteins involved in the uptake or export of drugs, hormones and arachidonic acid metabolites in dendritic cells (DC) and M$\emptyset$?compared to their precursors - blood monocytes - using cDNA microarray-, RT-PCR-, Western-blot- and immunostaining techniques. The transport proteins studied included members of the SLCO family and ABCC family. We found that only mRNAs for SLCO-2B1, -3A1, and -4A1 were present in monocytes, M$\emptyset$?and DC. Most interestingly the expression of SLCO-2B1 was markedly enhanced in M$\emptyset$as compared to monocytes and DC. The presence of mRNAs for ABCC1, 3, 4, 5 and6 in all three cell types was demonstrated. On protein level ABCC1 which has been identified as leukotriene C4 transporter was found to be the most abundant transporter in M$\emptyset$?and DC. Blocking the ABCC1 activity with the specific inhibitor MK571 resulted in a phenotypic change in DC but not in M$\emptyset$. Our data show that human blood monocytes and monocyte derived macrophages as well as DC express a specific profile of transporters involved in inlux and efflux of exogenous molecules like allergens or drugs, but also of endogenous substances in particular of inflammatory lipid mediators like leukotrienes and prostaglandins.

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Evidence that thymic peptides regulate human hair follicle biology

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Thymic peptides belong to a diverse family of polypeptides that exert multiple biological functions (e.g. stimulation of cell migration, angiogenesis, wound healing).Thymosin beta4 reportedly even enhances hair growth in rats, and topically applied thymus extracts have long been claimed (but never proven!) to stimulate human scalp hair growth *in vivo*. Therefore, we have explored whether thymic peptides alter human hair growth and/or pigmentation *in vitro*. Here we show that all three tested peptides (thymulin, thymosin alpha-1[TA1], thymosin-beta4 [TB4]) significantly inhibited hair shaft production of organ-cultured human scalp hair follicles (HF) under serum-free conditions (substantial interindividual variations were evident). Interestingly, thymulin or TA1-treated HF stayed longer in anagen than vehicle-treated control HF. Nevertheless, TA1-treatedHF showed fewer proliferating hair matrix keratinocytes than control HF. Quantitative Masson-Fontana histochemistry revealed that thymulin-treated HF had significantly more melanin granules than controls. TA1 and TB4 did not influence HF melanogenesis. Immunohistochemistry for all three thymic peptides appeared to show specific immunoreactivity in human scalp skin sections. Thymulin and TA1 follow similar patterns with strongest immunoreactivity seen in epidermis and in the HF inner roots heath (especially Henle's layer and medulla, distal to the hair bulb). TB4 immunoreactivity was much more widespread, and included also outer root sheath, dermal sheath and dermal papilla of the HF. Only hair matrix keratinocytes appeared to be negative for all three thymic peptides. These data provide first definitive evidence that thymic peptides can indeed alter human HF biology, and suggest that thymulin and TA1 might reduce telogeneffluvium by catagen inhibition, even though actual hair shaft formation is also inhibited. Furthermore we present the first evidence that human HF pigmentation is stimulated by thymulin. This suggests that the role of thymic peptides in human HF biology deserves systematic dissection.

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Thyrotropin (TSH) is produced by human epidermis and is controlled by its classical regulators, thyroid hormones and thyrotropin-releasing hormone (TRH)

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Pituitary thyrotropin (TSH) stimulates thyroid hormone production via its cognate receptor (TSH-R). TSH secretion is up-regulated by hypothalamicthyrotropin-releasing hormone (TRH), while increased serum thyroid hormone levels send negative feedback signals, e.g. by inhibiting TSH production. Since cultured human skin cells reportedly also transcribe elements of the central hypothalamus-pituitary-thyroid (HPT) axis, we have asked in the current study whether TSH is also translated in human epidermis *in situ*, and whether any intraepidemal TSH production is subject to TRH and/or thyroid hormone regulation, using scalp skin organ culture. TSH and TSH-R immunoreactivity (IR) were examined by the highly sensitive EnVison and the peroxidase based ABC methods. Intense TSH IR was found throughout the suprabasal, differentiated layers of normal human scalp epidermis, while TSH-R IR was completely absent in the epidermis, and was confined to mesenchymal compartments and nerve fibers of human skin. Full thickness human scalp skin was organ-cultured under serum-free conditions, and treated with vehicle or TRH (100 ng/ml) and TSH IR was assessed by quantitative immunohistomorphometry. We observed significantly up-regulated TSH protein expression after TRH-treatment, which even partially induced: weak basal layer keratinocytes TSH IR. In contrast, when skin biopsies were treated with thyroid hormones T3 (1 pM, 100 pM) and T4 (100 nM, 1 μM), the intraepidermal TSH immunoreactivity was dramatically down-regulated. Whether this regulations occurs at mRNA levels, will be determined by PCR analysis. Even though the function of TSH expression on the gene and protein level in human epidermal biology remains to be dissected, we here provide the first evidence that human scalp skin epidermis synthesizes TSH, which may stimulate intradermal TSH-Rs and that TSH regulation follows the rules appreciated from the central HP Taxis.

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Searching for new pointers to non-classical functions of erythropoietin (EPO) in human skin and hair biology

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Recently we reported that erythropoietin (EPO), the main growth and viability factor for red blood cells, plays role also in cutanoeus biology, beyond its classical hematopoietic function: Human scalp hair follicles (HF) produce EPO, and EPO-treatment is able to reduce chemotherapy-induced HF damage. By microarray analysis, we identified some differentially regulated candidate EPO-target genes (e.g. calmegin, a key molecular chaperon in testis biology), which were confirmed by quantitative PCR. In the current study, we aimed to further investigate possible functions of EPO in HF and scalp skin biology in term to pigmentation, mast cell functions and to further explore the candidate EPO-target genes. By immunhistochemistry we here provide the first evidence for extra-testicular calmegin expression by demonstrating that calmegin is expressed on the gene and protein level in the hair follicle epithelium. Consistent with our microarray and q-PCR data, after EPO treatment calmegin was down-regulated in HF organ culture. We are currently investigating whether calmegin is involved (similar to its role in testicular biology) in HF differentiation and/or protein folding. Since our microarray data had also revealed differential regulation of some melanogenesis-related genes (kinesinelight chain), we also investigated whether EPO may regulate HF pigmentation. By quantitative Masson-Fontana staining we found up-regulated melanin production in two from five female patients. Since skin mast cells reportedly express the EPO-receptor, we investigated EPO effect on skin mast cell number and degranulation. Intriguingly, EPO significantly reduced the number of c-kit+ mast cells in both the interfollicular dermis and the HF connective tissue sheath, yet did not appear to alter their granulation status. The current study explores several, as yet uncharacterized, non-classical functions of EPO in human skin, where this hormone may regulate e.g. melanin production and mast cell functions, and show that human skin is an extra-testicular site of calmegin expression. These obseobservations provide interesting new pointers in the search for the -as yet poorly understood - functions of EPO in biology.

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Comparison of ammonium ions of the skin surface with skin surface pH and transepidermal water loss at 65% relative air humidity

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A factor that is likely to affect a possible relation between skin surface ammoniumions (NH4+) and skin surface pH and transepidermal water loss (TEWL) is the relative air humidity (RH). Consequently it appears interesting to investigate such relations at an RH that is different from the standard 50% that is chosen in most investigations. Therefore the aim of the present study was to investigate the relation between NH4+ and pH and TEWL at 65% RH. Overall 39 volunteers aged 18–35 (Nine male and 10 female non-smokers, 10 male and10 female smokers) were enrolled. Room temperature was 20°C. RH was 65%.NH4+ was assessed using the Berthelot reaction. PH and TEWL were measured using a Tewameter®?- and a skin pH-meter® (both C&K Electronic GmbH, Cologne, Germany). Assessments were performed on the forearm (FA) and the forehead (FH).The explorative statistical analysis consisting of correlating NH4+ with pH and TEWL as well as correlating NH4+ normalized to TEWL with pH was performed for the following groups: Men (M), women (W), Smokers (SM), non-smokers (NSM), male non-smokers (MNSM), male smokers (MSM), female non-smokers (FNSM), female smokers (FSM). Correlation analysis of the values assessed on the FA revealed significant correlations between NH4+ and pH in W, SM and FSM. The highest correlation was found in W ($r = -0.574$; $P = 0.008$). Correlating NH4+ and TEWL revealed significant results in MSM ($r = 0.666$; $P = 0.035$). On the FH a significant correlation was assessed between NH4+ and pH in W and FSM with the highest correlation in W($r = -0.520$; $P = 0.019$). Correlation of NH4+ values normalized to TEWL with pH revealed significant correlations between the values assessed on the FH of W, NSM, FSM and FNSM. The highest correlation could be found in W ($P = -687$; $P = 0.001$). The results obtained in the present study show very different and partly significant results for the different groups analyzed. This indicates that at the present conditions factors such as site of investigation, gender and smoking habits have influence on the relation between NH4+ and pH and TEWL. Moreover, the significant results concerning the ammonium values normalized to TEWL suggest that ammonia from deeper regions of the skin might follow the water stream determining the TEWL before being converted into NH4+ in the acid milieu of the stratum corneum.

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Inhibition of cyclooxygenase-2 and epidermal growth factor-receptor by atriterpene extract from the outer bark of birch (Betulae cortex)

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Background: We have shown previously pro-apoptotic effects of a triterpene extractfrom the outer bark of birch (TE) in keratinocytes and skin cancer cell lines. TE contains betulin as the main component (80%), but also betulinic acid (3%), lupeol (2%), oleanolic acid (1%) and erythrodiol (1%) The anti-tumour activity of TE has previously been confirmed in the clinical setting by the successful treatment of actinickeratoses.

Methods: Here we have investigated the effect of TE on the expression ofcyclooxygenase-2 (COX-2) and epidermal growth factor-receptor (EGF-R), proteins known to be involved in skin carcinogenesis. Using immunocytochemical and flow cytometric methods, ultraviolet (UV)-B induced COX-2 expression was investigated in HaCaT keratinocytes, and EGF-R expression was studied in the skin cancer cell lineCal-39.

Results: TE inhibited UV-B-induced COX-2 expression in HaCaT keratinocytes. The effect was comparable to the COX-inhibitor indomethacin. Long term incubation with TE decreased the EGF-R expression in the EGF-R overexpressing Cal-39 cells. Testing the isolated compounds of the TE betulinic acid was responsible for the downregulation of EGF-R.

Conclusion: TE may be a promising anti-cancer agent, not only due to its pro-apoptotic cytotoxic activity, but also due to its specific effects counteracting the expression of tumour-associated molecules such as COX-2 and EGF-R.

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Discriminating catagen and anagen by Serial Analysis of Gene Expression(SAGE) of human hair follicles

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The disturbance of the hair follicle cycle constitutes the major cause of unwanted hairloss or excess. To gain a better understanding of the cycle alterations, acomprehensive analysis of the molecular changes underlying the progression through the hair cycle is essential. However, the involved mechanisms are as yet not completely understood, and, to make matters worse, most investigations on the molecular regulation are performed with hair follicles from mice. A non-critical transfer of these results to human hair follicles is arguable, considering the fact that gene homologues of each species sometimes tend to have a completely different function in the respective organism. In order to achieve a more comprehensive overview of the expression differences between human hair follicles in catagen and anagen, hair follicles of these stages were isolated and analysed by 'Serial Analysis of Gene Expression' (SAGE), indicating the involvement of the angiopoietin-like protein CDT6, the 14-3-3 protein family and DPP IV-family in the progression of the hair cycle. The results of this approach open new perspectives for the understanding of the hair cycle.

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Thyrotropin Releasing Hormone (TRH) modulates human hair follicle pigmentation and growth via its cognate receptor (TRH-R)

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TRH is the most proximal member of the hypothalamic-pituitary-thyroid (HPT) axis and regulator of thyroid hormone synthesis. RNA transcripts for members of this axis were detected in cultured human skin cell populations. Since human melanoma cell lines express TRH and are promoted to growth by TRH, and TRH is produced in amphibian skin where it modulates pigmentation, we now have assessed, whether (a) normal human skin and scalp hair follicles (HF) express TRH and/or its receptor, (b) whether exogenous TRH stimulates human HF growth, cycling and/or pigmentation (c) which genes may be the intrafollicular targets of TRH-R mediated signalling. TRH and TRH-R transcripts were detected in microdissected scalp skin HFs byRT-PCR. These appear to be translated, since intense TRH and TRH-R immunoreactivity (IR) was seen in human HF epithelium. TRH (1–10 ng/ml) significantly elevated the melanin content in microdissected HFs after 7 days of organ culture, as demonstrated by quantitative Masson-Fontana histochemistry. TRH significantly reduced the number of TGF-beta2-induced catagen HFs inorgan-culture, and compensated the TGF-beta2-induced decline of the HF melanincontent. TRH also promoted HF elongation in organ-culture, most effectively at 5 ng/ml. Finally, by micro array analysis (Agilent/Miltenyi) several differentially up-, or down-regulated TRH-target genes were detected (e.g. neurofilament3 (NEF3), keratin 6B (KRT6B), keratin, hair, acidic1 (KRTHA1), keratin, hair, basic6 (KRTHB&). Thus, human scalp HFs are a source and target of TRH, and respond to TRH stimulation by defined changes in HF cycling and pigmentation. Therefore, not only thyroid hormones, but also TRH-R mediated signalling can directly modulate human HF functions.

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