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Abstracts

P001

Drug-induced anaphylaxis in spontaneous adverse drug reaction reports

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Background: Drug-induced anaphylaxis is a maximal variant of a (pseudo-) allergic immediate-type reaction. In Germany, numerous cases of suspected adverse drug reactions (ADRs) are reported spontaneously to the Federal Institute for Drugs and Medical Devices (BfArM) and registered in a large ADR database. The aim of the present study was to analyze cases of drug-induced anaphylaxis reported to the BfArM with regard to the drugs administered and basic epidemiological parameters.

Methods: All cases of anaphylaxis, anaphylactic shock, anaphylactic/anaphylactoid reaction reported to the BfArM between 1993 and March 2004 were identified and assessed with regard to correctness of the diagnosis anaphylaxis and the causal relationship with the drug according to international agreed criteria. Further analyses were performed in defined subgroups. Estimations of exposure with the various drugs were derived from published utilization data.

Results: From 2239 cases reporting the aforementioned terms, 1144 were randomly extracted and analyzed in detail. The diagnosis anaphylaxis and the causal relationship with the drug were assessed as certain or probable in 386/1144 cases. Among these 386 anaphylaxis cases, 84 (22%) referred to antibiotics, 81 (21%) to contrast media, 45 (12%) to non-steroidal anti-inflammatory drugs, 36 (9%) to anti-neoplastics, and 24 (6%) to analgesics. Among the antibiotics, 52/84 cases (62%) referred to quinolones, and this was not matched by a comparable large estimation of exposure.

Conclusions: In our investigation, most anaphylaxis cases were confined to drug groups which have also been described in literature as being frequently associated with such reactions. Quinolones accounted for a relevant number of anaphylaxis cases among the group of antibiotics.

P002

The kinetics of immunoglobulin E-mediated signalling and mediator release in human basophils crucially depend on the magnitude of stimulation and are controlled by the phosphatase SHIP

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Dose-dependent immunoglobulin (Ig)E-mediated activation of basophils and mast cells usually gives rise to a bell-shaped mediator release curve. Previously, it was thought that the suppression of mediator secretion using supraoptimal antigen or anti-IgE concentrations is due to saturation of IgE-binding sites, thus reducing crosslinking ability. However, we observed that supraoptimal stimulation of human basophils gives rise to a rapid release of histamine but with lower maximum levels than seen for optimal anti-IgE-induced triggering. Here, we addressed the mechanisms responsible for this effect in comparison with lower degrees of anti-IgE stimulation. At the level of various signal transduction enzymes [e.g. Syk, p38 mitogen-activated protein kinase (MAPK) and ERK-1 and ERK-2], supraoptimal anti-IgE stimulation led to a sharp induction of phosphorylation but also faster inactivation than seen with optimal and suboptimal anti-IgE concentrations. The swift deactivation of signals generated by supraoptimal anti-IgE triggering correlated closely with a high degree of SHIP phosphorylation, known to control PI3-kinase-dependent signalling, an important regulator of IgE-mediated basophil activity. In contrast, the bacterial peptide fMLP failed to markedly increase basophil SHIP phosphorylation and also did not produce a bell-shaped dose–response curve, indicating that SHIP-mediated control may be limited to IgE-dependent basophil activation. Interestingly, interleukin-3 priming of basophils gave rise to a suppression of SHIP activity caused by supraoptimal anti-IgE challenge and increased mediator release. This further underlines that SHIP may be responsible for down-regulating IgE-dependent activation. In summary, signal transduction in basophils displays fundamentally different rates in kinetics, depending on the magnitude of IgE-receptor triggering. Our data also suggest that SHIP operates as the gatekeeper of these FceRI effector functions and therefore is a crucial target for anti-allergic therapy.

P003 (V22)

A parasitic antigen (IPSE) secreted by schistosoma mansoni eggs causes immunoglobulin E-dependent basophil activation in non-sensitized individuals without crosslinking immunoglobulin E

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Schistosoma mansoni eggs cause a pronounced T-helper 2 (Th2) immune response and IgE synthesis. We have recently identified a secretory molecule from Schistosoma mansoni eggs (IPSE) that may be responsible for Th2 induction as it rapidly triggers human basophils to release substantial quantities of interleukin-4, even in non-sensitized individuals. Here, we addressed the mechanism of IPSE-mediated basophil activation. Stripping of surface IgE from basophils and subsequent resensitization with purified polyclonal IgE demonstrated that IPSE stimulates basophils in an IgE-dependent manner. Furthermore, as with anti-IgE stimulation, IPSE-induced histamine release from basophils was insensitive to inhibition by pertussis toxin. The phosphorylation of various signalling enzymes, including Syk, p38 mitogen-activated protein kinase, ERK1 and 2, SHIP-1 and SHIP-2, were also similar for IPSE-induced basophil activation compared with anti-IgE and different to IgE-independent stimuli. However, when IPSE was pre-incubated with IgE, it formed complexes capable of activating IgE-stripped basophils, whereas anti-IgE was not. Maximum basophil mediator release was achieved by incubating IPSE with IgE at a ratio of 2:1, which is not in keeping with a crosslinking arrangement of at least 1:2. Additionally, IPSE bound to both Fc and Fab immunoglobulin regions. Nevertheless, it caused basophil activation only with whole IgE molecules and not IgE-Fc fragments, suggesting that binding to both regions is important for an activatory configuration. Finally, IPSE failed to crosslink IgE in sandwich blot assays. This further demonstrates that this molecule activates basophils by an entirely novel IgE-dependent mechanism that does not involve crosslinking. The functional monovalency of IPSE is unlike that of other known non-specific IgE-binding agents such as lectins and superantigens. IPSE therefore represents a new class of IgE-FceRI activating factors

that may play a significant role in polarizing immune responses to a Th2 phenotype.

P004

Interleukin-10-treated dendritic cells do not inhibit T-helper 2-immune responses in ovalbumin/alum-sensitized mice

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Background: It is well known that the immunoregulatory cytokine interleukin (IL)-10 inhibits the accessory function of human dendritic cell (DC) *in vitro*. Recently, we have shown that these IL-10-DCs inhibit the production of Th1 and Th2 cytokines by T cells from atopic individuals *in vitro*. The current study was set out to analyze whether IL-10-DCs also exert inhibitory effects *in vivo* in a murine model of allergy to ovalbumin (OVA).
Methods: OVA-pulsed or unpulsed bone marrow-derived DCs, which had been treated with IL-10 or left untreated during generation, were injected intravenously into BALB/c mice on days 9, 2 and 12. Then, mice were immunized by intraperitoneal injection of OVA adsorbed to the adjuvant aluminium hydroxide on days 0 and 14, and sera and immune responses of mesenterial lymph node cells were analyzed 4 days later *in vitro*. Additionally, bronchoalveolar lavage was performed 24 h after intranasal challenge with OVA on days 25–27.
Results: Treatment of BALB/c mice with OVA-pulsed DC prior to and during OVA sensitization led to an enhanced proliferation as well as Th2 (IL-4 and IL-5), Th1 [interferon (IFN)-γ] and IL-10 cytokine production after restimulation of lymph node cells with OVA *in vitro*. As compared with OVA immunization alone anti-OVA IgG1 and IgG2a antibody titers but not anti-OVA IgE were also increased after transfer of OVA-pulsed DC. In contrast, using OVA-pulsed IL-10-DC for transfer, production of IL-4, IL-10, and IFN-γ by lymph node cells were not enhanced, while IL-5 production and proliferation were only slightly enhanced. Furthermore, OVA-specific IgG1 and IgG2a production after transfer of OVA-pulsed IL-10-DCs were also significantly increased, whereas anti-OVA IgE production, airway eosinophilia, and inflammation remained unchanged.
Conclusions: Our data indicate that IL-10 treatment of DC only decreases the Th1-and Th2-cell stimulatory capacity of DC but does not actually inhibit allergen-specific Th2 responses *in vivo* in a murine model of allergy.

P005 (V33)

Administration of proinflammatory interleukin-1α during sensitization decreases T-helper 2-mediated allergic asthma

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Using an infectious disease model (leishmaniasis), we have previously demonstrated that genetically determined release of interleukin (IL)-1α from activated dendritic cells contributes to the development of characteristic T-helper (Th)1/Th2 immunity. With the intention to determine the role of IL-1 in other Th2-predominant immune responses, we investigated the influence of proinflammatory IL-1α on ovalbumin (OVA)/alum-induced allergic asthma. BALB/c mice were sensitized intraperitoneally with OVA/alum complexes on days 0 and 14 and challenged intranasally 2 weeks later. IL-1α (500 ng) was administered during sensitization on days 0, 1 and 2. Airway hypersensitivity responses (AHR) to methacholine were measured by invasive plethysmography. A bronchoalveolar lavage (BAL) was performed to determine the composition of the inflammatory infiltrate and cytokine profile, and lungs were collected for H&E staining. Mice treated with OVA/alum and IL-1α showed significantly decreased AHR compared with mice treated with OVA/alum alone ($P = 0.05$, $n = 9$). Lungs of IL-1α-treated BALB/c mice contained twofold decreased numbers of infiltrating cells. The percentage of eosinophils in the BAL was significantly decreased in IL-1α-treated mice ($33 \pm 3\%$) compared with controls ($51 \pm 2\%$, $P = 0.002$, $n = 9$) as was the number of infiltrating macrophages. In contrast, the percentage of neutrophils was increased by sevenfold in IL-1α-treated groups compared with control groups. In lung histology, IL-1α-treated mice displayed reduced peribronchial inflammatory infiltrates ($57 \pm 3\%$ infiltrated bronchi) as compared with control animals ($81 \pm 8\%$, $P = 0.05$, $n = 9$). Finally, we detected dramatically increased levels of interferon-γ and reduced levels of the Th2 cytokines IL-4, IL-5 and IL-13 ($P = 0.05$, $n = 9$) in IL-1α-treated asthma groups. In conclusion, treatment with IL-1α during the early sensitization phase of allergic asthma is effective in suppressing the course of disease by shifting the immune response towards Th1. Future studies will have to elucidate the therapeutic value of IL-1α in asthmatic patients.

P006 (V08)

$CD11c^+$ dendritic cells mediate 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 show that suppressor $CD8^+$ T cells (Tc2) of LZT inhibit the development of Tc1-mediated CHS. As skin-related dendritic cells (DCs) (epidermal Langerhans cells, dermal DC) and B cells were previously excluded to be involved in the development of LZT, we analyzed the role of antigen-presenting cells (APC) located in the lymphatic system for tolerance induction to allergens. FACS analysis revealed a significant increase in the number of $CD11c^+$ DC in the lymph nodes of mice toler ized by subimmunogenic doses of contact allergens (trinitrochlorobenzene ×5, 4.5 μg) as compared with control animals. To study whether $CD11c^+$ DC contributed to LZT, we use CD11c-DTR mice. In these mice, systemic administration of diphtheria toxin leads to a transient and rapid decline in $CD11c^+$ DC in lymph nodes and spleen. Notably, depletion of $CD11c^+$ DC by injection of diphtheria 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 with phosphate-buffered saline-treated CD11c-DTR animals, an unrestricted CHS reaction was observed in these mice resulting in an marked ear swelling mediated by the development of Tc1-effector T cells of CHS. Furthermore, adoptive transfer experiments of purified $CD11c^+$ DC obtained from lymph nodes of tolerized mice induced LZT in the recipients. Our findings identify $CD11c^+$ DC as key APC of LZT to allergens that are necessary and sufficient for Tc2 priming during induction of LZT and validate DC as a novel target for directed therapy of allergic diseases.

P007

The allergenic potential of sesquiterpene lactones in phytomedicines from *Arnica* – an immunologic revision

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Preparations of *Arnica montana* flowers have been used in traditional medicine since a long time to treat a variety of

inflammatory diseases. The secondary metabolites that mediate the anti-inflammatory effects are sesquiterpene lactones (SLs) of the 10 -methylpseudoguaianolide type like helenalin and 11&#61537, 13-dihydrohelenalin, and their ester derivatives. Several studies have shown that SLs exert this effect in part by inhibiting activation of the transcription factor NF-& #61547; B. Despite the proven anti-inflammatory effects, Arnica preparations are often considered as strong contact sensitizers and inducers of allergic contact dermatitis. This bad reputation is based on results from a guinea pig model where different preparations from *A. montana* and their isolated SLs turned out to be strong inducers of skin erythema and on case reports in the literature. In contrast to these findings, we had no success in causing contact hypersensitivity (CHS) to the same SLs and preparations in a well-accepted mouse model; on the contrary, we observed an anti-inflammatory effect of Arnica tinctures in an allergic ear swelling reaction caused by the strong contact sensitizer trinitrochlorobenzene (TNCB).

Further studies were undertaken to find out if $CD4^+CD25^+$ regulatory T cells (Treg) actively prevent CHS to Arnica tinctures. We injected anti-CD25 mAb or the cytostatic cyclophosphamide, which is known to deplete the $CD4^+CD25^+$ T-cell pool selectively. Although we failed causing CHS in $CD4^+CD25^+$ T-cell-depleted mice, our preliminary studies using MHC II 0/0 mice indicate that CHS to Arnica can be induced. As in CHS to TNCB and other allergens, $CD8^+$ T cells are the effector cells. Our results show that immunosuppressive mechanisms such as the action of Treg cells prevent CHS to Arnica. According to these findings, SLs and tinctures from Arnica have to be classified as weak contact sensitizers.

P008 (V15)

Failure of the IL-12/IL-12R system results in Toll-like receptor-4-dependent hapten-induced contact hypersensitivity

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The so-called irritant effect of contact sensitizers is crucial for the induction of allergic contact dermatitis (ACD). This effect is based on the ability of such allergens to activate the innate immune system by largely unknown mechanisms. One of the results of this innate immune response is the emigration of dendritic cells (DC) from skin to draining lymph nodes where the priming of naïve allergen-specific T cells takes place. A striking observation is the activation of the transcription factor NF-κB #61547; B and of mitogen-activated protein (MAP) kinases by contact sensitizers as both pathways are also activated by Toll-like receptor (TLR) triggering. However, up to now, it is unknown whether TLRs play a role in ACD. We have now analyzed two components of innate immunity and their role in contact hypersensitivity (CHS) to trinitrophenyl (TNP) in C57BL/6 mice and mutant mouse strains.

Using interleukin (IL)-12 p35/p40 knockout (KO) mice or IL-12R & #61538; 2 KO mice, we found that TNP-induced CHS was significantly increased. In contrast, TLR-4-deficient mice developed CHS similar to wildtype mice. However, mice deficient for both IL-12R & #61538; 2 and TLR-4 did not develop any CHS. These data clearly show that in the absence of a functional IL-12/IL-12R & #61538; 2 system, TLR-4 is obligatory for induction of CHS.

We are now in the process of analyzing the basis for the defective CHS response in the double-deficient mice. Our results indicate that the defect is due to DC dysfunction and is only relevant in the sensitization phase of CHS.

Our data reveal a role for TLR-4 in CHS to chemicals and may help to elucidate the mechanisms by which contact sensitizers activate the innate immune system.

P009

Significance of specific immunoglobulin E to natural rubber latex in sera of patients reactive to other allergens: crossreactive carbohydrate determinants and recombinant rubber latex components in *in vitro* allergy diagnostic

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There are reports on the detection of immunoglobulin (Ig)E specific to natural rubber latex (NRL) in sera of patients with clinically relevant allergy to other natural allergens. This study aimed at the detection of anti-NRL-IgE in sera of stinging insect allergy and to invent more specific *in vitro* tests. One hundred and eleven sera of patients with additional IgE to the crossreactive carbohydrate determinant (CCD)-screening allergen horseradish peroxidase (HRP) were also investigated for anti-NRL-IgE. Subsequently, recombinant (r) NRL components rHev b 1, 2, 3, 5, 6.01, 6.02, 8, 9, 11 were used. They were produced in *Escherichia coli* and were fusion proteins with the maltose-binding protein (MBP), except rHev b 5. MBP, therefore, served as a negative control. IgE detection was performed with the CAP FEIA (UniCAP, Pharmacia, Freiburg, Germany). Results >0.35 kU/l were considered as positive. A questionnaire on NRL allergy and an additional skin test with NRL extracts (ALK Scherax) was used to evaluate the clinical significance. Fifteen NRL allergic individuals served as positive controls. Eighty-eight of 111 CCD-reactive sera had specific (s) IgE to NRL. Forty-two of 61 patients were sIgE-negative for recombinant NRL allergens; 19/61 had sIgE to at least one recombinant NRL component. Sixteen of 61 NRL-IgE-positive hymenoptera venom allergic patients had a history positive for atopic diseases. Nine of 19 patients with sIgE to at least one recombinant NRL component completed the questionnaire and underwent skin prick test with negative results. All nine sera were additionally investigated with HRP and MBP. Five of them were reactive to MBP, all but one to HRP. Sera of hymenoptera venom allergic patients were included in the reciprocal inhibition assay with HRP revealing a complete HRP inhibition of sIgE to NRL which was in contrast to sera of patients with clinically relevant NRL allergy, indicating that the detection of sIgE to NRL does not necessarily indicate NRL allergy and that HRP is a useful screening allergen for anti-CCD-IgE. The reciprocal inhibition increases CAP FEIA specificity in anti-CCD-IgE containing patient's sera.

P010

Uptake of grass pollen allergens by dendritic cells

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Dendritic cells (DC) are the most effective antigen uptaking and presenting cells in the immune system. Furthermore, they have an important influence on the differentiation of naïve T cells toward T-helper (Th)1 or Th2 phenotype. Timothy grass pollen is the major allergen source in Europe and contains a variety of structurally different allergen molecules. For example,

the allergen Phl p 1 is a 28-kDa glycosylated protein, whereas Phl p 6 is a 12-kDa protein without post-translational modifications. We analyzed the mechanism of uptake of these two allergens by monocyte-derived DCs and the functional presentation by the proliferation of specific T-cell clones. The uptake of allergens was investigated with fluorescein isothiocyanate-labeled natural Phl p 1 and Phl p 6 using flow cytometry. To analyze the mechanism of uptake, we used methyl-α-D-mannopyranoside as inhibitor of the mannose receptor and amiloride and cytochalasin D as inhibitors of the macropinocytoses. The presentation of the processed allergens was analyzed by T-cell proliferation assays.

DC internalised both allergens Phl p 1 and Phl p 6 in a time- and dose-dependent manner – non-mature DCs significantly more compared with lipopolysaccharide-matured DCs. The maximal uptake was reached after 4 h. The allergen uptake occurred primarily by macropinocytoses as shown by use of amiloride and cytochalasin D as inhibitors. The mannose receptor was not involved in this process as methyl-α-D-mannopyranoside had no inhibitory effect.

In spite of the highly different structures of the allergens Phl p 1 and Phl p 6, we did not observe a significant difference in the uptake by DC.

P011

Regulatory CD4$^+$ and CD8$^+$ T cells inhibit Tc1-mediated skin inflammation after systemic presentation of allergens

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The induction of tolerance may be a promising target of strategies aimed 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 show that LZT is a systemically induced state of acquired peripheral tolerance. Low, subimmunogenic doses of the contact allergen trinitrochlorobenzene applied systemically (orally, intravenously) induced a significant tolerance reaction *in vivo* and *in vitro* comparable with epicutaneously tolerized mice. In detail, analysis of the immune response in all models of LZT revealed the generation of interleukin-10-secreting, regulatory CD4$^+$ T cells during induction of tolerance that are absolutely required for the development of hapten-specific CD8$^+$ Tc2 cells. These CD8$^+$ T cells of LZT are found as well in peripheral skin draining as in mesenteric lymph nodes and in the spleen of tolerized animals, independent of the route of tolerization. Adoptive transfer experiments identified these CD8$^+$-suppressor T cells as effector T cells of systemically induced LZT inhibiting the development of CHS-promoting Tc1 cells and consequently the manifestation of CHS. These data indicate that uptake and systemic presentation of small amounts of haptens (e.g. contact allergens, drugs, and metals) as a physiologically occurring process induce the development of LZT and thus prevent unwanted activation of the immune system and protect from allergic diseases.

P012

Pollen-associated lipid mediators induce T-helper 2 chemokines in human keratinocytes

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Exposure to pollen can induce eczematous skin inflammation. The underlying mechanisms are thought to be triggered by T-helper 2 (Th2)-cell recruitment into the skin. However, the mechanism leading to the dermal migration of Th2 cells in the early phase of acute eczematous reactions to pollen grains is not well understood. We recently demonstrated that pollen grains not only function as allergen carriers but are also a rich source of pollen-associated lipid mediators activating and attracting human granulocytes and modulating dendritic cell function that results in an enhanced capacity to initiate Th2 responses *in vitro*.

Here, we investigated the effects of water-soluble factors from birch and grass pollen grains on epithelial cells. Human primary keratinocytes from non-allergic and allergic (lesional/non-lesional) were exposed to aqueous pollen extracts (APE). In an *in vitro* inflammatory skin model, primary human keratinocytes were pre-stimulated with interferon (IFN)-γ (300 U/ml) and subsequently incubated with APEs. Effects on chemokine and cytokine production were investigated by real-time polymerase chain reaction. APE significantly induced the Th2 chemokines TARC (CCL17) and MDC (CCL22), while Th1 chemokines such as IP-10 (CXCL10) and i-TAC (CXCL11) were not expressed after APE stimulation. Furthermore, APE blocked the IFN-γ-induced production of CXCL10 and CXCL11, while the IFN-γ-induced expression of CCL22, CCL5, and CCL17 was further enhanced by APE.

Our data suggest that pollen-derived factors provide a signal to human keratinocytes which leads to an enhanced Th2 milieu promoting the infiltration of Th2 cells and eosinophils into the skin of pre-disposed individuals.

P013

Bullous allergic hypersensitivity to bed bug bites mediated by immunoglobulin E against salivary nitrophorin

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In Central Europe, bites from the common bed bug (*Cimex lectularius*) are nowadays rather uncommon. However, an increase of bed bug infestations has been observed over the past decade world wide, and potential hypersensitivity reactions may pose patients at risk following repeated exposure. Infestations are sometimes observed in old framehouses and by immigration due to international travel and migration. The clinical picture of bug bites substantially varies between individuals, depending upon previous exposure and the degree of an immune response. The potential antigens present in the saliva of *C. lectularius* or specific antibodies have not been characterized thus far. Here, we describe a patient with bullous bite reactions after sequential contact with *C. lectularius* over the period of 1 year. In skin tests, we observed immediate reactions to salivary gland preparations of *C. lectularius* which were followed by a pronounced partially blistering late-phase response. Immunoblot analysis of the patient's serum with salivary gland extracts and recombinant *C. lectularius* saliva proteins revealed specific immunoglobulin E (IgE) antibodies against the 32-kDa *C. lectularius* nitrophorin but not to *C. lectularius* apyrase. Our data demonstrate that bullous cimicosis is the late-phase response of an allergic IgE-mediated hypersensitivity reaction to *C. lectularius* nitrophorin.

P014

Impact of interleukin-13 on epidermal inflammation in atopic dermatitis

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Allergic eczematous skin diseases are characterized by an infiltration of activated T lymphocytes of the T-helper 2 (Th2) phenotype in the acute phase of inflammation. The aim of our study is to delineate the response of human primary keratinocytes (HPKs) to the Th2 lymphokine interleukin (IL)-13 in terms of initiation and chronification of eczema. In migration assays, we could observe that IL-13-stimulated HPKs preferentially recruit $CCR4^+$ ($CRTH2^+$) $CD4^+$ T cells. Interestingly, $CCR4^+$ $CD4^+$ T cells from atopic dermatitis patients showed a higher chemotactic response than those from healthy individuals toward IL-13-stimulated HPKs. We observed a marked and significant increase in the expression of CCL-22/MDC but not CCL-17/TARC in IL-13-stimulated HPKs, and neutralization of CCL-22/MDC could block the migration of $CCR4^+$ $CD4^+$ T cells. Moreover, IL-13 stimulation of HPKs induced selective expression of MMP-9 at mRNA level but not of MMP-3 in quantitative real-time polymerase chain reaction. MMP-9 induced by IL-13 in HPKs was functional as detected by biotrak activity assay system. In addition, IL-13 down-regulated the expression of E-cadherin on HPKs. Finally, we have demonstrated the concomitant mRNA expression of MMP-9 and IL-13 in biopsies from patients of acute eczematous skin diseases. Our results suggest that induction of MMP-9 and down-regulation of E-cadherin by IL-13-stimulated HPKs may play a role in the initiation and chronification of skin inflammation by facilitating emigration of Langerhans cells into the lymph node and infiltration of inflammatory cells into the epidermis.

P015

Involvement of the molecular triad RANKL/RANK/OPG in the pathogenesis of osteoporosis associated with mastocytosis

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Systemic mastocytosis is often associated with osteoporosis. Recent identification of the molecular triad receptor activator of NF-κB ligand (RANKL), its cognate receptor RANK, and its decoy receptor osteoprotegerin (OPG) has led to new insights into bone biology. RANKL expressed on osteoblasts stimulates maturation of osteoclasts via RANK binding, resulting in bone resorption, a mechanism that is counteracted by OPG. To investigate whether the RANKL/RANK/OGP triad is involved in the pathogenesis of secondary osteoporosis in mastocytosis, we examined expression of these molecules in human mast cells and in bone marrow sections from patients with mastocytosis. Using reverse transcription polymerase chain reaction, cultured cord blood-derived mast cells (CBMC) and mast cell lines derived from patients with systemic mastocytosis (HMC-1 and KU-812) were found to express RANKL and RANK mRNA. Significant amounts of OPG mRNA were only detected in CBMC but not in cell lines. Consistently, the ratio of RANKL/OPG protein as measured by immunoblotting and flow cytometry was increased in cell lines compared with CBMC. Coculture experiments with HMC-1 cells and peripheral blood mononuclear cell-derived macrophages resulted in formation of TRAP-positive osteoclasts, confirming functional expression of RANKL in mast cells. *In vivo*, infiltrates of mast cells in mastocytosis bone marrow were associated with specific expression of RANKL protein, whereas RANK and OPG staining was found in accumulations of mast cells as well as in bone tissue. Using a semiquantitative score to analyze the immunoreactivity for RANKL and OPG in bone marrow sections from 36 patients, correlations between the percentage of mast cell infiltration and tryptase levels with an enhanced RANKL/OPG ratio were observed. We conclude that human mast cells are able to express RANKL, RANK, and OPG and that alterations in the ratio of RANKL/OPG may contribute to osteoporosis often observed in systemic mastocytosis.

P016

Behavioral changes as a response to stress exposure in a mouse model of experimental allergic dermatitis

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Atopic dermatitis is a common, chronically relapsing, inflammatory skin disorder, accompanied with intense pruritus and eczematous skin lesions. Stress seems to play an important role in the pathogenesis and aggravation of the disease. Affected individuals, chronically exposed to distressing symptoms, show a specific behavioral profile characterized by depression, tension, and anxiety. To examine whether atopic sufferers respond to stress differently from healthy individuals, we used a mouse model of experimental allergic dermatitis (AD) and stress. C57BL/6 mice were intraperitoneally sensitized and subcutaneously challenged by ovalbumin and additionally exposed to sound stress for 24 h. Changes in depression- and anxiety-like behavior, locomotor activity, exploration, and 'approach/avoid conflict' behavior were assessed using elevated plus maze and tail suspension test. Results show that AD induction itself does not cause changes in anxiety-like behavior compared with control (non-treated) mice. Interestingly, stress seems to have an anxiolitic effect and to promote locomotion, exploration and inhibit an 'avoid conflict' behavior. This effect of stress is reduced in mice with AD. Taken together, our data suggest, that AD impairs coping with a new stressful situation as represented by the plus-maze. Enhancement of stress-coping skills therefore appears a useful measure to balance AD-induced behavioral changes.

P017

Contact hypersensitivity is significantly increased in cannabinoid receptor knockout mice: evidence for the involvement of the endogenous cannabinoid system in the regulation of cutaneous immune responses

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Background and aim: Cannabis preparations have been used in traditional medicine for the treatment of inflammatory diseases. The major active constituent of the plant Cannabis sativa is 9-tetrahydrocannabinol (THC). Two specific receptors mediate the effects of cannabinoids (CB1 and CB2 receptors). The discovery of endogenous CB receptor ligands proved the existence of an endogenous CB system with many physiological functions. A participation of CB receptors in the down-regulation of inflammatory processes was demonstrated in experimental models for atherosclerosis or colitis. Therefore, we investigated the role of the CB system in experimental contact hypersensitivity (CHS).
Methods: Experimental CHS was induced by application of 0.2% DNFB on the shaved abdomen and elicited by painting the ears with 0.3% DNFB. To evaluate the effect of exogenous CB, we

treated C57BL/6 mice ears with THC locally before 24 h and 48 h after challenge. Histopathological analyses were performed on inflamed skin, and the activity of neutrophils was evaluated in myeloperoxidase (MPO) assays. To assess the role of CB receptors, we used C57BL/6 mice lacking the CB1 and CB2 receptor (CB1/2$^{-/-}$ mice).
Results: Topical treatment with THC decreased ear swelling in wildtype (wt) C57BL/6 mice and showed effectivity even after repeated challenges. Histological examinations and MPO assays demonstrated a reduced tissue infiltration of neutrophil granulocytes in inflamed skin. Interestingly, contact allergy in C57BL/6CB1/2$^{-/-}$ mice was increased more than 100% in comparison with wt mice. Treatment with THC was also effective in CB1/2$^{-/-}$ mice, indicating that the effects are independent of the CB receptors.
Discussion and future perspective: Our results show that the endogenous CB-system is involved in down-regulating the cutaneous CHS response. Exogenously applied CB can restrict allergic inflammation in a receptor-independent manner. Future experiments will have to address how the endogenous CB system participates in the regulation of cutaneous immune responses. Furthermore, novel synthetic CB receptor ligands may be developed for the anti-inflammatory treatment of the skin.

P018 (V05)

Skin-selective homing of CD4$^+$ T lymphocytes by epicutaneous priming

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Tissue-selective imprinting and homing of antigen-specific T lymphocytes play an important role in the pathogenesis of contact hypersensitivity (CHS) and possibly also atopic dermatitis. Recent studies have shown that dendritic cells from selected organs can imprint antigen-specific T cells *in vitro* for specific homing into their corresponding organs after *in vivo* transfer. We asked if cutaneously administrated antigens may induce a skin-selective imprinting *in vivo*.

To investigate migration and homing of antigen-specific CD4$^+$ T lymphocytes, we used the ovalbumin (OVA) T-cell receptor transgenic mouse strain DO11.10. T lymphocytes were either naive, activated by subcutaneous application of OVA in Complete Freund's adjuvant (CFA) or T lymphocytes were activated in the context of protein CHS. Naive OVA-T-cell receptor (TCR) transgenic CD4$^+$ T lymphocytes were adoptively transferred into naive BALB/c mice. After subcutaneous challenge with OVA peptide in CFA, specific enrichment of OVA-specific T cells was observed in the draining lymph nodes, whereas challenge with phosphate-buffered saline (PBS) in CFA led to random distribution of OVA-specific T lymphocytes in all lymphoid organs of the recipient. Antigen-specific homing was associated with a down-regulation of L-selectin and P-selectin ligand (PSGL-1) expression on OVA-TCR transgenic T lymphocytes. When we transferred epicutaneously primed OVA-TCR transgenic T lymphocytes from skin-draining lymph nodes or spleen into naïve mice, these T cells showed a random distribution after epicutaneous PBS challenge. In sharp contrast, epicutaneous exposure to OVA peptide showed a selective migration, predominantly, into all skin-draining lymph nodes. These skin primed T lymphocytes also expressed lower levels of L-selectin and PSGL-1 after epicutaneous OVA peptide challenge. Importantly, these T lymphocytes did not only migrate to the skin-draining lymph nodes. They had also acquired and preserved the capacity to cause protein-contact dermatitis upon epicutaneous challenge with OVA peptide as shown by skin histology.

Thus, epicutaneous antigen administration may instruct T lymphocytes to home preferentially into the skin-associated lymphoid tissue and to cause allergic contact dermatitis.

P019

Genetic background affects susceptibility to milk-induced allergic lung disease

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Cow's milk allergy is a serious health problem in infancy, which may influence the propensity to allergic disease later in life. We developed a novel mouse model of milk-induced allergic asthma and tested the effect of genetic background on susceptibility in BALB/c and C57BL/6 (B6) mice. To establish milk-induced allergic asthma, we injected BALB/c and B6 mice with 10 mcg of low-fat milk powder dissolved in phosphate-buffered saline (PBS) intraperitoneally 3 weeks apart. One week later, we nebulized mice with 2% milk powder dissolved in PBS. Mice were evaluated for acute onset of disease (day 31) or were allowed to recuperate and were then re-exposed to a second milk aerosol challenge (>day 90) to generate disease relapse. We observed increased lung inflammation, mucus secretion and milk-specific immunoglobulin (Ig)E and IgG1 at acute onset disease in both B6 and BALB/c mice. However, milk-induced severe airway inflammation with approximately 1 000 000 vs. 80 000 cells per ml in B6 mice and BALB/c mice, respectively. Eosinophils comprised $70 \pm 0.7\%$ of airway infiltrate in B6 and $31 \pm 3.5\%$ in BALB/c mice compared with an absence of eosinophils in naïve mice. The number of perivascular and peribronchial infiltrates and eosinophils within the lungs in tissue sections reflected the differences observed in the airways. B6 mice had more inflammation and greater numbers of infiltrating eosinophils in the lungs than BALB/c mice. Similarly, mucus hypersecretion and milk-specific IgE and IgG1 levels were higher in B6 compared with BALB/c mice. Taken together, these results demonstrate a novel mouse model of cow's milk allergy in B6 mice. Moreover, we have observed genetic background differences in the immune response to milk. BALB/c mice are generally considered Th2-type responder animals, whereas B6 is a Th1-type strain. Thus, it is surprising that B6 mice have a significantly greater Th2 response to milk than BALB/c mice. Here, we present a useful, inexpensive, and clinically relevant mouse model of cow's milk allergy in B6 mice and demonstrate genetic differences between B6 and BALB/c to milk.

P020

Proteomic identification of new metal–protein interactions in human antigen-presenting cells reveals a potential link to metal-specific immune responses in human nickel allergy

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Background: Nickel (Ni) represents the most common occupational as well as public contact allergen, affecting 10–15% of the human population. However, the molecular events underlying this allergic disease still have to be elucidated. Moreover, Ni has been shown to be toxic and cancerogeneic. We have previously demonstrated that such Ni–protein interactions involving human serum albumin (HSA) lead to functional, Ni-specific human T-cell clone

activation. T-cell receptor – transfected cell lines were also activated in a Ni-dependent and HLA-restricted manner by such HSA-Ni metalloprotein-complexes (Thierse HJ *et al.* 2004, J Immunol, 172, 1926). With the aim of identifying unknown cellular Ni–protein interactions in human blood-derived professional antigen-presenting cells, we used *in vitro*-generated human dendritic cells (DC), as a model system for a metal-specific subproteomic approach (metalloproteome). Results were compared with Ni-interacting proteins in human B cells, which have just recently been described by our laboratory (Heiss K *et al.* 2005, Proteomics, 5, 3614).
Methods: Ni–protein interactions were detected via Ni-NTA-enrichment, 2-D electrophoresis, mass spectrometry, and database analysis. If possible, data were confirmed by Western blotting, graphite furnace atomic absorption spectrometry, and/or Biacore analysis.
Conclusions: In DCs, 32 of 57 isolated Ni-interacting proteins were identified. Comparative analysis of both cell types revealed differential Ni-interacting molecules in B cells and *in vitro*-generated human DCs. Among others, several chaperones/heatshock proteins were detected, which may be involved in Ni-epitope presentation and/or cellular stress responses toward heavy metal Ni. Functional understanding of these metal–protein interactions will potentially help to elucidate development and pathophysiology of T-cell-mediated human Ni allergy.

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P021

Activity of distinct vascular endothelial growth factor-2 gene regulatory sequences in ischemia-induced and inflammatory neovascularization

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Vascular endothelial growth factor (VEGF) and its high-affinity tyrosine kinase receptor VEGFR-2 are key players during embryonic vascular development and adult neovascularization, mediating proliferation, migration, and survival of endothelial cells. We previously established transgenic mouse lines that express the LacZ reporter gene under the control of distinct promoter/enhancer sequences of the *VEGFR-2* gene. Consistent with the endogenous VEGFR-2 expression, these mice showed endothelium-specific reporter gene expression throughout vascular development, which is down-regulated in most adult vessels. Moreover, the identified VEGFR-2 gene regulatory sequences conferred endothelium-specific LacZ reporter gene expression to the tumor vasculature as observed in three different tumor models *in vivo*. In this study, we analyzed the activity of the identified VEGFR-2 promoter/enhancer sequences in ischemia-induced and inflammatory angiogenesis *in vivo*. In the hindlimb ischemia model, most of the blood vessel in the ischemic region of the muscle tissue showed a strong endothelium-specific reporter gene expression. In contrast, in normoxic control tissue, there were nearly no LacZ-expressing capillaries detectable. Wound healing involves the formation of new blood vessels under inflammatory conditions. Therefore, we generated cutaneous punch wounds on the back of transgenic mice and examined the wound biopsies at different time points after wounding. At day 7 after wounding, the majority of the newly formed blood vessels in the wound tissue showed strong LacZ reporter gene expression. These results indicate that the regulatory sequences mediating the up-regulation of VEGFR-2 during ischemia-induced and inflammation-associated angiogenesis are contained in the promoter/enhancer sequences used. Taken together, our results suggest that the VEGFR-2 regulatory elements characterized in this study should allow the analysis of the transcriptional control mechanisms involved in the induction of VEGFR-2 expression in pathological neovascularization.

P022

Type I interferon-associated cytotoxic inflammation in lichen ruber planus

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Introduction: Lichen ruber planus (LP) is an inflammatory autoimmune skin disease of unknown origin. Evidence has accumulated that autoaggressive cytotoxic $CD8^+$ T lymphocytes cause destruction of keratinocytes. Recent studies suggested that type I interferons (IFNs) play a central role in cytotoxic skin inflammation by increasing the expression of IP10/CXCR10 and recruiting effector cells via CXCR3. Here, we investigated whether type I IFNs are also involved in the pathogenesis of LP.
Patients and methods: Skin biopsies of altogether 17 donors (seven LP and 10 healthy controls) were analyzed by immuno-histochemistry using monoclonal antibodies against CD3, CD4, CD8, CD20, CD68, CXCR3, granzyme B, IP10/CXCL10, CD123, and the MxA protein, which is specifically induced by type I IFNs.
Results: Our analysis revealed a significant expression of the MxA protein in all LP skin biopsies, indicating involvement of type I IFNs. Expression of MxA was closely associated with the recruitment of $CXCR3^+$ and granzyme B^+ lymphocytes, indicating a Th1-biased cytotoxic immune response. Strong expression of the CXCR3 ligand, the IFN-inducible protein IP10/CXCL10, links type I IFN expression and recruitment of $CXCR3^+$ lymphocytes. Plasmacytoid dendritic cells (DCs) appear to be a major source of type I IFNs in LP.
Discussion: Our observations support the hypothesis that lesional type I IFN produced by plasmacytoid DC plays an important role in chronic cytotoxic inflammation of LP by recruiting cytotoxic effector lymphocytes via IP10/CXCR3 interactions.

P023

A type I interferon-associated recruitment of 'skin homing' cytotoxic lymphocytes is characteristic for scarring skin lesions in discoid lupus erythematosus

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Introduction: Infiltrating T lymphocytes are considered to play a major pathological role in skin lesions of cutaneous lupus erythematosus (CLE), an autoimmune disease of unknown etiology. Earlier histological studies revealed that the inflammatory infiltrate in CLE skin lesions is predominantly composed of T lymphocytes but failed to explain the development of scarring skin lesions, characteristic for chronic discoid LE (CDLE). Because recent investigations highlighted the relevance of cytotoxic lymphocytes in autoimmune tissue destruction, we hypothesized that the scarring CDLE lesions might be caused by cytotoxic T lymphocytes.
Methods: All together, 15 CLE patients and five healthy controls were included. Skin biopsies were characterized by immunohistochemistry including staining for the cytotoxic molecule granzyme B, the skin homing molecule cutaneous lymphocyte antigen (CLA), the chemokine receptor CXCR3, and the protein MxA, which is, specifically induced by type I interferons

(IFN). Peripheral blood mononuclear cells were analyzed by flow cytometry.
Results: We found a strong coexpression of granzyme B and CLA on lesional lymphocytes of patients with scarring lCDLE and dCDLE, which was significantly enhanced when compared with non-scarring SCLE and healthy controls. These T cells were $CXCR3^+$. The increased expression of granzyme B was closely associated with the lesional expression of the type I IFN-induced proteins MxA and IP10/CXCL10. Interestingly, circulating $CXCR3^+$ cytotoxic T cells were significantly reduced in patients with widespread scarring skin lesions.
Discussion: Our results provide evidence that lesional activity of type I IFNs and potentially autoreactive cytotoxic lymphocytes targeting adnexal structures are highly associated with scarring LE lesions and might be responsible for their scarring character. The reduced numbers of $CXCR3^+$ cytotoxic T cells in the peripheral blood of patients with disseminated CDLE might refect the recruitment of these cells into the skin via CXCR3/IP10 interactions.

P024

Heterogeneity of T-cell clones infiltrating primary melanomas

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It is established that primary malignant melanomas (pMM) can be infiltrated by T-cell populations with predominantly one T-cell clone. As pMM generally express multiple tumor-associated antigens (TAA), here we used laser-capture microdissection (LCM) to isolate different tumor-infiltrating lymphocyte (TIL) clusters to determine whether pMMs are infiltrated only by one single clone or whether the TAA may attract various T-cell populations.

As T-cell receptor clonality is a useful tool for the demonstration of specific T-cell clones, we analyzed 56 pMM, three cutaneous melanoma metastases, and 15 pairs of pMM with a sentinel lymph node (SLN) for clonal rearrangements of the T-cell receptor ? chain gene (TCR).

We detected clonality of TCR γ chain gene in 25 of 56 pMM and in 10 of 17 SLN studied. In four of the 15 pairs of primary tumor and SLN, we found clonal TCR γ in both the melanoma and the SLN, with two pairs harboring the identical clone. We subsequently performed LCM in 21 pMM with multiple lymphocytic clusters for the presence of focal clonal T cells in different regions of the identical primary melanoma. In seven melanomas, both clusters of TIL showed the same rearranged TCR γ chain gene, and in five of the seven biopsies the clonal rearrangement occurred in different variable regions of the TCR γ chain gene. These tumors showed infiltration by more than one clone. In 10 biopsies, TCR clonality was restricted to one cluster, while the second microdissected sample of the infiltrate was polyclonal. In conclusion, within one pMM, several T-cell clones with different rearrangements may occur. The balance between these clones may decide on the progress of melanoma.

P025

The dying melanocyte in the aging hair follicle displays oxidative stress damage: a model for tissue-specific aging

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Increased oxidative stress is a common result of inflammatory as well as psychoemotional stress-exposure and has been associated with aging. Graying hair is one visible milestone on this road. We hypothesize that the continuous melanin synthesis in the growing (anagen) hair follicle generates high levels of free radicals selectively in hair bulb melanocytes. This endogenous oxidative stress may render hair bulb melanocytes highly susceptible to additional stressors. Their subsequent premature aging can serve as an indicator of the individual oxidative stress load and coping capacity. To test this hypothesis, we dissected human scalp skin anagen hair follicles from graying individuals and subjected them to macroscopic and immunohistomorphometric analysis and organ culture experiments. We found evidence of increased oxidative stress and melanocyte apoptosis in the pigmentary unit of hair follicles from graying individuals. Also, the 'common' deletion, a marker mitochondrial DNA deletion for accumulating oxidative stress damage, most prominently occurred in isolated graying hair follicles but not in pigmented and only rarely in unpigmented hair follicles of the same donors. Moreover, the unpigmented hair follicles showed better hair growth in culture than pigmented hair follicles of the same donor, while cultured pigmented hair follicles exposed to exogenous oxidative stress (hydroquinone) showed increased melanocyte apoptosis in the hair bulb. Against this background, we propose an 'oxidative theory of graying'. This process may be triggered and hastened by exogenous oxidative stress for example during inflammation or psychoemotional stress. Protection from oxidative stress should proof a useful measure to slow down graying.

P026

Skin and hair follicle innervation in experimental models: a review and guide for the exact and reproducible evaluation of neuronal plasticity

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Peripheral nerve fibers keep close contacts to and communicate with a wide variety of cells, which allows their signaling beyond the classical afferent and efferent functions. Upon depolarization, they expose their neighboring cells to a multitude of neuronal signaling molecules such as the neurotransmitters acetylcholine and noradrenaline or the neuropeptides substance P (SP), calcitonin gene-related peptide, peptide histidine methionine, neuropeptide Y, and many more. These mediators display a plethora of biological activities from immune modulation to growth factor activity and have lately gained considerable attention in the analysis of inert and specific cutaneous immune responses. In the skin, for example, the neuropeptide SP is involved in the regulation of hair growth, in neurogenic inflammation and in lymphocyte cytokine production. Inflammatory diseases and wound healing have been associated with plasticity of peripheral nerve fibers and nerve–immune interactions. However, quantitative analysis of peripheral innervation and nerve fiber–target cell interaction is rarely performed, and appropriate methods have been defined rudimentary and heterogeneously. We here review the available literature on quantification and functional analysis of cutaneous and hair follicle innervation in mammals and introduce a simple, fast, and highly reproducible quantitative method based on fluorescence immunohistochemistry for the exact quantification of peripheral nerve fibers. With our approach, nerve fiber–target cell interaction can be easily analyzed in relation to physiological and pathological tissue remodeling processes and in inflammation, and this method is transferable to other innervated peripheral organs.

P027

MAGE-A4 staining pattern differs in epithelial skin tumors of immunosuppressed and immunocompetent patients

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The human MAGE gene family encodes proteins (cancer/testis antigens) which are expressed in normal testis and in a variety of tumors. MAGE-derived peptides are recognized by $CD8^+$ T cells and represent targets for immunotherapy. Monoclonal antibody 57B predominantly detects MAGE-A4 protein. Studied mainly in melanoma, recent data suggest MAGE-A4 expression in epithelial cutaneous neoplasms. Frequency of many epithelial skin tumors is increased in immunosuppressed patients. We examined mAb 57B immunoreactivity (IR) in 119 formalin-fixed, paraffin-embedded specimens of epithelial skin tumors of actinic keratosis, bowenoid actinic keratosis, Bowen's disease, and squamous cell carcinoma ($n = 17$, 25, 61, and 16, respectively), in immunocompetent patients ($n = 84$), and organ transplant recipients ($n = 35$). Positive IR was defined as 5% or more positive tumor cells. In all positive samples, staining pattern and polarity of IR in the epidermis was assessed. Normal skin was immunonegative. All four epithelial skin tumors showed comparable IR ($P = 0.361$) ranging from 25 to 71% of samples. Absolute 57B IR was similar in the immunocompetent and immunosuppressed group, but scattered staining pattern was more frequent in immunosuppressed patients ($P = 0.025$). In squamous cell carcinoma, there was a polarity of IR within the basal layer ($P = 0.002$) compared with intraepithelial tumors. In conclusion, MAGE-A4 expression may help diagnosis and delineation of epithelial skin tumors. Invasiveness of tumors seems accompanied by increased MAGE-A4 expression on the leading cell front, possibly reflecting pronounced anaplasia. Cancer antigen expression as judged by MAGE-A4 expression displays subtle differences for organ transplant recipients only, suggesting other mechanisms underlying the increased cutaneous carcinogenesis in these high-risk immunosuppressed patients such as transforming growth factor (TGF)-β and vascular endothelial growth factor induction by calcineurine inhibitors.

P028

Epidermal structure and function in ichthyosis vulgaris

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Although a decreased/absent granular layer (GL) and reduced (pro) filaggrin expression are hallmarks of ichthyosis vulgaris (IV), its genetic etiology is still unknown, and it is unclear to what extent these alterations correlate with structural and functional abnormalities. In a cohort of 17 IV patients and 25 age- and sex-matched normal controls, we observed a persistence of corneodesmosomes upto higher stratum corneum (SC)-layers in IV on electron microscopy. Furthermore, there was a thinning of the cornified envelopes (CE) in the lower SC, which was associated with a reduced mechanical resilience of isolated CE exposed to ultrasound. Accordingly, SC integrity, assessed as protein removed per D-squame disc, was also abnormal in IV. Whereas skin surface pH was significantly increased in IV, the pH-gradient approached neutral values in deeper SC layers, and SC hydration was decreased in IV vs. controls. In addition, we noticed a significant increase in baseline transepidermal water loss as well as a delay in barrier recovery after acute barrier disruption by tape-stripping in patients vs. normal subjects. These functional abnormalities correlated with increased lanthanum tracer penetration into the extracellular domain of the SC and a disturbed architecture of the extracellular lamellar membranes. These observations were made regardless of GL presence with the exception of the thinning of the CE, which was predominantly observed in those IV subjects with absent GL. Finally, haplotype analysis in a pedigree with absent GL confirmed linkage to chromosome 1q21-22. Further dissection of this locus should provide the basis for identifying the molecular cause of the structural and functional abnormalities in IV.

P029

Towards a molecular classification of skin diseases: establishment of a reference database based on expression profiles of more than 800 patients

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Gene expression profiling has become increasingly important in the development of new tools and methods for early diagnosis, monitoring of disease progression, and for increasing the effectiveness of existing therapies. In line with this, strong efforts are being made to enable a more accurate skin disease diagnosis by applying combinations of marker genes, allowing for a classification of patients, and finally improving prognosis. In 2002, a most comprehensive expression analysis of the human skin transcriptome was started, with the aim to develop a fully automated microarray-based diagnostic and prognostic system for human skin diseases. Ten different skin conditions and three different expression profiling methods were included in the study setup to unravel the gene expression profile of human skin. Besides healthy human skin, congenital nevi, skin affected by three types of skin cancers, and skin biopsies of four types of inflammatory skin diseases were analyzed. Based on an extensive whole genome analysis, a topic-defined microarray was configured that was subsequently used to generate patient and disease specific expression profiles. The study focussed on deciphering diagnostic or prognostic relevant gene expression patterns in human skin diseases. In addition, a technological setup for a fast and reproducible gene expression profiling system was developed. The correlation of expression data and medical histories enabled a molecular classification of skin diseases that could help to evaluate, e.g. the metastasizing potential of primary melanomas, finally leading to an improved diagnosis or prognosis. By gaining new insights in the aetiology of the diseases, the development of drugs for the treatment of skin diseases could be facilitated. The expression patterns are going to be implemented in a reference database that will eventually allow a diagnostic or prognostic evaluation of new patient expression profiles, and in the future, it will help to improve existing diagnostic tools, for example by selecting an optimal therapy for a given patient.

P030 (V21)

Mosaicism of activating FGFR3 mutations in human skin causes epidermal nevi

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Epidermal nevi of the non-epidermolytic type are common congenital skin lesions with an incidence of about one in 1000

people, but their genetic basis remains elusive. Germline mutations of FGFR3 cause autosomal dominant skeletal disorders such as achondroplasia and thanatophoric dysplasia. Some of these patients also show acanthosis nigricans of the skin. As epidermal nevi and acanthosis nigricans share some clinical and histological features, we analyzed the presence of activating FGFR3 mutations in non-epidermolytic epidermal nevi.

We used a SNaPshot multiplex assay to screen for nine previously described activating FGFR3 point mutations in 33 patients with clinically and histologically confirmed epidermal nevi of the non-epidermolytic type.

Activating FGFR3 mutations were identified, almost exclusively at codon 248(R248C), in nine of 33 (27%) epidermal nevi. The mutations were not present in adjacent normal skin, which was available in three patients.

Our results provide evidence that a significant proportion of epidermal nevi is caused by a mosaicism of activating FGFR3 mutations in the human epidermis, resulting from a postzygotic mutation in early embryonic development. The R248C mutation appears to be a hotspot for FGFR3 mutations in epidermal nevi.

P031

Adenoviral-mediated reconstitution of enzymatic protoporphyrinogen oxidase activity in a cell culture model of variegate porphyria

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Variegate porphyria (VP) arises from a decrease in the activity of protoporphyrinogenoxidase (PPOX), the seventh enzyme in the pathway of heme biosynthesis. This rare disorder is inherited in an autosomal dominant fashion and caused by mutations in the *PPOX* gene. Clinically, skin symptoms on the sun-exposed areas of the body and life-threatening acute porphyric attacks might manifest. Protection from UV light can prevent the occurrence of cutaneous symptoms and acute attacks are treated symptomatically with heme-arginate infusions, but, currently, there is no causal therapy for the disease. One possible causal treatment would be the reconstitution of enzymatic activity by gene therapy. As a initial step, we reconstituted PPOX activity in a cell culture model of VP by gene transfer using a recombinant adenoviral system. The cell culture model consisted of Epstein–Barr virus-immortalized peripheral blood mononuclear cells from patients with VP. In all cell lines, decreased enzymatic activity was shown by *in vitro* assays. An adenoviral vector containing the coding sequence of the human PPOX cDNA under transcriptional control of a CMV promoter was constructed. Transfection of the vector into the cultured cells resulted in expression of the recombinant human PPOX mRNA and, subsequently, reconstitution of enzymatic activity. Titration curves correlating transfection dose, mRNA expression, and enzymatic activity were determined. For the first time, we herein demonstrate adenoviral-mediated restoration of PPOX activity in a model system of VP as an initial step toward gene therapy for this disorder.

P032 (V04)

Bathing suit ichthyosis is associated with transglutaminase 1 deficiency

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Bathing suit ichthyosis (BSI), a unique clinical type of lamellar ichthyosis (LI), has been especially described for LI individuals in the African population. Its molecular pathology is unclear so far.

We ascertained three independent families from Germany and Turkey with individuals suffering from BSI. Affected individuals were born with collodion membrane and developed large, dark scales on the trunk, whereas the four limbs and the face were almost completely spared. Sequencing analyses revealed different missense mutations in TGM1, the gene encoding transglutaminase-1 (TGase-1). One patient showed the homozygous new mutation Tyr276Asn. The three-dimensional in silico modeling of Tyr276Asn based on the known atomic structure of human factor XIIIa did not appear to introduce any structural modifications or errors in protein folding. However, the *in situ* monitoring of the TGase activity, which was performed with skin samples taken from the involved areas of the trunk of the Tyr276Asn patient, showed a deficiency of TGase activity. Interestingly, immunohistochemistry using a monoclonal anti-TGase-1 antibody showed a markedly reduced expression of the protein. In contrast, samples of healthy skin of the limbs revealed a normal distribution zone of TGase-1 protein as well as activity. Ultrastructural analyses of the affected skin revealed morphological signs (such as cholesterol clefts in the SC) typically seen in LI, but the ultrastructure of healthy skin areas was again completely normal.

We conclude that BSI is a distinct phenotype of LI, which is associated with mutations in TGM1. The BSI phenotype poses a biological riddle as far as the difference between healthy and diseased skin areas is concerned. The *in vivo* lack of TGase activity may be attributable to altered protein–protein interactions, solvent molecules, or to pH of different skin areas.

P033

A large genomic deletion in the *KIND-1* gene – a novel mutational mechanism in the Kindler syndrome

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Kindler syndrome (KS) is a rare autosomal recessive genodermatosis characterized by skin blistering, photo- sensitivity, and progressive poikiloderma. It is caused by mutations in the *KIND-1* gene located on 20p12.3. So far, over 20 mutations were reported: deletions and insertions of one nucleotide or point mutations that lead to PTC and absence of the KIND-1 gene product, kindlin-1 protein. However, conventional mutation detection strategies failed to disclose mutation in a number of KS families. In this study, we report for the first time a large Alu-mediated genomic deletion in the *KIND-1* gene in two unrelated Italian patients with KS. By quantitative real-time polymerase chain reaction (PCR), we demonstrated that the patient 1 was homozygous for the genomic deletion, while her parents and patient 2 and her father were heterozygous. Using long-range PCR and subcloning, we characterized the 3.9-kb deletion spanning from IVS9 −2165 and IVS11 +449. The breakpoint was embedded in two identical 30-bp sequences, both parts of AluSx repeats, suggesting that the deletion resulted from homologous recombination, as Alu elements are often involved in genomic rearrangements and insertions. The KIND-1 sequence spanning exon 9 to exon 12 is composed of

42.5% of repetitive elements. The phenotype of the KS patient with the large deletion did not show any particularities. These results emphasize the need to design alternative mutation detection strategies for genes rich in Alu repeats. In particular, this holds true for KS.

P034

Congenital ichthyosis: mutation distribution between the genes *TGM1*, *ALOX12B*, *ALOXE3* and *ichthyin*

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Autosomal recessive congenital ichthyoses (ARCI) form a group of rare, severe disorders of keratinization with a prevalence of one in 100 000–200 000 in the European population and in the US. Phenotypes and genetic etiology are both extremely heterogeneous. Up to date five different gene loci were described: TGM1 on 14q11.2, ALOX12B and ALOXE3 on 17p13.1, ABCA12 on 2q34-q35, and the gene for ichthyin on 5q33. Two loci were described on chromosome 19 but corresponding mutations underlying ARCI are still unknown. Mutations in TGM1 were found in approximately 40% of all patients with ARCI. We could recently show that at least 12% of all patients with a European background have mutations in one of the genes *ALOX12B* or *ALOXE3*. Our latest investigations demonstrates that also at least 12% of all ARCI patients from Central and Eastern Europe, Turkey, and India had mutations in the gene coding for ichthyin on chromosome 5q33.3. We analyzed 244 samples from unrelated ARCI patients. Only a small part of these patients presented a consanguineous family background. We found six different mutations, of which two have been described previously. Mutations in ABCA12 were only found in patients from North Africa so far. Summarizing TGM1, ALOX12B, ALOXE3, and the gene for ichthyin covers about 60% of all ARCI cases.

Our main focus is a better understanding of the correlation between the phenotype and the underlying genotype. This will be done by further analyzing the metabolic pathways and cellular localizations of wildtype and mutated proteins.

P035

Variations of the *MC1R* and the *GSTM1* and *T1* genes in cutaneous malignant melanoma

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Variations in the melanocortin-1 receptor gene (*MC1R*) and in the glutathione-S transferase genes μ1 (GSTM1) and θ1 (GSTT1) have been reported to influence UV sensitivity and to be associated with melanoma risk. *MC1R* is one of the major genes that determine skin pigmentation because the melanocortin-1 receptor regulates eumelanin synthesis. GSTT1 and GSTM1 are enzymes expressed in the skin that detoxify products of oxidative stress reactions occurring in response to UV irradiation. In this study, we investigated whether variations in MC1R, and homozygous deletions in GSTM1 and GSTT1, are associated with melanoma and assessed the relation of these variations with phenotypic risk factors of cutaneous malignant melanoma.

The region of the *MC1R* gene containing the majority of genetic variations was sequenced and the D294H polymorphism analyzed by RFLP in 347 healthy controls and 322 melanoma patients recruited in Germany. GSTM1 and GSTT1 null genotypes were analyzed by multiplex polymerase chain reaction. In addition, phenotypic characteristics of the study participants were recorded.

Of the recorded phenotypic characteristics, fair skin type, high nevus count, and high age were independently associated with melanoma ($P < 0.001$). We found an association of melanoma risk with carriage of the rare alleles of the MC1R D84E and R151C variants [odds ratio = 4.96, 95% CI (1.06–23.13), $P = 0.0315$, and odds ratio = 1.69, 95% CI (1.12–2.55), $P = 0.0130$, respectively]. Melanoma risk significantly increased with the total number of non-synonymous MC1R variants ($P = 0.0030$). In our study population, only the D84E polymorphism influenced melanoma risk independent of phenotypic risk factors ($P = 0.0468$). There was no association of GST null variants with overall melanoma risk. In contrast to earlier results, there was also no significant effect of the GSTM1 null variant on melanoma risk in the subgroup of individuals with reddish or fair hair.

P036 (V36)

Organ-specific leukocyte recruitment through the multifunctional junctional adhesion molecule-B

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Junctional adhesion molecules (JAM)-A, JAM-B, and JAM-C have been shown to mediate leukocyte transmigration. However, more recently, JAM-C has been shown to additionally mediate firm adhesion of leukocytes, and JAM-B has been demonstrated to bind the leukocyte integrin VLA-4, which mediates rolling and firm adhesion in skin microvasculature through an interaction with VCAM-1. We therefore hypothesized that in addition to mediating transmigration, JAM-B is also involved in leukocyte rolling and firm adhesion. To address this, using a flow chamber, isolated human T cells were perfused over JAM-B-coated slides, showing JAM-B-dependent rolling and sticking interactions of T cells at low shear stress (0.2 dyn). At higher shear forces (1.0 dyn), no interactions were observed, indicating that JAM-B is only functionally active at low shear stress. We next used blocking antibodies in our flow chamber system. Preliminary analysis ($n = 2$) shows a dependency of JAM-B-mediated rolling and sticking on T-cell VLA-4 expression, as both an α4- and a β1-integrin function blocking antibody reduced the interactions. Combination of both antibodies had no additional effect, indicating that VLA-4 not other integrins are involved. Using intravital microscopy of murine skin microvasculature, we also obtained preliminary results, indicating that rolling interactions of murine leukocytes *in vivo* is also affected by antibody blockade of JAM-B. To test, if JAM-B mediated leukocyte–endothelium interactions are functionally relevant, a contact hypersensitivity reaction was elicited in mice using DNFB. Injection of a function blocking JAM-B antibody prior to challenge consistently reduced the inflammatory response

evoked by DNFB in a dose-dependent fashion. In contrast, using adoptive transfer experiments, no effect of JAM-B blockade is observed in the sensitization phase, indicating that JAM-B organ specifically governs leukocyte transmigration through multifunctional activities.

P037

Critical involvement of P-selectin in the sensitization phase of cutaneous hypersensitivity

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Cutaneous hypersensitivity reactions depend on a sensitization and effector phase. During sensitization, naïve T cells home into lymph nodes, where antigen presentation and clonal expansion occur. While expression of L-selectin is high on naïve T cells, effector T cells have only a small amount of this adhesion molecule expressed on the cell surface. Hence, L-selectin has been implied as the most important adhesion molecule for entry of lymphocytes into peripheral lymph nodes. We here however provide evidence that P-selectin contributes to an at least similar extent to lymphocyte extravasation to lymph nodes. Wildtype (wt), L-selectin-deficient (L-sel$^{-/-}$) or P-selectin-deficient (P-sel$^{-/-}$) mice were sensitized with DNFB. Five days after DNFB-exposure, mice were sacrificed and leukocytes were isolated from peripheral lymph nodes and spleens and i.v. injected into recipient wt mice, which were subsequently exposed to DNFB on the right ear. As previously described, L-selectin significantly contributes to sensitization in the model of DNFB-induced cutaneous contact hypersensitivity. However, P-selectin also is critically involved in generating immunity towards DNFB. By comparing the relative contribution of L-selectin and P-selectin in this process, the contribution of P-selectin seems more detrimental (Δear swelling: 40 ± 12, 31 ± 10 and $19 \pm 11\,\mathrm{cm} \times 10^{-3}$ for wt, L-sel$^{-/-}$ and P-sel$^{-/-}$ mice, respectively). To further elucidate the relative contribution of endothelial vs. platelet P-selectin, using bone marrow transplantation, we have generated mice selectively deficient in platelet-P-selectin expression. In addition, P-selectin dependency of lymphocyte interaction with the microvasculature of lymph nodes are currently investigated. From the data gained so far, we were able to show that P-selectin is critical for generation of immunity toward DNFB. Hence, homing to peripheral lymph nodes relies not only on L-selectin expression but also on P-selectin.

P038 (V20)

Chronification of allergic contact dermatitis decisively depends on the T-helper 1 cytokine osteopontin

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We have demonstrated that the T-helper (Th)1 cytokine osteopontin (OPN) is crucial during the sensitization phase of contact hypersensitivity (CHS), for guiding skin dendritic cells into lymph nodes, simultaneously instructing them to adopt a Th1-inducing phenotype. In mice, OPN deficiency is associated with a reduced acute CHS response. We now investigated OPN functions during the chronification of allergic contact dermatitis. Immunohistochemistry revealed low OPN expression in normal human skin. In acute allergic contact dermatitis predominantly perivascular leukocytes and endothelial cells stained positive for OPN. In chronic eczema, OPN was highly expressed in the inflammatory infiltrate and by keratinocytes. Double immunohistochemistry revealed that CD45RO^{+} infiltrating T cells, antigen-presenting cells, and microvascular endothelial cells express OPN and its receptors CD44v and avß3 integrins. This regulated expression correlated with high-quantitative OPN mRNA expression in contact dermatitis compared with unaffected skin of the same donor. To determine OPN secretion by nickel-specific T cells, CD4^{+} T-cell clones were generated from allergic donors, and their secretion of interferon (IFN)-γ, interleukin (IL)-4, and OPN was measured upon $NiSO_4$ stimulation. These T-cell clones constitutively secreted OPN, which was not modulated upon antigen restimulation. However, when comparing the cytokine patterns of different clones, IFN-γ high, IL-4 low clones produced little OPN, but IFN-γ low IL-4 high clones secreted fourfold the amount, indicating that in antigen-specific T-cells, OPN may function as a redundant Th1 cytokine. Because OPN was highly up-regulated by keratinocytes in chronic eczema, their modulated expression of OPN by inflammatory cytokines was determined. Among a panel of cytokines tested, only IFN-γ specifically induced high OPN expression. To test the *in vivo* function of OPN for the chronification of CHS, OPN wildtype or OPN-deficient mice were repetitively challenged for 1 month. OPN-deficient animals constantly displayed a reduced chronic inflammation measured by ear swelling. In conclusion, regulated OPN secretion and OPN receptor expression by different cells types is essential in the chronification process of allergic contact dermatitis.

P039

Regulated osteopontin production by dendritic cells is crucial for their migratory capacity

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We have previously demonstrated that osteopontin (OPN)-deficient mice are impaired in their capacity to mount a contact hypersensitivity (CHS) response. When analyzing the source of OPN during the sensitization phase of CHS, we found that DC in draining lymph nodes express OPN mRNA. We therefore investigated OPN secretion by DC during their differentiation and analyzed its functional relevance for their migration. When dendritic cells (DC) were differentiated from murine bone marrow (BM) by GM-CSF and interleukin (IL)-4, OPN highly accumulated in such cultures. When quantifying OPN mRNA and the secreted protein from DC-rich and DC-depleted BM cells, we identified DC as the major source of OPN throughout BM culture. Immature DCs on day 6 of culture were matured by IL-4, interferon (IFN)-γ, TNF-α & #61484; IL-1α or lipopolysaccharide (LPS) in the presence of GM-CSF. In the absence of cytokines, DCs secrete low amounts of OPN, which was increased by a combination of GM-CSF and IL-4. When IL-4 was replaced by TNF-α or IL-1α, which are both established pro-migratory cytokines, a strongly up-regulated OPN secretion was observed. In contrast, DC immobilized following terminal maturation by LPS did not secrete OPN. Furthermore, when *in vitro* comparing OPN-deficient DC with DC from OPN wildtype (wt) mice, OPN knockout cells were diminished in their chemotactic ability. We tested the *in vivo* relevance of these findings by i.c.

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injecting OPN-deficient DC into OPN wt mice and found that these cells were impaired in their ability to migrate into lymph nodes. In conclusion, OPN production by DC is differentially modulated by inflammatory and migration-promoting cytokines, and loss of OPN production leads to impaired DC migration *in vivo*.

P040

Immunoglobulin G autoantibodies targeting distinct regions of the desmoglein 3 ectodomain correlate with the activity and clinical phenotype of pemphigus vulgaris

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Pemphigus vulgaris (PV) is an autoimmune blister disease caused by autoantibodies primarily against the desmosomal adhesion molecule Dsg3. The aim of the study was to relate immunoglobulin (Ig)G reactivity of PV sera against defined regions of the extracellular domain of Dsg3 with disease activity and clinical phenotype of PV. We thus developed an enzyme-linked immunosorbent assay utilizing baculovirus-encoded recombinant proteins, representing the five extracellular domains (EC1 to EC5) of Dsg3, including a recently described NH2-terminal immunodominant epitope within the EC1 which were tested with sera of PV patients and controls. Overall reactivity and the titers of IgG reactive with the Dsg3 ectodomain (aa1–566) significantly correlated with disease activity of the PV patients. In addition, the presence of IgG against the EC2, EC3, and EC4 was correlated with disease activity while IgG against the COOH-terminus (EC5) was not. Titers of domain-specific IgG were not correlated with disease activity. Investigation of IgG reactivity of PV patients with different clinical phenotypes (mucosal, mucocutaneous, and cutaneous) showed that IgG antibodies against the EC1–4 were less prevalent in cutaneous (i.e. chronic) than in mucosal (i.e. acute onset) PV. In mucosal and mucocutaneous PV, IgG antibodies against the EC1, EC2, EC3, EC4 were more prevalent than in cutaneous PV.

These data suggest that (1) there is a prevalence of IgG reactive with the NH_2-terminus (EC1) of Dsg3 in PV, (2) the prevalence of IgG against EC2, EC3, and EC4 tends to decrease in the course of the disease, (3) the prevalence of IgG against the EC1-4 of Dsg3 seems to decrease upon transition of mucosal and mucocutaneous PV to cutaneous PV. These findings may be extremely useful in defining predictive disease markers and for understanding the relative contribution of IgG autoantibodies in PV.

P041

Dimethyl fumarate blocks T-cell adhesion to selectins *in vitro*

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Esters of the fumaric acid are commonly used to treat moderate to severe psoriasis. Thus far, the modes of action by which fumaric acid esters act in improving symptoms of this mainly Th1-driven inflammation are only partially understood. So far, evidence is accumulating for the therapeutically active dimethyl fumarate (DMF) exerting signals via activation of the transcription factor NF-κB. We investigated the effects of DMF on T-cell activation via the pathophysiologically relevant stimulation by the bacterial superantigen TSST-1. Using flow cytometry on $CD3^+$ T cells, we show that DMF dose-dependently reduces surface expression of the skin-homing receptor 'cutaneous lymphocyte antigen' (CLA) and CD25. Reduced CLA expression results in decreased binding to P-selectin and E-selectin. No alteration was observed for HLA-DR and CD54 (ICAM-1). An increased expression was shown for the early T-cell activation marker CD69 causing synthesis of the anti-inflammatory cytokine transforming growth factor -β. Using a cytometric bead array assay, we investigated the cytokine and chemokine expression pattern of DMF-treated human peripheral blood mononuclear cells. No changes were observed for the expression of Th1-cytokines interleukin (IL)-2 and interferon-γ, whereas expression of Th2 cytokines such as IL-4, IL-6, and IL-10 was increased following DMF treatment. Increasing concentrations of DMF reduced dose-dependently the expression of MCP-1/CCL2 and MIG/CXCL9 and led to a strong up-regulation of RANTES/CCL5. Further on, we show the regulation of important inflammatory mediators of the prostanoid pathway following DMF treatment. Taken together, our results support a more diverse role of DMF in immune regulation and inflammation. In addition, we provide evidence that expression of adhesion molecules, as well as binding to selectins, is impaired after DMF treatment, thus interfering with the process of lymphocyte extravasation. These effects may act synergistically in the treatment of psoriasis.

P042 (V02)

Altered lymphocyte trafficking and differentiation leads to an increase in $CD3^+$ $CD4^-CD8^-$ double-negative T cells in β2 integrin-deficient mice

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β2 integrins form four different heterodimeric membrane receptors in combination with four α-chains (CD11/CD18). These are differentially expressed only on leukocytes and contribute crucially to transmigration and re-circulation also of lymphocytes. Using mice deficient in CD18 ($CD18^{-/-}$), we showed that loss of CD18 led to a defective homing of lymphocytes to secondary lymphoid organs, whereas an aberrant localization to (non-lymphoid) tissues within the lung and liver occurred.

To investigate the reasons underlying an aberrant homing of lymphocytes, we now analyzed phenotypic and functional changes of re-circulating T lymphocytes in primary and secondary lymphoid tissues of $CD18^{-/-}$ mice using *in vivo* adoptive cell transfers, flow cytometry, fluorescence microscopy, and *in vitro* proliferation assays. We found that a decrease in mature $CD4^+$ and $CD8^+$ T cells, which was due to a defective homing, was contrasted by high numbers of $CD3^+$ $CD4^-CD8^-$ (DN) T lymphocytes in the peripheral lymphoid organs of $CD18^{-/-}$ mice. These DN T lymphocytes had either αβ or γδ T-cell receptor (TCR) composition. As also TCR-αβ T cells lacked the coreceptors CD4 and CD8, this clearly classified them as either aberrantly differentiated, accumulated, or aberrantly located T cells. Interestingly, these cells showed an activated, antigen-experienced phenotype characterized by high expression of CD44 and CD25 and down-regulated CD45RB. When cultured *ex vivo*, they were highly responsive to exogenous interleukin-2 (IL-2) supplementation. Despite their expression of CD25 and response to IL-2, TCR-αβ DN cells did not express the transcription factor Foxp3, thus widely ruling out that they were regulatory T cells.

We here show that β2 integrins not only guide transmigration and re-circulation but also critically influence T-lymphocyte maturation and differentiation. Adoptive transfers of $CD18^{-/-}$ cells onto RAG-deficient vs. athymic nude mice are now on the way to elucidate the role of thymus in the development of the abnormal T-cell phenotype in $CD18^{-/-}$ mice.

P043

Ex vivo analysis of autoaggressive desmoglein 3-reactive T cells in pemphigus vulgaris using peptide-HLA class II tetramers

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Pemphigus vulgaris (PV) is a severe autoimmune bullous skin disorder associated with T-cell-dependent immunoglobulin G autoantibodies against desmoglein 3 (Dsg3). $CD4^+$ T-cell epitopes of Dsg3 have been shown to be restricted by the PV-associated HLA class II alleles, DRB1*0402, and DQB1*0503. The goal of this study was to directly analyze functionally relevant Dsg3-reactive T-cell subsets in PV. We thus developed a HLA class II tetramer-based detection system using DRB1*0402 tetramers loaded with Dsg3 peptides known to be recognized by Dsg3-reactive T-cell clones (TCC). Dsg3-responsive TCCs were incubated with PE-labelled tetramers (10 μg/ml) and varying concentrations of Dsg3 peptides (0–50 μg/ml) for 1 h in RPMI 1640 medium and 10% human serum at room temperature, washed, and analyzed by flow cytometry. TCC showed specific staining with tetramers loaded with their specific Dsg3 peptide while incubation of the TCC with irrelevant Dsg3 peptide tetramers did not show any specific binding. A similar degree of tetramer staining was observed with murine Dsg3-reactive T-cell hybridomas, generated from HLA-DR0402-DQ8 transgenic mice. As the characterized epitopes of Dsg3 share similar binding motifs for the PV-associated HLA-DR0402 molecule, crossreactivities between different peptides and tetramers had to be excluded. Therefore, we stained the TCC together with their Dsg3 peptide-specific tetramers and increasing concentrations of exogenous specific ± unrelated Dsg3 peptides. We saw a dose-dependent specific binding of TCC to the tetramers with the agonistic Dsg3 peptide which was not inhibited by increasing concentrations of unrelated Dsg3 peptides. To estimate the sensitivity of detecting Dsg3-reactive T cells using HLA class II tetramers, we added different numbers of TCC to peripheral blood mononuclear cell (PBMC) from a healthy control. Dsg3-specific T cells were detected at a frequency of >1 per 100 PBMC (1%). HLA class II tetramers may thus provide a unique approach to monitor *ex vivo* the cellular immune response during the clinical course of PV.

P044

CD137 ligand induced differentiation of human dendritic cells to potent activators of antigen-specific T cells

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Tumor necrosis family receptor protein CD137 is a potent T-cell coactivator, and CD137 agonists are successfully used in cancer therapy models. Recent evidence suggests bidirectional modes of CD137 receptor/ligand signaling, enabling immune cell crosstalk. We investigated the bidirectional function of CD137 ligand in human monocyte-derived dendritic cells (MoDCs) and its role in T-cell activation and priming. We find that CD137 ligand activation on immature MoDCs leads to partial cell differentiation, with up-regulation of maturation markers CD83, CXCR4, and CCR7, as well as of HLA-DR and the T-cell costimulatory molecule CD86. In addition, we detect significant levels of secreted TNF-α. Cocultivation of allogenic T cells with CD137 ligand differentiated MoDCs leads to interferon-α release, whereas secretion of interleukin-10 decreases. Importantly, such MoDCs provide strong T-cell costimulatory signals in an autologous and antigen-specific T-cell/DC reaction, leading to T-cell proliferation and shifting the T-cell response toward a Th1 cytokine pattern.

Our findings deliver new insights into the relatively unknown bidirectional signaling capacity of the CD137 receptor/ligand system related to T-cell and DC interaction and may also enable improved therapeutic strategies in cancer, infection, and autoimmune diseases that take CD137 ligand signaling into account.

P045

Balance between Langerhans cells and inflammatory dendritic epidermal cells in eczema

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Two different dendritic cell subtypes are the key players in the epidermal skin lesions of atopic eczema (AE): Langerhans cells (LC), which are localized in the lesional and non-lesional skin and inflammatory dendritic epidermal cells (IDEC), which are recruited into the skin at the initiation of AE and only present at inflammatory epidermal sites. Considering the pathophysiology of AE, IDECs are regarded as the main amplifiers of immunogenic allergic inflammatory immune responses in the skin, while LCs have been shown to be capable to some degree to induce immuno-tolerance in a constantly antigen-exposed epidermal environment. Data supporting the idea of a critical balance between LC and IDEC in AE in this context are still lacking. In the last decade, calcineurin inhibitors such as tacrolimus (FK506) have been successfully introduced as an effective treatment of AE. Furthermore, it has been described that tacrolimus treatment diminishes the number of IDEC cells in the skin and restores the physiological supremacy of LC, although the underlying immune mechanism leading to this phenomenon remains unknown. We observed that treatment of differentiating DC from patients with AE with tacrolimus induces the production of transforming growth factor (TGF)-β by DC, which triggers the generation of high numbers of LC in a TGF-β-related manner. Further on tacrolimus modifies the expression coinhibitory molecules involved in the induction of tolerogenic mechanisms such as B7 molecules on the surface of LC while decreasing the stimulatory capacity of LC toward autologous and allogeneic T cells in T-cell proliferation assays. Interestingly, the down-regulation of the stimulatory capacity of tacrolimus-treated LC corresponds with the induction of T cells with a high IL-10 and TGF-β-producing capacity. Besides, we could show that the suppression of T-cell responses initiated by tacrolimus-treated LC is dependent on the presence of $CD4^+CD25^+$ regulatory T-cell subsets within the T-cell fraction. From our data, a picture emerges that the tacrolimus-promoted differentiation of LC from precursor cells recruited into the skin and DC present within the skin might skew the balance from immunogenic IDEC toward tolerogenic LC aimed at targeting the function of LC as potential natural silencers of allergic inflammatory immune response.

P046

Tissue targeting of cytotoxic T cells by dendritic cell immunization in melanoma defense

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We have recently shown that different routes of dendritic cell (DC) immunization lead to the generation of effector/memory $CD8^+$ T cells expressing different arrays of tissue-specific homing receptors. This polarization is driven *in vivo* by tissue-specific DC and involves soluble factors. Homing receptor polarization can be flexibly switched by such tissue-specific DC even on memory T cells. In our current study, we have begun to investigate the role of homing receptor polarization by DC vaccination for antitumor immunity in the mouse model of B16.F10 melanoma given subcutaneously or intravenously in both protective and therapeutic settings. We use the parental tumor as well as B16.F10GP33 which expresses a T-cell epitope from the glycoprotein of lymphocytic choriomeningitis virus.

We examined the role of the route of DC immunization for the homing of T-cell receptor transgenic P14 effector/memory T cells which recognize GP33 and its correlation with the ability to control tumor growth.

We adoptively transferred naive P14 T cells and activated them *in vivo* with GP33-pulsed DC via the intracutaneous (i.c.), intravenous (i.v.), or intraperitoneal (i.p). route. After subcutaneous inoculation of B16.F10 or B16.F10GP33 melanoma cells into the left and right flanks of C57BL/6 mice, respectively, tumor size was measured every 2 days. GP33-pulsed DC prevented the growth of B16.F10GP33 tumor in all groups independent of the immunization route. Thus, induction of skin-specific homing receptors upon i.c. DC injection or the lack of a tissue-specific polarization or flexible reprogramming when DCs are injected i.v. or i.p., respectively, may allow efficient tumor defense.

Currently, we are determining the frequency and homing receptor profiles of effector T cells in spleen and various lymph nodes after DC vaccination. Furthermore, we analyze the resulting efficiency of tumor immunity under suboptimal conditions, i.e. a limiting number of tumor-specific effector T cells to determine whether skin targeting of T cells via i.c. DC immunization is superior to i.v. or i.p. immunization or whether the flexible reprogramming of homing receptor patterns will enable efficient tumor immunity independent of the route of DC vaccination. Our findings are relevant for the biological therapy of human cancers.

P047

The impact of a chronic delayed-type hypersensitivity reaction on leukocyte migration in alopecia areata

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Induction and maintenance of a mild chronic eczema can be curative in alopecia areata (AA). The underlying mechanism is unknown. We here explored whether treatment with the contact sensitizer SADBE may have an impact on leukocyte migration such that either AA antigen carrying dendritic cell (DC) migration toward the draining lymph node or that the recruitment of activated T cells toward the skin becomes hampered.

SADBE treatment of AA-affected mice was not accompanied by a significant decrease in skin infiltrating leukocytes nor draining lymph node cells. Instead, there was a striking increase in chemokine and chemokine receptor expression in the skin and the draining lymph nodes. In addition, the distribution of leukocyte subsets was changed with a dominance of monocytes in the skin and a reduced percentage of DC in the draining lymph nodes. Irrespective of SADBE treatment, lymph mode cells (LNC) from AA-affected mice showed high migratory activity *in vitro* and intravenously applied fluorescent dye-labeled LNC of AA-affected and of SADBE-treated mice readily homed in skin draining lymph nodes and the skin of SADBE-treated, AA-affected mice. Labeling of the skin with a fluorescent dye revealed that in SADBE-treated mice, a reduced number of antigen-presenting cells migrated from the skin toward the draining lymph node. In addition, a high percentage of $CCL20^+$, $CCR6^+$, and $CCR8^+$ leukocytes was retained in the skin. In line with these findings was the poor recovery of DC in draining nodes of AA-affected, SADBE-treated mice after subcutaneous application of a dye-labeled mixture of $CD11b^+$ and $CD11c^+$ cells.

Thus, induction of a chronic contact eczema apparently has no impact on effector T-cell migration toward the skin. Instead, in AA-affected mice, that received topical application of a contact sensitizer, the migration of DC toward the draining lymph node is severely impaired. Reduced antigen transfer and presentation with the consequence of insufficient T-cell activation could well contribute to delayed-type hypersensitivity-induced prevention of AA progression and to hair regrowth.

P048

A new way to generate cytolytic tumor-specific T cells: electroporation of RNA coding for a T-cell receptor into T lymphocytes

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Effective T-cell receptor (TCR) transfer until now required stable retroviral transduction. However, retroviral transduction poses the threat of irreversible genetic manipulation of autologous cells. We therefore used optimized RNA electroporation for transient manipulation. The transfection efficiency, using EGFP-RNA, was $\geq$90%. The electroporation of primary T cells, isolated from blood, with TCR coding RNA resulted in functional cytolytic T lymphocytes (CTLs) ($\geq$60% killing at an effector : target ratio of 20:1) with the same HLA-A2/gp100 specificity as the parental CTL clone. The TCR-transfected T cells specifically recognized peptide-pulsed T2 cells, or dendritic cells (DCs) electroporated with gp100-coding RNA, in an interferon-γ-secretion assay and retained this ability, even after cryopreservation, over 3 days. In addition, the transfected T cell maintained their functionality after cryopreservation. DCs electroporated with gp100-coding RNA were also specifically recognized. Most importantly, we show here for the first time that the electroporated T cells also displayed cytotoxicity and specifically, lysed peptide-loaded T2 cells and HLA-A2-positive/gp100-positive melanoma cells over a period of at least 72 h. Peptide-titration studies showed that the lytic efficiency of the RNA-transfected T cells was similar to that of retrovirally transduced T cells and approximated that of the parental CTL clone. Functional TCR transfer by RNA electroporation is now possible without the disadvantages of retroviral transduction and forms a new strategy for the immunotherapy of cancer.

P049 (V31)

Functional chimeric E/L-selectin expression by RNA transfer into dendritic cell: targeting of dendritic cells from blood to lymph nodes

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For vaccination strategies, it is essential that dendritic cells (DC) reach peripheral lymphatic tissue, usually peripheral lymph nodes, to efficiently exert their immunogenic function. However, only a small portion of intracutaneously injected DC reach the draining lymph node. In contrast to T lymphocytes, unmodified monocyte-derived DCs are unable to extravasate from the bloodstream into the lymph nodes. Therefore, we used RNA transfections to manipulate human DC to express E/L-selectin, a chimeric protein consisting of the extracellular domain of E-selectin and the transmembrane and intracellular domain of L-selectin. The transfection efficiency was $\geq$90%, and surface expression could be detected for more than 48 h. Mock- and E/L-selectin-transfected DC displayed the same expression pattern of the surface markers CD25, CD80, CD83, CD86, HLA classes I and class II and had the same CCR7-mediated migratory capacity *in vitro*. E/L-selectin RNA and MelanA RNA coelectroporated DC expanded MelanA-specific T cells like DC transfected with MelanA RNA alone. In contrast to mock-transfected DC, the E/L-selectin-transfected cells attached to and rolled on sialyl LewisX, L-selectin's natural ligand, in a parallel plate flow chamber assay. To confirm E/L-selectin function *in vivo*, E/L-selectin electroporated murine DC were injected intravenously in mice. The E/L-selectin transfected DC but not the mock-electroporated DC were able to enter the lymph nodes, while both were found in the spleen. The presented method is easily clinically applicable, as no genetic manipulation or viral transformation is involved and allows intravenous injection of the DC vaccine for targeting of all peripheral lymph nodes. This could make DC vaccination easier and more efficient.

P050

Lichen planus: an immunological skin disorder of late age with a characteristic immune fluorescence pattern

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Lichen planus (LP) is a common skin disease with a population prevalence of 0.2%. In 1970, Barthelmes and Haustein reported an inhomogenous and irregular pattern of fibrin deposition in LP, especially at the dermal–epidermal junction. Purpose of this study was to document demographic data of the population involved and to exactly identify the fluorescence pattern of this immune dermatosis. Ninety-five subsequently clinically and histologically diagnosed cases with LP were evaluated regarding their demographic characteristics and the identity and pattern of immune complexes deposited in skin sections. Frozen sections from involved skin areas were incubated with fluorescein isothiocyanate-conjugated monoclonal antibodies against immunoglobulin (Ig)G, IgM, IgA, C3, and fibrinogen in room temperature, washed thrice with phosphate-buffered saline, fixed and evaluated under a fluorescence microscope. A 1:2.2 male : female ratio (30 males and 65 females) was documented. The disease occurred in most patients (73%) after the age of 50 years. The duration of the disease at diagnosis varied between 14 days and 30 years, with a recurrent course. The prevalence of clinical variants was LP of the oral mucosa in 46 patients (48.4%), LP exanthematicus in 27 patients (28.4%), LP of the genital mucosa in nine patients (9.5%), localized LP in six patients (6.3%), and rare variants in seven patients: LP atrophicans (three), lichen verrucosus (two), and lichen follicularis (two). In immune histology, linear fray deposits of fibrinogen at the dermo–epidermal junction were mostly documented, followed by IgA deposits at the dermal vessel wall and fibrinogen cytoid bodies. In contrast to previous reports, cytoid bodies, being characteristic for the deposition of Ig, C3 and fibrinogen, were found in a smaller specimen number by fluorescence microscopy compared with conventional histology, probably due to phagocytosis of the apoptotic bodies. In conclusion, LP is an immunological disorder of the aged population with a female predominance. Marked linear fray fibrinogen deposits can even be documented in older lesions, in contrast to previous reports of fluorescence fading with time, making this specific fluorescence pattern valuable for diagnostic purposes independently of the disease.

P051

Dermal fibroblast potently induce maturation of dendritic cells

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For effective triggering of T-cell-mediated immune response, dendritic cells (DCs) have to migrate from peripheral tissues such as the epidermis via the dermis into locally draining lymph nodes where they present antigen to naive T cells. During this migration, DCs undergo distinct phenotypic and functional changes termed collectively DC maturation, which can be recapitulated during *in vitro* DC culture. To date, little is known about the signals delivered to DC by the various cellular microenvironments that DC encounter during their travel from peripheral epithelia to lymphoid tissues. In a first attempt to address this issue, we studied the interaction of DC with fibroblasts, the major cellular component of the dermal microenvironment. Here, we report that *in vitro* human DCs have the capacity to adhere specifically to human dermal fibroblasts via the interaction of the b2-integrins (on DC) and Thy-1 (CD90) and ICAM-1 (on fibroblasts). Moreover, in the dermis of an evolving cutaneous immune response such as allergic contact dermatitis, b2-integrin-positive DCs are found in close apposition to Thy-1/ICAM-1-positive fibroblasts. To study the effects of such contacts on DC-immune functions, we generated human DCs from $CD14^+$ peripheral blood mononuclear cell using GM-CSF and IL-4-containing media and were cocultured for 24 h with allogeneic or autologous fibroblasts. By flow cytometry, we showed that many maturation markers among them CD80, CD83, and HLA-DR were induced or up-regulated on DC upon coculture with fibroblasts. Separation of DC and fibroblasts by transwell inserts revealed that both a direct cell–cell contact as well as soluble mediators are responsible for the fibroblast-induced maturation of DC. On the basis of preliminary results, we suppose that adhesion mediated by b2-integrin (on DC) and Thy-1/ICAM-1 (on fibroblasts) results in a dramatic stimulation of autocrine TNF-α secretion by DC, which in turn induces full DC maturation.

In summary, we demonstrate that human dermal fibroblasts potently induced differentiation of immature DC to mature DC via b2-integrin/Thy-1/ICAM−1interactions. Our data are consistent with the notion that the dermal cellular microenvironment actively participates in the regulation of DC-immune functions.

P052 (V09)

Switch of syndecan-1 and syndecan-4 expression controls maturation-associated dendritic cell motility

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Dendritic cells (DCs) need to mobilize within the extracellular matrix (ECM) during maturation and migration from peripheral

sites to lymphoid organs. Syndecans mediate their interactions with the ECM. Here, we investigated the influence of syndecans on DC motility and morphology. Langerhans cells of the epidermis and monocyte-derived DCs as their *in vitro* model undergo a switch of SDC expression during maturation. SDC1 is down-regulated and SDC4 is strongly up-regulated within the first hours of lipopolysaccharide stimulation. Syndecan (-1, -4) expression was analyzed using quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and flow cytometry. DC motility was evaluated by videomicroscopy tracking and modulated by antibody targeting and RNA silencing. Cytoskeletal linkage of syndecan-4 and DC morphology on ECM substrata fibronectin were determined by confocal microscopy. Syndecan-1 is down-regulated and syndecan-4 is up-regulated during DC maturation and Langerhans cell emigration from human skin as shown by flow cytometry and qRT-PCR. Syndecan-4 is connected to the cytoskeleton via up-regulated a-actinin as revealed by flow cytometry and confocal microscopy. Impairment of syndecan-4 through antibody targeting or siRNA knockdown decreases DC motility. A functional consequence of differential syndecan expression is shown, in that syndecan-1 down-regulation is inhibited by Syndecan-4 knock down. We conclude that a switch of Syndecan expression during DC maturation controls their motility that appears to be crucial for their mobilization from peripheral sites and subsequent migration to lymphoid tissues.

P053

Anti-CD20 (rituximab) treatment of pemphigus vulgaris affects both autoaggressive B and T lymphocytes

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The treatment of pemphigus vulgaris (PV), a severe autoimmune bullous skin disorder primarily associated with autoantibodies (auto-ab) against desmoglein 3 (Dsg3) remains challenging. A new therapeutic approach using the anti-CD20 ab, rituximab, to target anti-Dsg3 immunoglobulin (Ig)G-secreting B cells is quite attractive as rituximab leads to rapid and prolonged B-cell depletion. In the present study, we sought to investigate an additional mode of action, i.e. modulation of autoaggressive T-cell activation. Qualitative and quantitative changes of Dsg3-reactive $CD4^+$ T-cell subsets were analyzed in four PV patients with extensive and recalcitrant PV on rituximab standard regimen consisting of four infusions of 375 mg/m^2 at weekly intervals. Before each rituximab infusion and during a 3-month follow-up period, different subtypes of Dsg3-reactive T cells were analyzed using the MACS secretion assay: as previously reported, T cells were characterized by their cytokine profiles as T-helper (Th)1 (interferon-γ), Th2 (IL-4), and type 1 regulatory-like (Tr1) T cells (IL-10). After the first rituximab infusion, Dsg3-reactive Th1 and Th2 cells increased 1.5–9.5-fold in three patients, whereas IL-10^+ Dsg3-reactive T cells mounted moderately up to 2.9-fold. The fourth patient with very high numbers of Dsg3-specific Th1 and Th2 cells experienced a dramatic decrease (5.0–6.7-fold) of these T cell subsets while the Dsg3-reactive Tr1 cells increased 1.5-fold after the first rituximab infusion. One month after the last rituximab infusion, the numbers of all studied Dsg3-reactive T-cell subsets were on pre-treatment levels again. After the second rituximab infusion, there was a complete and sustained B-cell depletion. Four weeks after rituximab, the titers of Dsg3-reactive IgG remained unchanged or slightly increased (2.0–2.5-fold) in the studied PV patients. Strikingly, three of four patients showed a good clinical response with healing of existing erosions/absence of *de novo* blisters beginning 4 weeks after treatment. The present findings thus strongly suggest that rituximab has a dual immunomodulatory effect. A not-yet-identified mode of action includes a delayed modulation of autoaggressive T cells which may explain why rituximab treatment of PV has a delayed mode of onset and is clinically effective despite persisting auto-ab titers.

P054

Structural requirements and mechanism of antimicrobial activity of dermcidin-derived peptides

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Dermcidin (DCD) is a recently described antimicrobial peptide, which is constitutively expressed in eccrine sweat glands and transported via sweat to the epidermal surface. Using postsecretory proteolytic processing in sweat, the DCD protein gives rise to several truncated DCD peptides with different biochemical properties and yet unknown functional activities. In this study, we analyzed (1) the spectrum of activity and (2) the mechanism of antimicrobial activity of several naturally occurring DCD peptides which differ in length and charge. Using circular dichroism measurements, we show that irrespective of charge, the DCD peptides adopt an α-helix in helix-inducing solvents. Interestingly, the different DCD peptides have a diverse and partially overlapping spectrum of activity. Furthermore, we investigated the mechanism of antimicrobial activity of DCD peptides using carboxyfluorescein-loaded liposomes, membrane permeability studies, and electron microscopy examinations. Using all three methods, we could not detect damage or pore formation of the bacterial membrane by different DCD peptides. In contrast, immune-EM studies suggested that DCD peptides bind to the bacterial membrane. These studies indicate that irrespective of their charge, DCD peptides do not act by membrane desorption or pore formation but probably by receptor interaction on the bacterial membrane.

P055

CD4-mediated activation of human $CD4^+CD25^+$ regulatory T cells

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Naturally occurring $CD4^+CD25^+$ T-regulatory cells ($CD25^+$ Tregs) play a central role in the maintenance of peripheral self-tolerance by suppression of auto-aggressive T cells. The immune-regulatory function of $CD25^+$ Tregs depends upon their activation. Agents capable of activating their function should prove to be efficient therapeutic tools in autoimmune and other inflammatory diseases. Here, we report that the monoclonal anti-CD4 antibody B-F5 triggers the suppressive activity of human $CD25^+$ Tregs in a T-cell receptor-independent manner. No suppressive activity is induced in conventional $CD4^+CD25^-$ T cells by this antibody. Activation of $CD25^+$ Tregs by B-F5 requires crosslinking of CD4 on the surface of $CD25^+$ Tregs and is dose-dependent. Among 14 others tested, no other monoclonal antibody comparably activated $CD25^+$ Tregs. In summary, our data demonstrate the functional activation of $CD25^+$ Tregs by CD4 crosslinking and help to understand the tolerogenic function of anti-CD4 treatment *in vivo*.

P056

Inhibition of proinflammatory cytokines and modulation of the T-cell stimulatory capacity of dendritic cells through application of the nasal decongestant oxymetazoline

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Response to viral upper respiratory tract infections play a critical role for the development and maintenance of local nasal inflammation. As the nasal decongestant oxymetazoline (OMZ) is frequently used in the topical treatment of rhinitis, the aim of this study was to examine its influence on the functional activity of human immune cells. Peripheral blood mononuclear cells (PBMC) from buffy coats of healthy volunteers were isolated, stimulated in the presence and absence of OMZ *in vitro* and were analyzed concerning their proliferation and cytokine profiles. In addition, monocyte-derived dendritic cells (DC) – well known as professional antigen-presenting cells – were generated and submitted to different concentrations of OMZ. DC phenotype and their T-cell stimulatory properties were analyzed. The vasoactive substance OMZ showed a concentration-dependent inhibitory effect on proliferation and activation of polyclonal stimulated T cells. In addition, the secretion of proinflammatory mediators such as the cytokines IL-1β, TNF-α, IL-6, and IL-8 produced by PBMC and T cells was also selectively inhibited in the presence of physiological doses of OMZ. Furthermore, non-toxic concentrations of OMZ were able to inhibit the immunostimulatory capacity of human DC including their cytokine production without influencing their phenotype. Our data suggest that anti-inflammatory properties of OMZ are partially mediated by the inhibition of proinflammatory cytokines resulting in a repressed stimulation of T cells and by the modulation of the immunostimulatory capacity of human DC. These findings may explain the anti-inflammatory and immunomodulatory effects of OMZ in acute viral rhinitis seen *in vivo*.

P057

Gedächtnis T-zellen im spontan melanom-maus modell

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Die palliative Behandlungssituation beim fortgeschrittenen Melanom bedarf dringendneuer Therapiekonzepte (u.a. im Bereich der Immuntherapie). Man weiß vonArbeiten beim Mammakarzinom, dass die Gedächtnis-T-Zellen (MTC) die Fähigkeithaben, schneller und effektiver auf Tumorantigene zu reagieren, denen sie zuvorbereits begegnet sind. Sie könnten deshalb als eine Quelle für die Generierung vontherapeutischen Effektorzellen dienen. Wir verwenden ein Ret-transgenes (Ret-tg) Mausmodell, bei dem ca. Thirty percent der Mäuse innerhalb von drei Monaten spontanMelanome entwickeln, die Melanomassoziierte Antigene (MAA) 'tyrosinase-related protein-1' (TRP-1), TRP-2, gp100 und tyrosinase exprimieren und die humaneMelanome gut widerspiegeln. Phänotypische Analysen zeigten, dass tumorfreieRet-tg Mäuse die ähnliche Anzahl von CD4 und CD8 T-Zellen im Knochenmark undin der Milz wie wildtyp Tiere haben. Die tumortragende Ret-tg Mäuse im Bezug aufihren Anteil CD4- und CD8-T-Zellen-Anteil im Knochenmark (KM), in zwei Gruppenunterteilt werden konnten: Einige Tiere zeigten im Vergleich mit den Kontrollgruppeneinen deutlich erhöhten Anteil an T-Zellen, die meisten jedoch wiesen keinensignifikanten Unterschied auf. Antigenunspezifische Aktivierung der T-Zellen aus derMilz und Lymphknoten mit Concanavalin A oder mittels Antikörper gegen CD3 undCD28, sowie die Immunisierung mit dem Modellantigen Ovalbumin oder mit demMAA TRP2 konnten zeigen, dass das Immunsystem Ret-tg Mäuse funktionell aktivist. Dann untersuchten wir die phänotypische Zusammensetzung der MTC im Blut, inder Milz und im KM in verschiedenen Krankheitsstadien. Hierbei war auffällig, dasstumorfreie Ret-tg Tiere einen höheren Anteil an Gedächtnis-T-Zellen aufwiesen als wildtyp Tiere. Eine Analyse der MTC zeigte, dass bei Ret-tg Tieren der Anteil an 'central memory' T-Zellen (CM) im Vergleich zur wildtyp Gruppe etwa gleich blieb, wogegen die Zahl der 'effector memory' T-Zellen (EM) erhöht war.

Unsere immunologischen Untersuchungen liefern uns die Grundlage für zukünftigeimmuntherapeutische Ansätze, die an unserem Mausmodell durchgeführt werdenkönnen.

P058 (V16)

$CD25^+/CD4^+$ regulatory T cells suppress maturation of dendritic cells *in vitro* and *in vivo* and induce an immunosuppressive phenotype of dendritic cells

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Previous results have shown that immature dendritic cells (DCs) play a crucial role in the activation of regulatory T cells (Treg). Vice versa, only sparse data is available addressing the effect of Treg on DC development. Therefore, we set out to investigate the effects of Treg on DC maturation, activation, and phenotype *in vitro* and *in vivo*. To address the effect of Tregs on DC maturation *in vitro*, we cultured immature bone marrow-derived DC as well as magnetic-bead separated lymph node DC in the presence of isolated $CD4^+/CD25^+$ Treg and assessed their maturation status by FACS and mixed lymphocyte reactions. In these assays, it became evident that DC, after coculture with Treg, retained their immature status as indicated by low expression of CD80, CD86 and inferior T-cell stimulatory capacity as compared with controls. This reduced T-cell stimulatory capacity was largely due to up-regulation of the inhibitory molecule B7-H3, and addition of B7-H3-blocking antibodies could indeed re-establish vigorous T-cell proliferation. These effects required cell–cell contact, as supernatant of cultivated Treg affected DC maturation only slightly. We next injected isolated Treg into mice and assessed DC maturation and antigen presentation *in vivo*. Similar to *in vitro* experiments, DCs displayed reduced expression of costimulatory molecules and showed reduced MHC-class II–peptide complex formation, which altogether resulted in reduced T-cell stimulation as compared with controls. Thus, this data indicate that Treg can impair the maturation of developing DC. In context of data showing that immature DC stimulate generation of Treg, our data indicate that Treg themselves in a 'feedback-loop' suppress DC maturation keeping them immature and thus ensuring their Treg-activating properties.

P059

Soluble factors produced by $CD4^+CD25^+$ regulatory T cells suppress the effector phase of contact hypersensitivity reactions and block the influx of $CD8^+$ T cells into inflamed tissues

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We previously demonstrated that the injection of $CD4^+CD25^+$ regulatory T cells leads to suppression of inflammatory reactions in the *in vivo* model of trinitrochlorobenzene (TNCB)-induced contact hypersensitivity (CHS) reactions. To elucidate the function and the migration pattern of injected Tregs and endogenous leukocytes during CHS, we established a novel model using

intravital fluorescence microscopy in awake C57BL/6 mice implanted with a skinfold chamber. This model enables us to visualize the leukocyte flux directly at the side of antigen challenge. In our experiments, mice were sensitized by epicutaneous application of 1% TNCB. Six days later, syngeneic $CD4^+CD25^+$ T cells were adoptively transferred, and mice were challenged with 0.1% TNCB. Here, we could observe that Tregs significantly reduced the inflammatory reaction as determined by an enormous reduction of the leukocyte influx into the inflamed tissue. Analysis of the inflamed tissue showed that the inhibition of CHS was associated with a decreased immigration of $CD8^+$ T cells. However, we were notable to detect any of the injected Tregs in this area. Instead, Treg accumulated in the draining lymph nodes and in the spleen within few minutes after injection. Notably, suppression only occurred when Tregs were injected at least 6 min before challenging. Simultaneous injection of Treg and TNCB challenging, respectively, did not prevent the ear-swelling reaction. Thus, this data indicate that Treg need a certain time to acquire their suppressive properties *in vivo*. Moreover, when Treg-derived tissue culture supernatant was injected, CHS reactions as well as leukocyte influx was suppressed too. These effects could be blocked by administration of anti-IL-10 antibodies while neutralization of TGF-β was inefficient. Thus, these data show that Tregs suppress CHS reactions by blocking the influx of $CD8^+$ effector T cells and the leukocyte–endothelium interaction in inflamed tissues *in vivo* by an IL-10-dependent pathway.

P060

Detection of the antimicrobial proteins psoriasin (S100A7) and RNase 7 in the peripheral blood of psoriasis patients and healthy controls

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Recently, we identified the S100 protein Psoriasin as well as the ribonuclease RNase7 as potent antimicrobial proteins derived from healthy human stratum corneum extracts. The antimicrobial activity of Psoriasin is mainly directed against *Escherichia coli*, whereas RNase 7 exhibits a broad spectrum of antimicrobial activity. Both proteins can be isolated in significant higher concentrations from psoriatic scale extracts when compared with healthy human stratum corneum underlining that Psoriasin and RNase 7 are inducible. This study was initiated to investigate if the antimicrobial proteins Psoriasin and RNase 7 can be detected in serum samples of healthy controls and patients with psoriasis vulgaris.

Enzyme-linked immunosorbent assay (ELISA) systems for the identification of both antimicrobial proteins were established and standardized: for the detection of Psoriasin a sandwich-ELISA with two monoclonal antibodies generated against natural Psoriasin, for RNase7 an ELISA with affinity-purified polyclonal antibodies against natural RNase 7. Serum samples of 40 healthy controls and 40 patients hospitalized with psoriasis vulgaris were included in the study.

In the healthy controls, a Psoriasin level above the detection limit could be detected in two/40 persons, whereas RNase seven could be measured in 15/40 individuals. In the psoriasis patients, both parameters showed a significant higher detection level: Psoriasin was elevated in 21/40 patients with a median concentration of 0690 ng/ml, RNase 7 was shown to be increased in 25/40 patients (median concentration: 0698 ng/ml).

In summary, this study demonstrates for the first time that the antimicrobial proteins Psoriasin and RNase 7 are detectable in human peripheral blood and that elevated serum levels of these antimicrobial proteins can be detected in patients with psoriasis vulgaris. Further investigations have to identify the cellular source and biological significance of these serum-derived antimicrobial proteins.

P061

Detection of tumor-specific T cells beyond the sentinel lymph node in the lymphatic draining region

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Cell-immune responses are normally initiated in the lymph node. For viral infections, the importance of the draining lymph node for priming of T cells is unequivocally proven. In contrast, the role of the sentinel lymph node (SLN) for the initiation of immune responses against tumors has not been examined in detail especially as in mice a direct priming of T cells could be observed. Therefore, we analyzed the T-cell clonality of primary tumor and corresponding SLNs of 49 melanoma patients by clonotype mapping. To this end, we detected identical T-cell clones in primary tumors and SLN in 12 patients by comparative clonotype mapping although most of the SLN consisted of a polyclonal T-cell population. In addition, increasing the sensitivity of the detection method by utilizing specific primers of T-cell clones present in primary tumors sustained this finding as several clones could be detected in corresponding SLNs. As more than half of these patients did not display any sign of micrometastases in their lymph nodes, this data support the role of the SLN as the primary organ for T-cell priming also for antitumor-immune responses. Notably, we previously demonstrated in a clinical case report that T-cell clones present in the SLN can participate at the immune responses to subsequent tumors despite the excision of the SLN. Therefore, we analyzed in three patients the presence of T-cell clones detected in the primary tumor in lymph nodes of the lymphatic draining region by specific primers for these clones. For each patient, we had four lymph nodes and examined three T-cell clones. In one of these patients, the expansion of the respective T-cell clones was below the detection threshold. In the two others, some of the T-cell clones could be detected not only in lymph nodes bearing micrometastases but also in lymph nodes without micrometastases. Thus (1) the SLN seems to be important for T-cell priming of antitumor-immune responses and (2) lymph nodes of the lymphatic draining region are involved at least in the maintenance of an antitumor-immune response.

P062

Functional analysis of Cytip in dendritic cells

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The β2 integrin LFA-1 is important for transendothelial migration of leukocytes. The binding avidity of LFA-1 is regulated by the cytosolic proteins Cytohesin-1 and Cytip. To be active, we found that Cytohesin-1 aggregates LFA-1 at the plasma membrane, resulting in enhanced LFA avidity, whereas Cytip complexes Cytohesin-1 and removes the complex from the inner membrane. Kinetic studies reveal enhanced Cytip expression in dendritic cell (DC) during maturation. Thus, it might be hypothesized that LFA-1 function in DC is regulated by Cytip expression. To determine the relevance of LFA-1 for antigen presentation by DC, we investigated bone marrow-derived DC (BMDC) after Cytip siRNA transfection. In Cytip-specific-siRNA-transfected BMDC, we detected reduced

amounts of Cytip by confocal microscopy. Analysis of *in vitro* Cytip siRNA-transfected DC show significantly longer contact times to $CD4^+$ T cells in a three-dimensional collagen gel. Surprisingly, although the average contact time between DC and $CD4^+$ T cells is increased, the antigen-specific proliferation of $DO11CD4^+$ T cells induced by Cytip siRNA-transfected DC is substantially reduced. We speculate that due to the reduced Cytip expression, induced by siRNA transfection, Cytohesin-1 is able to aggregate with LFA-1, which might result in enhanced binding of LFA-1 to ICAM-1. Thus, transfected DC might form longer contacts to T cells, which disables the latter to proliferate. In ongoing studies, we are investigating LFA-1-mediated adhesion of Cytip-siRNA transfected DC.

P063

In vitro model for oral mucosal-like Langerhans cells

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Sublingual immunotherapy (SLIT) has been shown to be efficient in the treatment of allergic rhinitis. Data from classical subcutaneous immunotherapy suggest tolerance induction to be a key immunological mechanism. However, only few information is available about the immunological mechanisms underlying SLIT. It is more than likely that dendritic cells (DC) bearing the high-affinity receptor for immunoglobulin (Ig)E such as oral mucosal Langerhans cells (oLC) play a central role. Nevertheless, functional data are difficult to acquire due to limited material obtained from oral mucosal biopsies. Hence, we intended to develop an *in vitro* model for oLC using cord blood $CD34^+$ stem cell-derived DCs (CD34dDC). Thereby, we could show that specific concentrations of transforming growth factor (TGF)-β1 in combination with Mercapto-ethanol and GM-CSF, TNF-α and SCF not only gave rise to $CD1a^+$ CD34dDC but also to the generation of DC displaying a phenotype very similar to oLC expressing the high-affinity receptor for IgE (FcεRI), Langerin, CD14, and CD11b. Furthermore, we could demonstrate that *ex vivo*- and *in vitro*-generated oLC expressed not only coactivating B7 molecules such as CD80 and CD86 but also coinhibitory molecules, which has been shown to be involved in allergen-specific tolerance induction. Moreover, *in vitro*-generated oLC sufficiently bound IgE and were able to stimulate allogeneic T cells after allergen uptake via allergen-specific IgE. This novel *in vitro* model for oLC described here enables us to study functional mechanisms involving FcεRI-bearing oLC and pathways leading to allergen-specific tolerance induction in SLIT.

P064 (V12)

Impaired type I allergic responses in mice that exhibit defects in sphingomyeline hydrolysis

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Phospholipid metabolites derived from processing of sphingomyeline, e.g. ceramide generated by hydrolysis of sphingomyelin by acid sphingomyelinase (ASMase) have been shown to importantly modulate the activation of various types of immune cells including mast cells (MCs). To assess the specific effects of ASMase, which is localized in the granules of MCs, on MC-driven immune responses *in vivo*, we induced localized or systemic anaphylactic responses in genetically ASMase-deficient mice. As anaphylactic responses in mice are virtually entirely MC dependent, we first compared MC numbers in $ASMase^{-/-}$ mice and normal littermates. Skin and peritoneal MC populations in both types of mice were found to be very similar in size and phenotype as assessed by quantitative histo/cytomorphometry. Notably, skin inflammatory responses in $ASMase^{-/-}$ mice subjected to passive cutaneous anaphylaxis were markedly reduced: increases in skin thickness were significantly delayed and reduced by up to 60% as compared with wildtype (wt) mice. Also, $ASMase^{-/-}$ mice exhibited impaired passive systemic anaphylaxis as assessed by measuring core body temperature drops. Hypothermia in wt mice was characterized by an earlier onset, markedly larger temperature drops during the entire course of the response and a significantly longer duration as compared with $ASMase^{-/-}$ mice. Our findings suggest that ceramide generated via hydrolysis of sphingomyelin by ASMase can promote the development of allergic responses, possibly by acting directly on MCs, which may – in the long run – facilitate the development of novel antiallergic therapies.

P065

New insights into the immune privilege of the hair follicle: natural killer cells and their possible relevance to alopecia areata

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The common autoimmune disease, alopecia areata (AA), likely results from a collapse of the normal immune privilege (IP) of the anagen hair follicle (HF). The HF-IP is, e.g., characterized by down-regulation of MHC classes I and II expression and local expression of potent immunosuppressants (e.g. α-MSH, TGF-β1). While, normally, NK cells attack cells with absent or low MHC class I expression, surprisingly few perifollicular NK cells are found around healthy human anagen HFs. Therefore, we asked how the anagen hair bulb escapes from NK cell attack and whether HFs of AA patients differ in this respect. Here, we report that normal human anagen HFs prominently express macrophage migration inhibitory factor (MIF), which suppresses NK cell activity. In contrast to healthy controls, besides $CD4^+$ and $CD8^+$ T cells, $CD56^+$ and $NKG2D^+$ NK cells prominently accumulate around AA-HFs. By FACS analysis, the mean intensity of NKG2D and NKG2C, which activate NK cells, on $CD56^+$ NK cells is significantly higher in AA patients than in healthy controls. Moreover, lesional AA-HFs strongly express MICA (activates NK-cells by interacting with $NKG2D^+$). In contrast, the expression of NK-inhibitory killer cell immunoglobulin-like receptor (KIR) on $CD56^+$ NK cells is significantly lower in AA patients compared with healthy controls. Therefore, normally, anagen HFs probably escape from NK cell attack by MIF expression and by low expression of NKG2D and MICA, along with high expression of KIR. The reverse is true in AA patients, thus priming their NK cells to attack the HF.

P066

Lymphocytes do not necessarily need to home into tumors to elicit efficient antitumor-immune responses

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Although $CD4^+$ T lymphocytes are able to regulate most antigen-specific immune responses, very little attention has

been given on how T-helper (Th) cells can eliminate cancer cells. In previous studies, we could show that adoptive transfer of tumor antigen-specific (Tag) interferon-γ-producing $CD4^+$ Th1 (Tag-Th1) cells delayed tumor development twofold in a mouse model of multistage carcinogenesis, the RIP1-Tag2 mouse. In this tumor model, Tag is expressed in all insulin-producing β cells, leading to the development of adenomas and finally carcinomas. At 15 weeks, mice die of hypoglycemia. Intraperitoneal (i.p.) injection of Tag-Th1 caused neither a major T-cell infiltrate in tumors nor major signs of tumor cell or vessel destruction. As Th1 cells can not recognize MHC class II-negative tumor cells directly, we wanted to know where Tag-Th1 traffics from the time of injection up to 4 days. We used *in vivo* non-invasive high-resolution positron emission tomography (PET) and invasive methods such as autoradiography combined with histology and fluorescence microscopy. For visualizing Th1 trafficking and homing up to 1 day, we labeled the cells *in vitro* with [64 Cu] PTSM. We injected 10×10^6 Th1 cells i.p. into RIP1-Tag2 and naïve C3H mice. At 30 min, 3, 8, and 24 h after injection, PET scans showed that Tag-Th1 cells homed in the pancreatic lymph node and the pancreas. Autoradiography combined with micrographic analysis of the pancreas and the lymph nodes revealed accumulation of tumor-specific Th1 cells either in the tumor draining lymph node or in lymphoid-like structures around the islet tumors. To visualize cell trafficking and homing for a longer period, we labeled Tag-Th1 lymphocyte with the carbocyanine fluorescence dye DiD. Injection of 10×106 labeled Th1 cells i.p. showed that part of the Th1 cells still accumulated in the pancreas and the draining lymph node even after 4 days. Together, the findings strongly suggest that the arrest of tumor development by Th1 cells is largely independent on Th1-cell infiltration into the tumor.

P067

The development of psoriasiform dermatitis depends on CD18 deficiency of $CD4^+CD25^+$ Tregs in a murine psoriasis model

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$CD4^+CD25^+$ regulatory T cells (Tregs) suppress autoimmune and inflammatory diseases through mechanisms that are only partly understood. Previous studies suggested that the cytotoxicity of Tregs is dependent on CD18 adhesive interactions. In addition, dysfunctional Tregs were identified in human patients with psoriasis. We previously reported that CD18 hypomorphic (CD18hypo) PL/J mice with reduced levels of the common chain of β2 integrins (CD11/CD18) develop an autoimmune skin disease strongly resembling human psoriasis. In this study, we show that CD18 deficiency causes the reduced number of $CD4^+CD25^+$ Tregs in peripheral lymphoid organs of CD18hypo and CD18 completely deficient (CD18 null) PL/J mice. CD18 deficiency does not influence expression of the transcript factor Foxp3 of Tregs. However, CD18-deficient $CD4^+CD25^+$ Tregs are significantly impaired in their inhibitory functions *in vitro*. Adoptive transfer of Tregs from CD18 wildtype (CD18wt) into affected CD18hypo PL/J mice resulted in a complete resolution of the psoriasiform dermatitis as assessed by an adapted PASI score. Furthermore, in both *in vivo* and *in vitro* experiments $CD4^+CD25^-$ responder T cells from spleen of affected CD18hypo PL/J mice exhibit increased early proliferative responses, compared with CD18wt mice. These $CD4^+CD25^-$ T cells revealed an antigen-experienced phenotype (CLA^+ $CD62L^-$ $CD45RB^-$ $CD44^+$) in the dermal–epidermal junction of lesional skin as well as in blood of affected CD18hypo mice. Taken together, our data suggest that reduced expression of CD18 results in the reduced suppressor activity of Tregs. This is in conjunction with accelerated proliferation of $CD4^+CD25^-$ pathogenic T cells which leads to the development of the psoriasiform skin disease in CD18hypo PL/J mice.

P068

Efomycine M inhibits L-selectin-mediated lymphocyte adhesion and diminishes lymphocyte sensitization in the pathogenesis of allergic responses

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Selectins are attractive targets for the development of specific anti-inflammatory compounds such as the recently described small-molecule macrolide, efomycine M, which has been shown to block adhesive functions of E- and P-selectin and to alleviate inflammatory disorders in preclinical models of psoriasis. Its activity toward L-selectin was thus far unknown.

Using *ex vivo* isolated human lymphocytes as well as an L-selectin-transfected lymphocyte line (NALM-6) in dynamic flow chamber video microscopy experiments, we demonstrate here that efomycine M significantly blocks L-selectin-mediated lymphocyte adhesion and rolling on sialylated Lewis X, an action that was confirmed on the molecular level by plasmone resonance spectroscopy (Biacore).

Given that the recruitment of naïve lymphocytes to peripheral lymph nodes depends, at least to a considerable extent, on L-selectin-mediated adhesion to high endothelial venules, intravital microscopy was performed. Indeed, these *in vivo* experiments revealed a significant reduction of lymphocyte rolling in peripheral lymph nodes of mice treated with efomycine M. As L-selectin-dependent recruitment of naive lymphocytes is a pre-requisite not only for lymphocyte sensitization in normal T-cell-mediated immune reactions but also in the pathogenesis of allergic responses, C57BL6 mice were treated systemically with efomycine M or an L-selectin specific antibody during contact sensitization with DNFB. We could demonstrate that the capacity of their T cells to induce a contact hypersensitivity response after adoptive transfer into non-sensitized recipient mice was significantly reduced in either case ($P = 0.0002$ and $P = 0.0001$, respectively).

These data demonstrate that it is possible, in principle, to diminish T-cell-mediated allergic reactions through antibody-mediated or small-molecule-mediated interference with L-selectin functions during the sensitization phase.

P069

Untersuchung von regulatorischen T-zellen im spontan melanom-maus modell

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Die Aufgabe regulatorischer T-zellen (Treg) besteht in der Unterdrückung aktivierterautoreaktiver Effektor-T-Zellen, um Immunreaktionen gegen körpereigene Antigeneauszuschließen. Es wird vermutet, dass die Immunantwort auf Tumorantigene durchEffektor-T-Zellen durch Treg gehemmt wird. Daher werden neue Therapieansätze,die auf der Depletion oder Unterdrückung dieser Treg basieren, dringend gebraucht.

Als Melanom-Mausmodell stehen Ret-transgene (Ret-tg) Mäuse zur Verfügung. Ca.30% dieser Mäuse entwickeln nach kurzer Latenz spontan Melanome, die diegenetische, histopathologische und klinische Situation menschlicher Melanomen gutimitieren. Wir untersuchten die CD4 Treg, die sich durch konstitutive Expression vonCD25, CTLA-4 und des

Transkriptionsfaktors FoxP3 auszeichnen. Die Anzahl vondiesen Treg wurde im Blut, im Knochenmark (KM) und in der Milz in tumorfreien undtumortragenden Ret-tg Mäusen gemessen. Als Kontrollgruppe dienten C57BL6 wildtyp Mäuse. Es wurde im KM Ret-tg Mäuse ein höherer Anteil an Treg an derGesamt-T-Zellpopulation beobachtet als im KM der Kontrollmäuse. Anders verhieltes sich mit der Milz, wo der Anteil von CD4 Treg an der Gesamt-T-Zellpopulation inallen drei Gruppen vergleichbar war. Außerdem wurde im Blut von transgenenMäusen, die über längere Zeit beobachtet wurden, ein Anstieg des Anteils Treg ander Gesamt-T-Zellpopulation festgestellt.

In einem in vitro Stimulationsassay wurde die unterdrückende Wirkung der Treg aufaktivierte T-Zellen untersucht. Hierbei wurden aus der Milz der Ret-tg und wildtyp Mäuse gewonnene Treg und Effektor-T-Zellen in Ko-Kultur drei Tage lang stimuliert, wobei eine Unterdrückung der Proliferation von Effektor-T-Zellen beobachtet werdenkonnte.

Das Erproben von immuntherapeutischen Strategien imSpontanmelanom-Mausmodell, basierend auf der Depletion von Treg soll Gewissheitüber die Auswirkung von Treg auf den Krankheitsverlauf beim malignen Melanombringen und auf mögliche neue Therapieformen hinweisen.

P070 (V24)

Human $CD4^+CD25^+$ regulatory T cells express galectin-10, essential for their anergic state and suppressive capacity

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$CD4^+CD25^+$ regulatory T cells ($CD25^+$ Tregs) are essential negative regulators of multiple immune functions. However, little is known about the molecular mechanisms and the proteins contributing to the hyporesponsive state and regulatory activity of Tregs. A better understanding of their function at the molecular level should provide more insights into a potential therapeutic exploitation of these cells in allergic and autoimmune diseases.

Using differential proteomics, we identified galectin-10, a member of the lectin family, as predominantly expressed in human $CD25^+$ Tregs. Comparing protein expression in $CD25^+$ Tregs and $CD4^+CD25^-$ T cells, we found galectin-10 predominantly expressed in $CD25^+$ Tregs among resting T cells but almost exclusively expressed in $CD25^+$ Tregs after T-cell activation. Specific inhibition of galectin-10 by small interfering RNA restored the proliferative capacity of Tregs, virtually to the level of conventional $CD4^+CD25^-$ T cells. Most notably, inhibition of galectin-10 expression in human $CD25^+$ Tregs abrogated their suppressive activity on cocultured $CD4^+CD25^-$ T cells. In summary, galectin-10 expression is essential for the anergic state and the suppressive capacity of human $CD25^+$ Tregs.

P071

NOD2/CARD15 is essential for muramyl dipeptide-mediated induction of human β-defensin-2 in primary keratinocytes

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Production of inducible antimicrobial peptides offers a first and rapid defense reaction of skin and other epithelia against invading microbes. Human β-defensin-2 (hBD-2) is an antimicrobial peptide induced in various epithelia upon extracellular as well as intracellular bacterial challenge. Nucleotide-binding oligomerization domain protein 2 (NOD2/CARD15) is a cytosolic protein involved in intracellular recognition of microbes by sensing peptidoglycan fragments (e.g. muramyl dipeptide). The aim of this study was to gain more insight into the biological significance of NOD2 regarding the induction of hBD-2 in primary keratinocytes. Using reverse transcription polymerase chain and Western blot analyses, we were able to detect *NOD2* gene and protein expression in primary keratinocytes. Stimulation of primary keratinocytes with the NOD2 agonist muramyl-dipeptide (MDP) led to an induction of hBD-2 gene expression as well as hBD-2 peptide release. To verify that activation of NOD2 was responsible for the MDP-mediated hBD-2 induction, we used a siRNA approach to down-regulate NOD2 expression in the primary keratinocytes. The siRNA-mediated down-regulation of NOD2 expression in primary keratinocytes resulted in a defective induction of hBD-2 upon MDP treatment.

These data support the hypothesis that NOD2 serves as an intracellular pattern recognition receptor in keratinocytes to enhance cutaneous defense by inducing the production of antimicrobial peptides such as hBD-2.

P072

Neutrophils are required for the antibody-induced blister formation in a mouse model of epidermolysis bullosa acquisita

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Epidermolysis bullosa acquisita (EBA) is an autoimmune subepidermal blistering disease associated with tissue-bound and circulating autoantibodies directed against the dermal–epidermal junction. Patients' autoantibodies are directed to type VII collagen and induce dermal–epidermal separation in cryosections of human skin when coincubated with human leukocytes. Using a passive transfer mouse model, our group has recently shown that antibodies to murine type VII collagen cause a blistering skin disease that strikingly reproduces human EBA at the clinical, histological, ultrastructural and immunopathological levels. In the present study, we investigated the role of neutrophils for blister formation induced by antibodies to type VII collagen in this mouse model. For this purpose, BALB/c mice were treated with a monoclonal antibody (RB6-8C5) specific for the Gr1 receptor on murine neutrophils. In contrast to animals treated with a mock antibody, binding of RB6-8C5 to neutrophils triggered their apoptosis and caused a reduction of neutrophils in the peripheral blood of more than 95% as revealed by flow cytometry analysis. Subsequent injection of the pathogenic rabbit antibody against murine type VII collagen resulted in binding of IgG to the dermal–epidermal junction and local activation of complement in all animals. Importantly, neutrophil-depleted mice ($n = 8$) injected with the pathogenic IgG against type VII collagen failed to develop a blistering phenotype, both clinically and histologically. As expected, IgG against type VII collagen induced infiltration of neutrophils and subepidermal blisters in neutrophil-sufficient control animals ($n = 8$), and omission of the neutrophil-depleting antibody under continuous administration of the pathogenic antibody resulted in an increase in peripheral blood neutrophil counts, followed by the development of skin blisters. These findings demonstrate that neutrophils are essential for subepidermal blister formation in experimental EBA. This should facilitate the development of novel treatment strategies for EBA which target the infiltrating leukocytes.

P073 (V32)

The role of RNase 7 in the defense system of human primary keratinocytes

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Human skin is able to mount a fast response against invading microorganisms by the release of various antimicrobial proteins such as RNase 7, a broad spectrum antimicrobial protein.

To further explore the role of RNase 7 in the chemical defense system of human skin, we analyzed its expression in primary keratinocytes and compared it with the expression levels of the antimicrobial proteins hBD-2, hBD-3, Psoriasin, and RNase 7. In contrast to the other antimicrobial proteins real-time polymerase chain reaction (PCR) revealed high constitutive gene expression of RNase 7 in primary keratinocytes which was up-regulated in cells grown at higher confluence. This observation was in concordance with enzyme-linked immunosorbent assay experiments showing an up-regulation of RNase 7 protein expression during differentiation of the keratinocytes. Immunohistochemistry analyses confirmed high expression levels of RNase 7 in the more differentiated upper layers of human epidermis.

To further analyze the antimicrobial significance of RNase 7, we generated RNase7-specific antibodies which inactivated the antimicrobial activity of RNase 7 in an antimicrobial microdilution assay. Testing the capability of concentrated supernatants of cultured primary keratinocytes to kill microorganisms such as *Escherichia coli* revealed high antimicrobial activity present in the supernatants. Treatment of these supernatants with the RNase 7-specific antibodies resulted in a substantial decrease of killing activity against *E. coli*. Antimicrobial activity was not inhibited when equivalent concentrations of an irrelevant control antibody were used.

These findings demonstrate the important biological significance of RNase 7 as an antimicrobial factor produced by human keratinocytes and indicate that RNase 7 may play an important role in the innate immunity of human skin.

P074

Expression of functional in doleamine 2,3-dioxygenase in human dendritic cells is highly up-regulated under *in vitro* maturation: implications for tumor immunotherapy

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Immunologic responses to dendritic cell (DC)-based tumor vaccines strongly depend on DC subtype as well as its differentiation and maturation state. Indeed, immature or IL-10-modulated DCs rather induce tolerance via regulatory or anergic T lymphocytes than effective cytotoxic responses. Induction of immunological unresponsiveness may be mediated by the heme-containing enzyme in doleamine 2, 3-dioxygenase (IDO). IDO catalyzes the initial, rate-limiting step in tryptophan catabolism, and depletion of the essential amino acid tryptophan together with the production of directly toxic metabolites renders cytotoxic T cells susceptible to apoptosis.

Here, we demonstrate that expression of active IDO in human DCs during *in vitro* maturation may be of importance for the successful application of DC in cancer immunotherapy. IDO expression was strongly up-regulated in DC after induction of maturation by stimulation with IL-1β, TNF-α, IL-6, and PGE-2 over a time course of 24 h. This induction was detected both on RNA and on protein level, i.e. analyzed by reverse transcription-polymerase chain reaction and Western blot. Furthermore, functional enzymatic activity of IDO was demonstrated by tryptophan chromatography of supernatants collected from DC during maturation. A decline in tryptophan concentration was observed after application of the maturation cocktail. Control culture medium of immature DCs did not exhibit such a tryptophan depletion. Immunohistochemical analysis of biopsies of cutaneous sites of DC application revealed a diffuse infiltrate of IDO-positive cells, underlining the significance of a potential, local immune modulative effect of *in vitro* matured DC *in vivo*. These findings were confirmed in a large series of DC obtained by leukapheresis for therapeutic purposes. An interpersonal and intrapersonal variation in IDO RNA levels was observed without correlation between enzyme expression and differentiation markers such as CD83 or MHC class I.

In summary, our study demonstrates that tolerizing IDO-expressing DC may be induced under *in vitro* maturation of DC. Therefore, vaccine response may be improved by IDO inhibitors such as 1-methyl-tryptophan. Further investigations are needed to elucidate the clinical relevance of IDO-expressing DC for therapeutic vaccinations.

P075

The epidermis represents an immunological barrier for the induction of $CD8^+$ cytotoxic T-cell responses: lessons from cutaneous DNA vaccination studies using a luciferase fusion protein as model antigen

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Background: Adaptive immunity in the skin must be tightly controlled because the epidermis is continuously exposed to a large number of potentially immunogenic proteins. We studied the induction of cutaneous antigen-specific $CD8^+$ cytotoxic T cells (CTL), which are of particular importance for the defense of viral infection, using recombinant DNA vaccines. Particle bombardment with plasmid DNA using the gene gun is ideally suited to investigate the induction of $CD8^+$ CTL following antigen expression in the epidermis.

Methods: A fusion protein between EGFP, the H2-Kb-binding peptide OVA aa257–264 and luciferase (gLuc) was applied to compare gene gun immunization with intracutaneous injection of adenovirus (Ad) or Ad-transduced dendritic cell (DC) (Ad-DC). First, we performed expansion and cytotoxicity studies with adoptively transferred T cells. Then, we correlated $CD8^+$ T-cell stimulation with the time course and distribution of antigen expression using *in vivo* bioluminescence imaging. Non-specific activation was measured by flow cytometry. Finally, the enhancement of gene gun immunization by CpG oligonucleotides was investigated.

Results: Gene gun immunization was considerably less effective in stimulating Ova-specific $CD8^+$ T cells than intracutaneous injection of Ad or Ad-DC. Experiments with T-cell receptor transgenic (OT-1) T cells confirmed that the gene gun promotes a slow predominantly regional $CD8^+$ T-cell response while injection of Ad or Ad-DC induce a rapid systemic $CD8^+$ T-cell expansion. *In vivo* bioluminescence imaging revealed that the comparatively weak T-cell stimulatory capacity of the gene gun was not simply due to inefficient *in vivo* gene transfer. As an alternative explanation, we determined that gene gun immunization only induced a weak non-specific activation of the immune system when compared with Ad injection.

Conclusions: We believe that these results reflect a very important immunological function of the epidermis which must severely restrict the induction of $CD8^+$ CTL responses to maintain peripheral tolerance against harmless foreign or self antigens.

P076

Differential expression of acetylcholine receptors in T-helper 1- or T-helper 2-polarized macrophages

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The autonomous cholinergic system plays a significant modulatory role in the regulation of macrophage activation. It was previously shown that macrophages express muscarinic- (M1–M5) and nicotinic- (α7 and α10) acetylcholine receptors

(AChR) and that the activation of these receptors leads to a reduced inflammatory response to lipopolysaccharide (LPS). In this study, we analyzed if the responsivity of macrophages to cholinergic stimulation depends upon their polarization. We analyzed the expression of nicotinic and muscarinic cholinergic receptors in human macrophages, differentiated in the presence of T-helper (Th)1, Th2 cytokines and/or dexamethasone using reverse transcription polymerase chain reaction (RT-PCR). In accord with the published data, we detected the expression of mRNA for the α7 nAChR in all populations of differentiated macrophages ($n = 10$). Real-time RT-PCR analysis showed that the expression of α7 mRNA may be suppressed by both IL-4 and interferon (IFN)-γ in a donor-dependent manner, while dexamethasone had little or no effect. The expression of M5 mRNA was detected mainly in macrophages stimulated by IL-4 and/or dexamethasone and by the combination of IL-4 and IFN-γ, though it was not induced by IFN-γ alone. Similarly, α3 was expressed mainly in macrophages differentiated under Th2 conditions. In contrast, α1 was detected in macrophages stimulated by the Th1 cytokine IFN-γ. We found that mRNAs coding for α2, α4, α5, α6, β2, and β3 were not expressed in the differentiated macrophages examined, and mRNAs coding for M1, M2, M3, M4, α9, and β4 were detected in single cases without clear correlation with the type of macrophage stimulation. The significance of observed differential expression of cholinergic receptors will be investigated *in vitro* using primary macrophages-based model of LPS-induced inflammatory reaction. Donor- and stimulation-dependent regulation of the autonomous cholinergic system may impact on our understanding of stress-mediated modulation of immune reactions and autoimmune diseases.

P077

Macrophage-derived interleukin-10 controls the inflammatory response mediated by Toll-like receptor-4 but not the response to Toll-like receptor-9 ligation

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Interleukin (IL)-10 is an important regulator of immune responses secreted by a variety of cell types including macrophages, lymphocytes, epithelial, and mast cells. While complete IL-10 deficiency in IL-$10^{-/-}$ mice results in exaggerated T cell as well as innate responses, we have previously shown that mice with a selective inactivation of the *IL-10* gene in T cells suffer from deregulated T-cell responses exactly as in IL-$10^{-/-}$ animals. In contrast to IL-$10^{-/-}$ mice, the T-cell-specific mutants do not mount enhanced innate responses, which therefore must be subject to control by IL-10 from non-T cells. We now generated mice with a cell-type-specific IL-10 deficiency in macrophages and studied the local and systemic inflammatory response to CpG-oligonucleotides and lipopolysaccharide (LPS) mediated by TLR-9 and TLR-4, respectively, in these mutants. Subcutaneous injection of both TLR ligands in wildtype (wt) mice results in a moderate local inflammatory infiltration dominated by macrophages. The macrophage-specific IL-10 mutants developed an enhanced inflammatory infiltration as well as extensive tissue necrosis. The mutants also showed increased serum levels of proinflammatory mediators after intraperitoneal LPS injection in comparison with wt mice. In contrast, the local and systemic inflammatory responses of macrophage-specific IL-10 mutants to CpG oligonucleotides were indistinguishable from CpG responses in wt mice. These results show that different innate responses can be subject to control by IL-10 from different cellular sources. We are presently working on the identification of the IL-10-secreting cell type responsible for regulation of the CpG response. In addition, we are investigating innate responses in cell-type-specific IL-10 receptor mutants.

P078

T-helper 2 vaccination actively protects against inflammatory autoimmune disease

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Experimental autoimmune encephalomyelitis (EAE) is an inflammatory demyelinating disease of the central nervous system induced by the generation of an immune response against myelin epitopes. EAE is used as a model for multiple sclerosis and is a prototype of a T-helper (Th)1-driven, organ-specific autoimmune disease.

$CD4^+$ helper T cells (Th) can be divided into Th1 and Th2 subsets based upon the cytokines they produce. Th1 and Th2 cells have been found to be mutually antagonistic. Several data suggest that in chronic inflammatory autoimmune diseases, such as EAE, interferon γ-producing Th1 cells are pathogenic, while antigen-specific interleukin (IL-)4-producing Th2 cells have been shown to induce EAE only in immune-deficient mice. In the present study, we investigated whether transfer of antigen-specific Th2 cells is able to improve the clinical course of actively induced EAE in SJL mice through their cytokine pattern that curbed the encephalitogenic potential of myelin antigen (PLP)-specific Th cells.

SJL mice were immunized with PLP peptide in CFA and pertussis toxin. $CD4^+$ T cells were isolated from lymph nodes and spleen and cultured in the presence of PLP peptide with CpG and anti-IL-4 for Th1 differentiation or with IL-4 for Th2 generation. After a second round of restimulation, cytokine phenotype was determined. Adoptive transfer of antigen-specific Th1 cells led to severe disease, whereas PLP-specific Th2 cells did not induce any disease over a period of 90 days.

To show that antigen-specific Th2 cells may protect against active induction of EAE, we challenged mice that received PLP-specific Th2 cells with the autopeptide, CFA, and pertussis toxin. After transfer, mice were assessed daily for clinical signs of EAE.

Control mice developed severe encephalomyelitis after active immunization. In contrast, mice receiving Th2 cells remained healthy or developed only mild disease. Importantly, all mice vaccinated with Th2 cells survived.

Our data show that autoreactive Th2 cells do not induce inflammatory autoimmune disease. Moreover these antigen-specific Th2 cells seem to provide an anti-inflammatory memory phenotype protecting from EAE even after challenge under strong Th1-driving conditions. Thus, antigen-specific Th2 cells may be used as a cellular vaccine protecting from autoimmune disease.

P079

Mitogen-activated protein kinase p38-related interleukin-10 production does not mediate the suppressor function of regulatory T cell induced by tolerogenic dendritic cells

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We have previously shown that induced anergic regulatory T cells (iTreg), generated by stimulation of $CD4^+$ T cells with IL-10-modulated tolerogenic dendritic cells (IL-10DC) display an altered pattern of mitogen-activated protein (MAP) kinase activation as compared with effector T cells. MAP kinase p38 activity is enhanced in iTreg, whereas ERK and JNK activities are reduced. As shown by inhibition of p38 by the specific inhibitor SB203580, the activity of p38 is essential for both induction of the anergic state and the regulatory function of iTreg. MAP kinase p38 is known to be involved in the regulation of IL-10 in T cells, raising the question, if inhibition of IL-10 secretion might be responsible for the effect observed. Thus, we analyzed IL-10 expression in iTreg or effector T cells over time during restimulation (days 0–3) after primary culture, in the presence or absence of SB203580. We performed quantitative reverse transcription-polymerase chain reaction for IL-10 mRNA and quantified secreted IL-10 protein in high-sensitive enzyme-linked immunosorbent assay. Expression and secretion of IL-10

protein was low in both populations after restimulation though iTreg expressed higher levels of mRNA and secreted higher amounts of protein (0–207 pg/ml; 24.3 ± 43.3 pg/ml) as compared with effector T cells (0–17 pg/ml; 2.35 ± 4.9 pg/ml). iTreg pretreated with SB203580 showed reduced expression of IL-10 mRNA and secretion of IL-10 protein after restimulation (0–109 pg/ml; 14.7 ± 27.4 pg/ml). In contrast, effector T cells pretreated with SB203580 showed only slightly reduced mRNA expression, whereas secretion of IL-10 protein reached the level of untreated iTreg (0–209 pg/ml; 24.7 ± 49.5 pg/ml). In coculture of iTreg and effector T cells, production of IL-10 protein was within the range observed for iTreg alone (38–207 pg/ml; 120.3 ± 84.6 pg/ml). Pretreatment of iTreg with SB203580 before coculture with effector T cells resulted in reduced IL-10 production during coculture assays (33–49 pg/ml; 40.3 ± 8.0 pg/ml). Blocking experiments with antibodies against IL-10 or IL-10R did not show any effect on regulatory activity of iTreg. In summary, in our hands, production of IL-10 by iTreg is comparable with IL-10 levels of effector T cells treated with SB203580 and is far lower than reported for Tr1 cells. Our results suggest that p38-mediated IL-10 production of iTreg is not involved in the anergic state and suppressor function of Tregs induced by tolerogenic DC.

P080 (V11)

Epidermal Langerhans cells control peripheral homeostasis of regulatory T cells via CD254

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The CD265–CD254 (RANK–RANKL) interaction has various effects on immune cells including T-cell activation and inhibition of apoptosis. CD265 is expressed on dendritic cells (DC), whereas CD254 is found on activated T cells. Accordingly, epidermal Langerhans cells (LC) express CD265, and CD254 is detectable in skin upon inflammation. We have previously shown that increased epidermal CD254 expression in keratin-14-CD254 transgenic (tg) mice induced two to threefold elevated numbers of $CD4^+CD25^+$ regulatory T cells (Tregs). The increased number of Tregs was dependent on CD254-mediated signaling, as blocking this pathway with RANK-Fc resulted in a reduction of Tregs to normal levels. As keratin-14 is also expressed on thymic epithelium, we performed day 3 thymectomy followed by thymus transplantation to test if thymic CD254 expression in tg mice was responsible for the increased numbers of Tregs. Wildtype (wt) recipient mice of tg thymi developed normal numbers of $CD4^+CD25^+$ T cells, but increased numbers of $CD4^+CD25^+$ T cells were present in tg mice grafted with a wt thymus. Now, we asked the question how does local expression of CD254 in the skin alter the numbers of Tregs in lymphoid tissues as skin-specific overexpression of CD254 does not lead to enhanced serum concentrations. Interestingly, LC isolated from the epidermis of tg mice induced significantly enhanced proliferation of $CD4^+CD25^+$ T cells compared with LCs from wt. Importantly, LCs from CD254 tg mice show increased expression of CD205 which has been previously associated with DC-mediated induction of $CD4^+CD25^+$ Tregs. Furthermore, $CD4^+CD25^-$ T cells cocultured with CD254-stimulated bone marrow-derived DCs displayed enhanced expression of markers characteristic for Tregs (Neuropilin-1, CTLA-4). To analyze the relevance of epidermal LC to expand Tregs, LCs were depleted from the epidermis by topical mometasone fluorate treatment. Surprisingly, depletion of LC reduced the frequencies of $CD4^+CD25^+$ T cells in skin-draining lymph nodes of tg mice dramatically. Together, these data show that CD254-stimulated cutaneous LC/DC are important for the peripheral homeostasis and expansion of $CD4^+CD25^+$ Tregs *in vivo*.

P081

Interleukin-4 is an effector cytokine in contact hypersensitivity responses

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Contact hypersensitivity responses (CHR) against potent haptens such as trinitrochlorobenzene (TNCB) are prototypic delayed type hypersensitivity responses mediated by interferon (IFN)-γ-producing T cells. This type of response can be effectively suppressed by IL-4-producing T-helper (Th)2 cells. Less-potent haptens-like fluorescein isothiocyanate (FITC) may exert different types of CHR. We therefore defined an effective sensitization protocol, the resulting immunological phenotype, the expression dynamics of Th1 and Th2 cytokines, and the role of interleukin (IL)-4 in this model. To this end, BALB/c mice were sensitized epicutaneously, and while TNCB was effective after a single application, FITC elicited significant CHR only in mice sensitized twice per week in a 3-week protocol. Interestingly, FITC-CHR showed a biphasic pattern of cutaneous inflammation: an immediate phase maximal at 0.75 h and a delayed-type reaction peaking at 24–48 h. Intracytoplasmic FACS of skin-draining lymph nodes revealed both $IL\text{-}4^+$ and $IFN\text{-}\gamma^+$ $CD4^+$ T cells. High levels of FITC-specific IgE were detected in sensitized mice only, indicating a functional relevance of IL-4. Moreover, sensitization of $IL\text{-}4^{-/-}$ mice failed to induce significant FITC-specific IgE. To characterize a functional role of IL-4, dynamic cytokine expression was analyzed by real time polymerase chain reaction from lesional skin after FITC exposure. Interestingly, IL-4 mRNA expression peaked at 12 h, whereas IFN-γ levels increased later dominating that of IL-4 at 48 h. Consequently, the early peak of FITC-induced CHR was delayed and reduced in $IL\text{-}4^{-/-}$ mice. However, $IL\ 4^{-/-}$ mice showed increased IFN-γ production, and the late-phase CHR in $IL\text{-}4^{-/-}$ mice was reduced by only 25% indicating dominant Th1/Tc1-mediated skin inflammation mostly compensating the loss of IL-4 effect or functions. Indeed, reduction of CHR was doubled in wildtype FITC-sensitized mice treated with blocking anti-IL-4 Abs during the effector phase only. These findings indicate that (1) FITC-CHR is a suitable model for atopic dermatitis as both responses are associated with antigen-specific Th2 cells and IgE and display an early phase with IL-4 expression that is followed by a second IFN-γ-dominated phase. (2) IL-4 plays an important role as effector cytokine in CHR driven by less-potent allergens as it may be the case in atopic dermatitis and some types of contact allergy.

P082

Mass spectrometric analysis of antimicrobial active fractionated psoriatic scale extracts

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Psoriatic lesions are remarkably free of microbial infections. A number of skin-derived antimicrobial peptides have been discovered in the past; some of them were isolated from psoriatic scales. We wondered whether there might be additional skin-derived antimicrobial molecules contributing to a broad antimicrobial defense function of the skin.

To address this question, we fractionated psoriatic scale extracts and compared mass spectrometric profile with antimicrobial activity for each fraction. HPLC fractionation of heparin-affinity purified pooled scale extracts and subsequent testing for antimicrobial activity revealed high antimicrobial activity against various microorganisms in many fractions. The specificity of antimicrobial activity varied between different HPLC fractions. All major mass signals (revealed by ESI-QTOF mass spectrometric analysis) were found in antimicrobial active fractions; they correspond to known heparin-binding antimicrobial peptides/proteins identified as hBD2 [average Mass (Mr) = 4327.27, most intense mass signal], Psoriasin (Mr = 11364.92),

Lysozym (Mr = 14691.08), Rnase 7 (Mr = 14545.71), HNP-1 (Mr = 3441.45) and HNP-2 (Mr = 3370.51). Closer examination revealed some mass heterogeneity especially around major peaks of higher molecular weight indicating the presence of similar molecules (e.g. due to modifications or polymorphism). A number of less-intense mass signals in other HPLC fractions displaying antimicrobial activity (together with evidence of non-heparin-binding antimicrobials) indicate the presence of not-yet-identified antimicrobials in psoriatic scales.

Future mass mapping experiments after further purification and reevaluation of antimicrobial activity associated with these molecules may provide first clues on their identity.

P083

Short dendritic cell stimulation with the Toll-like receptor ligand peptidoglycan results in T-helper 1-shifting cells

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Toll-like receptor (TLR) ligands are powerful and versatile activators of dendritic cells (DC). Besides the upregulation of costimulatory molecules and the enhancement of the stimulatory capacity of DC, they also induce the release of different sets of cytokines. DC-derived interleukin (IL)-12 is considered an important cytokine in the induction of T-helper (Th)1 T cells. However, it is well known that IL-12 is only released during a narrow time window and afterwards the cells become exhausted. This leads to the presumption that for applications relying on the action of Th1-biased responses such as anti-tumoral immunotherapy, it would be helpful to inject DC still able to release IL-12 in the secondary lymphoid organs.

In this study, we compared several TLR ligands (PAM3CSK4, Peptidoglycan, MALP-2, Poly (I : C), lipopolysaccharide, R848, and CpG) to discover a stimulus, which activates DC within 2 h of incubation and results in cells able to induce a strong Th1-biased immune response. For this purpose, we stimulated bone marrow-derived DC (BMDC) for 2 h with the TLR ligands and checked the up-regulation of activation-dependent surface markers, the potential to activate T cells, and the release of cytokines (IL-12, IFN, and IL-10). Whereas all stimulated BMDC up-regulate surface markers, several showed a decreased stimulatory capacity in an allogenic MLR and also the release of cytokines was reduced. Peptidoglycan (PGN) proved to be the only examined TLR ligand able to activate BMDC after 2 h. In subsequent experiments, we confirmed the Th1 shifting capacity of PGN-stimulated BMDC.

P084 (V26)

Deficiency of the proteinase-activated receptor 2 results in a T-helper 2 switch in experimental leishmaniasis

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Resistance in cutaneous *Leishmania major* infection crucially depends on the development of a *L. major*-specific T-helper (Th)1 response while Th2 differentiation in BALB/c mice results in susceptibility. There is growing evidence that signaling substances produced early at the site of infection affect Th-cell differentiation, most likely by influencing the emigrating antigen-presenting cells. We therefore analyzed differential gene expression in infected skin of resistant and susceptible mice by microarray technology.

We found an increased expression of coagulation factor X (FX) 16 h after infection in resistant compared with susceptible mice. The activated FX has been described as a ligand of the proteinase-activated receptor (PAR)-2, and we recently demonstrated a proinflammatory role of PAR-2 in cutaneous inflammation.

Thus, we analyzed the course of *L. major* infection in PAR-2-deficient (PAR-$2^{-/-}$) mice. Compared with wildtype (wt) mice, PAR-$2^{-/-}$ mice developed significantly larger footpad lesions and harbored dramatically higher numbers of living parasites in footpads and local lymph nodes. In contrast to wt mice, we detected living parasites in the spleen of some PAR-$2^{-/-}$ mice. In agreement with these data, CD4-positive T-cells isolated from infected PAR-$2^{-/-}$ mice produced significantly more antigen-specific interleukin (IL)-4 and less interferon (IFN)-γ than T-cells isolated from wt mice. Thus, the *L. major*-specific Th1 type immune response is shifted toward a Th2 secretion pattern in PAR-$2^{-/-}$ mice.

Dendritic cell (DC)-derived IL-12 is known to be crucial for the induction of a Th1-type-immune response. We therefore analyzed secretion of IL-12 by DC *in vitro* and found a diminished capacity of PAR-$2^{-/-}$ DC to secrete IL-12 following lipopolysaccharide or IFN-γ stimulation.

We conclude that absence of the PAR-2 receptor results in a switch toward Th2 differentiation and increased susceptibility in experimental leishmaniasis due to diminished secretion of IL-12 by DC. It is tempting to speculate that PAR-2 agonists produced in the skin at an early stage of infection may stimulate DC IL-12 secretion and favor Th1 differentiation in resistant mice.

P085

Characterization of dendritic cells as major players in basal cell carcinoma lesions regressing upon imiquimod

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Imiquimod is a synthetic agonist to Toll-like receptor (TLR)-7. It acts as an immune response modifier and has been successfully used for the treatment of certain skin neoplasms. To get an insight into the underlying mechanisms, we analyzed the local cellular imiquimod-induced inflammatory immune response of basal cell carcinomas (BCC) ($n = 7$) at particular clinical time points of tumor regression (untreated BCC, after 2 and after 6 weeks of imiquimod therapy) by single- and multi-color immunostainings.

In untreated BCC, we found a sparse infiltrate, mainly consisting of T cells. Upon 2 weeks of imiquimod treatment, a massive infiltrate had occurred, which was dominated by $CD8^+$ T cells and considerable numbers of $CD11c^+$ myeloid dendritic cells (mDCs) and $CD14^+$ macrophages. We also observed interferon (IFN)-γ-producing $CD123^+/CD45RA^+$ plasmacytoid DCs (pDCs), which were often located in close proximity to natural killer (NK) cells. The search for death molecules (granzyme A, granzyme B, TRAIL and perforin) revealed $CD11c^+$ $HLA^-DR^+CD14^-iNOS^+$ mDCs, as opposed to NK cells and T cells, as main source of such lytic proteins. After 6 weeks of therapy, the tumors had resolved and the cellular infiltrate returned to baseline levels.

According to these results, $CD11c^+HLA^-DR^+CD14^-iNOS^+$ mDCs, referred to as Tip-DCs (TNF-iNOS-producing DCs), and IFN-α-producing pDCs represent candidate effector cells in imiquimod-induced tumor destruction.

P086

Cell volume regulation by the taurine transporter TAUT determines T-cell survival

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Mammalian cells trigger volume recovery during apoptotic cell shrinkage through accumulation of organic osmolytes. The β-amino acid taurine is a leukocyte-specific osmolyte and accumulated by the plasma membrane Na-Cl-dependent taurine transporter TAUT. The importance of functional TAUT transporter is observed in taut$^{-/-}$ mice, which show a loss of vision due to progressive loss of photoreceptor cells. The role of TAUT for lymphocytes has never been investigated. FACS analysis of naive taut$^{-/-}$ mice revealed marked or significant decrease of CD4$^+$ and CD8$^+$ T cells in peripheral blood and spleen compared with taut$^{+/+}$ mice; however no such differences could be observed in CD19$^+$ B cells from blood, lymph node, or spleen. Interestingly, especially activated CD69$^+$ CD4$^+$, and CD69$^+$ CD8$^+$ T cells were significantly decreased in spleen cells from taut$^{-/-}$ mice. However, lack of TAUT does not affect all cell types and in all compartments, as lymph node T- and B-cell numbers were unchanged comparing naive taut$^{+/+}$ and naïve taut$^{-/-}$ mice. As resting and naïve T cells present in lymph nodes of naïve mice are resistant to apoptosis, we investigated the role of TAUT in animals that were sensitized with haptens as an example of immunological stress. Contact hypersensitivity reaction to trinitrochlorobenzene was elicited and resulted in a significant decrease in the percentage of CD4$^+$ and CD8$^+$ T-cells populations in skin-draining lymph nodes and in reduced inflammatory responses in the skin of taut$^{-/-}$ mice. These findings indicated that intact memory and naïve T cells resting in lymph nodes are susceptible to apoptosis upon antigen-specific activation and that TAUT may play a crucial role in rescuing T cells from activation-induced cell death. Indeed, activation studies showed increased rates of apoptosis as demonstrated by enhanced annexin binding of taut$^{-/-}$ CD4$^+$ and CD8$^+$ T cells followed by reduced taut$^{-/-}$ CD4$^+$ and CD8+ T-cell survival compared with wildtype. These data show that cell volume regulation by TAUT is important in the decision of T-cell apoptosis vs. survival after activation and may therefore play a crucial role in the regulation of T-cell memory.

P087

Tumor necrosis factor-α induces a delayed anti-inflammatory response in lipopolysaccharide-stimulated macrophages mediated by transcription factor ARNT2

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Recognition of lipopolysaccharide (LPS), a major constituent of Gram-negative bacteria, by monocytes and macrophages initiates a rapid release of inflammatory mediators resulting in vascular activation and recruitment of immune cells. In contrast to immediate reactions, a reliable analysis of the late gene expression program in monocytes elicited by LPS is not yet done. Using oligonucleotide microarrays covering more than 13 000 genes, we performed a sophisticated analysis to define the delayed LPS-triggered gene expression profile in monocytes after 16 h. Functional clusters according to the Gene Ontology consortium classification like 'inflammatory response,' 'adhesion,' and 'apoptosis' were significantly over-represented among the 247 up- and 99 down-regulated genes. In addition, statistical analysis demonstrated that LPS induced a tumor necrosis factor (TNF)-α dependent up-regulation of anti-inflammatory rather than proinflammatory molecules at this delayed time point, which was confirmed by specific blockade of TNF-α in independent functional experiments. These data point to an important TNF-α-dependent feedback mechanism protecting against overwhelming inflammatory reactions, which may be responsible for the deleterious effects of anti-TNF-α therapy in human sepsis. We identified bHLH-PAS transcription factor ARNT2 as a mediator of this gene expression pattern which therefore may be an attractive target for modulation of the innate immune response to bacterial triggers.

P088

C57BL/6 mice develop stronger dermatitis in an antigen specific model of atopic dermatitis compared with BALB/c mice

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Development of an immune imbalance favoring T-helper (Th)2 responses is regarded to be an important underlying cause for atopic dermatitis (AD); however, 25% of AD patients are non-atopic in this respect. An important model to study the divergent and inherent immune response pattern is the comparison of Th2-prone BALB/c mice with C57BL/6 mice that mount Th1 responses. It was the goal of this study to define characteristic differences in the immune response of BALB/c and C57BL/6 mice after epicutaneous antigen exposure as it is relevant in AD. Development of immune responses and dermatitis in response to ovalbumin (OVA) application is believed to represent a good model for AD. To this end, mice of both strains repetitively received epicutaneous applications of OVA. Analysis revealed that both strains developed significant levels of Th2-associated OVA-specific immunoglobulin (Ig)E and IgG1; however, BALB/c IgE levels were three times higher, whereas IgG1 was always much higher in C57BL/6 mice. Cutaneous anaphylaxis using Evan's blue injections demonstrated unequivocal responses to OVA in both strains indicating that IgG1 compensated for IgE in C57BL/6 mice. Only C57BL/6 mice developed remarkable levels of OVA-specific IgG2a levels indicating the development of Th1 cells. Surprisingly, OVA-dermatitis was significantly greater in C57BL/6 mice displaying severe skin inflammation and epidermal acanthosis. Adoptive transfer experiments into ear skin of naive syngeneic mice showed that application of OVA-specific Th2 cells together with OVA induced strong ear swelling responses but not Th2 cells or OVA alone. The adoptive transfer of OVA-specific Th1 cells together with OVA induced stronger and more prolonged ear swelling responses compared with Th2-cell-mediated inflammation; however, there were no differences between BALB/c and C57BL/6 Th1 or Th2 cells and mice. These data demonstrate that the degree of OVA-specific dermatitis correlates with increasing Th1 cells rather than with increasing Th2 immunity explaining AD development independent of Th2 and IgE and the correlation of severe AD with IFN-γ expression.

P089

Mast cell tumor necrosis factor induces tissue inflammation through tumor necrosis factor receptor 1-expressing endothelia

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Signaling through tumor necrosis factor receptor 1 (TNFR1) plays a central role in the control of intracellular pathogens and the induction of inflammatory T-helper (Th)1-cell-mediated autoimmune diseases, such as non-obese diabetes or autoimmune encephalitis. Yet, the underlying mechanisms remain enigmatic, and various models have been proposed. Dissecting Th1-cell-mediated delayed-type hypersensitivity responses (DTHR) into single steps here, we show that the central defect is localized to the missing TNFR1 expression by endothelial cells (ECs). TNFR1 was not needed for *in vivo* priming of effector Th1 cells capable to induce strong DTHR, as shown by adoptive transfer experiments. In addition, mast cell-knock-in experiments, using $TNF^{-/-}$ or $TNFR1^{-/-}$ recipient mice showed that the signaling defect was downstream between mast cells and endothelia. Downstream analysis showed that $TNFR1^{-/-}$ mice are highly defective in inducing mRNA expression of P-selectin, ICAM-1, and VCAM-1 during DTHR. To definitely ask whether TNFR1-signaling on endothelia is required, we performed intravital fluorescence microscopy. In $TNFR1^{-/-}$ mice, lymphocyte rolling was strongly suppressed at 2 h post challenge. More importantly, firm adhesion was reduced to 20% even at 3.5 h a time when lymphocytes infiltrate the ear tissue. Thus, TNFR1 signaling from MC to TNFR1-expressing EC is critically needed for lymphocyte recruitment during Th1-cell-induced DTHR. Appropriate expression of P-selectin, ICAM-1, and VCAM-1 by EC is essential for recruitment of mononuclear and polymorphonuclear cells during Th1-cell-mediated inflammation. Thus, signaling at mast cell-derived TNF through TNFR1-expressing EC is essential for control of intracellular pathogens or the induction of inflammatory autoimmune diseases.

P090

M-DC8$^+$ blood dendritic cells are principal producers of early interleukin-12: studies on their control in blood by erythrocytes and their presence in the T-helper 1-dominated diseases psoriasis vulgaris and rheumatoid arthritis

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Early and high-level production of interleukin (IL)-12 is crucial for effective immune responses against pathogens; yet, the cells that provide this initial IL-12 have remained elusive. We show that a subset of human blood dendritic cell (DC) which is defined by a carbohydrate modification of P-selectin ligand-1 and recognized by the mAb M-DC8 is the principal and primary source of IL-12p70 when blood leukocytes are stimulated with the Toll-like receptor (TLR)-4 ligand lipopolysaccharide (LPS) or with CD40L. To respond to LPS, it was found that these DCs require a short phase of *in vitro* maturation which is completely blocked in the presence of erythrocytes. This maturation depends on the interaction of CD47 on erythrocytes and its ligand SIRP-α on M-DC8$^+$ DC. While tightly controlled in blood by erythrocytes, in tissues, the high IL-12- and TNF-α-producing capacity of M-DC8$^+$ DC may be critical for the defense against pathogens and, if uncontrolled, may lead to adverse inflammatory reactions. Accordingly, we found large numbers of M-DC8$^+$ DC within the cellular infiltrate of the T-helper (Th)1-dominated diseases psoriasis vulgaris and rheumatoid arthritis.

P091

Human cytotoxic lymphocytes release upon activation two different granule systems – RANTES comes first

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Natural killer cells and CD8$^+$ cytotoxic T lymphocytes (CTLs) contain lytic granules. We recently described the discovery and characterization of an additional and independent granule system within these cells characterized by RANTES as the so far sole known cargo. We now extended these studies and compared the release kinetics of both granule systems. Flow cytometry, laser scan microscopy, enzyme-linked immunosorbent assay, Western blotting, activation of cells using various stimuli, as well as activation of a CTL clone (TyrF8) by the melanoma cell line Mel93.04A12 were employed. RANTES granules were mobilized to the cell surface within 1 h after stimulation with a combination of phorbol ester and calcium ionophore and/or anti-CD3 crosslinking in the case of CTLs. Within this time interval, the release into the supernatant was independent of protein neosynthesis. In addition, RANTES granule release from cytotoxic peripheral blood lymphocytes was only in part influenced by the level of extracellular calcium. Release kinetics of RANTES granules was significantly faster as compared with perforin granules. This difference was also observed during the interaction of CTLs with melanoma targets and required the establishment of an immunological synapse.

Taken together, a new secretory cell organelle in cytotoxic lymphocytes is described which is stuffed with RANTES. RANTES granules are secreted before the lethal hit is performed. The relevance of this finding for immunoregulation and tumor defense has to be elucidated.

P092

Cyclosporin A abolishes CD28-mediated resistance to CD95-induced apoptosis via superinduction of caspase-3

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Costimulation of T cells via CD28 promotes both proliferation and resistance to apoptosis. Here, we show that the immunosuppressive drug cyclosporin A (CsA) fully reverses resistance to CD95-mediated cell death after T-cell receptor (TCR)/CD28 costimulation or superagonistic anti-CD28 mAb stimulation of primary T cells. This effect correlated with a pronounced superinduction of caspase-3 on both mRNA and protein levels, while its main antagonist, X-chromosome-linked inhibitor of apoptosis, was unaffected by inclusion of CsA. Apoptosis triggered by CD95 crosslinking was characterized by robust capase-3 activation. Pretreatment of these cells with a caspase-3-like protease inhibitor largely prevented CD95-triggered apoptosis, indicating that caspase-3-like activity is required for breaking apoptosis resistance in CsA-treated TCR/CD28 costimulated or superagonistic anti-CD28 mAb-activated T cells. Furthermore, CsA sensitization to CD95-mediated apoptosis of CD28-activated T cells did not alter mRNA stability of superinduced caspase-3 mRNA suggesting a transcriptional regulation of the *caspase-3* gene. Addition of $Ca2^+$ ionophores to TCR/CD28 or

superagonistic CD28-stimulated cells reduced caspase-3 levels, further supporting a role for $Ca2^{+}$-dependent signaling pathways in negatively regulating caspase-3.

Taken together, these findings suggest that CsA promotes sensitivity to CD95-mediated apoptosis in CD28-stimulated T cells by superinduction of the *caspase-3* gene via a mechanism involving suppression of the calcineurin pathway. The data presented here might shed a new light on additional potential mechanisms of immune suppression conferred by CsA treatment.

P093

Interleukin-4 determines the dendritic cell phenotype in humans by up-regulating interleukin-12 and inhibiting interleukin-10 production

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Interleukin (IL)-4 is known to be the major factor that initiates T-helper (Th)2 cell differentiation. Recently, it was demonstrated that IL-4 also potently promotes IL-12 production by dendritic cells (DC) consequently inducing interferon-γ (IFN-γ) producing Th1 cells. These findings are of importance for the understanding especially of the regulation of immune responses associated with IL-4 such as in atopic dermatitis or allergic asthma. In these diseases, early IL-4 dominated inflammation is followed by IFN-γ expression, and the regulation of this process is still not understood. Moreover, human monocyte-derived DC (MoDC) are commonly generated *in vitro* using IL-4 and promising DC-based vaccination strategies to treat cancer may also depend on regulatory effects of IL-4 on DC. To elucidate the role of IL-4 in humans, the phenotype of activated MoDC was analyzed as a function of IL-4. In all cases, $CD11c^{+}$ $CD14^{-}$ HLA-DR^{+}, $CD86^{+/-}$, $CD83^{-}$ immature MoDC were analyzed and activated with different stimuli and cytokines. As expected, HLA-DR and CD86 expression was vigorously up-regulated and CD83 appeared within 24 h upon stimulation with LPS and other stimuli. Pre-culture with different doses of IL-4 between 5-50 ng/ml did not significantly influence the DC phenotype as detected by expression of the indicated surface molecules. However, in contrast to low or intermediate doses of IL-4, high concentrations of IL-4 markedly altered the LPS-induced cytokine production of human DC. High-dose IL-4 significantly enhanced IL-12p70 production in human DC. Investigating the underlying mechanisms, we found that enhanced IL-12p70 production induced by high dose IL-4 depended on IL-4-regulated reduction of IL-10 in stimulated DC. In low-dose IL-4-treated DC, LPS activation induced an IL-12/IL-10 ratio <1, whereas in high-dose IL-4-treated DC, IL-12 was up- and IL-10 was down-regulated resulting in an IL-12/IL-10 ratio >6. Our data clearly show that there is a regulatory balance of IL-12 and IL-10 in human DC that is orchestrated by IL-4. This is of clinical relevance in regard to the role of IL-4 in atopic diseases and for the design of immunotherapies using *in vitro*-generated DC for vaccination.

P094

Evaluation of DNA vaccination with recombinant adenoviruses using bioluminescence imaging of antigen expression

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Recombinant DNA vaccines are able to induce strong $CD8^{+}$ T-cell-mediated immunity and have become increasingly attractive for the prevention and treatment of infectious diseases and cancer. The application route and the delivery method determine the *in vivo* distribution of antigen and critically influence the resulting immune response. Here, we report the construction of vectors expressing a fusion protein between enhanced green fluorescent protein, the H2-Kb-binding peptide OVAaa257–264 and green click beetle luciferase as a model antigen which allows for simultaneous quantitative assessment of antigen expression using fluorescence and bioluminescence imaging in correlation with $CD8^{+}$ T-cell stimulation *in vivo*. We applied this construct to evaluate the role of the application route for DNA vaccination with recombinant adenoviral vectors and assess the impact of using cultured dendritic cells for vaccine delivery. Antigen expression was non-invasively followed *in vivo* by visualizing bioluminescence with an ultrasensitive CCD camera. $CD8^{+}$ T-cell stimulation was detected with H2-Kb-OVAaa257–264 tetramers. We found that intravenous injection of adenovirus-transduced dendritic cells stimulated the strongest OVAaa257–264-specific CTL responses, although it delivered two orders of magnitude less antigen *in vivo* when compared with direct injection of recombinant adenovirus. We believe that our experimental approach has the potential to facilitate translational development of improved genetic immunization strategies designed to target and activate dendritic cells directly *in vivo*.

P095

CCL1–CCR8 interactions: an axis mediating the recruitment of T cells and Langerhans cells to sites of atopic skin inflammation

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Atopic dermatitis represents a chronically relapsing skin disease with a steadily increasing prevalence. Skin-infiltrating T cells, dendritic cells (DC), and mast cells are thought to play a crucial role in its pathogenesis. We report that the expression of the CC chemokine CCL1 is significantly and selectively up-regulated in atopic dermatitis in comparison with psoriasis, cutaneous lupus erythematosus, or normal skin. CCL1 serum levels of atopic dermatitis patients are significantly higher than in healthy individuals. DC, mast cells, and dermal endothelial cells are abundant sources of CCL1 and allergen challenge, and *Staphylococcus aureus*-derived products induce its production. *In vitro*, binding and cross-linking of immunoglobulin E on mast cells resulted in a significant up-regulation of this inflammatory chemokine. Its specific receptor, CCR8, is expressed on a small subset of circulating T cells and is abundantly expressed on DC. Activation of circulating T cells recruits CCR8 from intracytoplasmic stores to the cell surface. CCL1 synergizes with the homeostatic chemokine CXCL12, resulting in the recruitment of T cells and DC. These findings suggest that CCL1 plays a role in the initiation and amplification of atopic dermatitis.

P096 (V29)

Interleukin-31 – a new link between T cells and pruritus in atopic skin inflammation

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Interleukin-31 (IL-31) is a novel T-cell-derived cytokine, which induces severe pruritus and dermatitis in transgenic mice. In this report, we demonstrate for the first time that human IL-31 is significantly overexpressed in 'pruritic' atopic compared with 'non-pruritic' psoriatic skin inflammation. Notably, non-lesional skin of atopic dermatitis patients showed significantly increased IL-31 and IL-31RA expression as compared with healthy skin. Highest levels of IL-31 transcripts were detected in prurigonodularis, one of the most pruritic forms of chronic skin inflammation. *In vivo*, exposure to staphylococcal superantigen, a known trigger factor of atopic skin inflammation, rapidly induced IL-31 expression in atopic individuals. *In vitro*, staphylococcal enterotoxin B but not exposure to viruses or T-helper (Th)1 and Th2 cytokines induced IL-31 in leukocytes. In atopic individuals, activated leukocytes produced significantly higher levels of IL-31 transcripts compared with non-atopic subjects. Analysis of the tissue distribution (in 63 different human tissues) of the IL-31 receptor heterodimer demonstrated that IL-31RA showed most abundant expression in dorsal root ganglia representing the site within the peripheral nervous system where the cell bodies of cutaneous sensory neurons reside. Our findings provide a new link between increased *Staphylococcus aureus* colonization, subsequent T-cell recruitment/activation, and pruritus induction in atopic dermatitis patients. Taken together, IL-31 and its signaling pathway may represent interesting novel targets for antipruritic drug development.

P097

IL-4 determines the DC phenotype in humans by upregulating IL-12 andinhibiting IL-10 production

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Interleukin (IL)-4 is known to be the major factor that initiates Th2 cell differentiation.Recently, it was demonstrated that IL-4 also potently promotes IL-12 production bydendritic cells consequently inducing interferon g(IFN-g) producing Th1 cells. Thesefindings are of importance for the understanding especially of the regulation ofimmune responses associated with IL-4 such as in atopic dermatitis or allergicasthma. In these diseases, early IL-4 dominated inflammation is followed by IFN-gexpression and the regulation of this process is still not understood. Moreover,human monocyte-derived DC (MoDC) are commonly generated in vitro using IL-4and promising DC based vaccination strategies to treat cancer may also depend onregulatory effects of IL-4 on DC. To elucidate the role of IL-4 in humans, thephenotype of activated MoDC was analyzed as a function of IL-4. In all casesCD11c+ CD14- HLA-DR+, CD86+/-, CD83- immature MoDC were analyzed andactivated with different stimuli and cytokines. As expected, HLA-DR and CD86expression was vigorously upregulated and CD83 appeared within 24h uponstimulation with LPS and other stimuli. Pre-culture with different doses of IL-4between 5-50 ng/ml did not significantly influence the DC phenotype as detected byexpression of the indicated surface molecules. However, in contrast to low orintermediate doses of IL-4, high concentrations of IL-4 markedly altered the LPSinduced cytokine production of human DC. High dose IL 4 significantly enhancedIL-12p70 production in human DC. Investigating the underlying mechanisms, wefound that enhanced IL 12p70 production induced by high dose IL-4 depended onIL-4 regulated reduction of IL-10 in stimulated DC. In low dose IL-4 treated DC LPSactivation induced an IL-12/IL-10 ratio 6. Ourdata clearly show that there is a regulatory balance of IL-12 and IL-10 in human DCthat is orchestrated by IL-4. This is of clinical relevance in regard to the role of IL-4 inatopic diseases and for the design of immunotherapies using in vitro generated DCfor vaccination.

P098 (V07)

Papillomavirus-like particles for immunotherapy of prion disease

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Prion diseases, including bovine spongiform encephalopathy and Creutzfeldt–Jakob disease, are fatal neurodegenerative disorders caused by proteinaceous infectious pathogens termed prions (PrPSc). To date, there are no prophylactic and therapeutic modalities available. Recent studies have shown that passive immunization with monoclonal antibodies (Abs) recognizing the normal host-encoded prion protein (PrPC) abolishes PrPSc infectivity and delays onset of disease. While highly attractive, active immunization may be difficult to achieve due to established immunological tolerance against the widely expressed PrPC.

Immunization with papillomavirus-like particles (VLP) genetically engineered for surface expression of certain self-antigens has resulted in high-titer and long-persisting production of specific autoAbs. By recombinant baculo virus technology, we have now generated PrP-VLP that display a nine amino acid (aa) peptide, DWEDRYYRE, of the murine/rat prion protein (aa 144–152) in an immunogenic surface loop of L1 major capsid protein of the bovine papillomavirus 1. This peptide was selected based on its suggested central role in prion pathogenesis. Immunization with PrP-VLP induced high-titer, high-affinity anti-PrP Abs in rabbits and in rats, without inducing adverse effects. As determined by peptide-specific enzyme-linked immuno-assay, rabbit immune sera recognized not only the inserted murine/rat epitope but also cross-reacted with the rabbit/human epitope DYEDRYYRE differing in only one aa at position 2. In contrast, rat immune sera recognized the murine/rat peptide only. Sera of both the species recognized PrPC in its native conformation in mouse brain and on rat pheochromocytoma cells as determined by immunoprecipitation and FACS analysis. Importantly, rabbit (but not rat) anti-PrP Abs inhibited *de novo* synthesis of pathogenetic PrPSc in infected cells *in vitro*. If also effective *in vivo*, PrP-VLP vaccination opens a unique possibility for medically applicable immunologic prevention of currently fatal and incurable prion-mediated diseases.

P099

Dendritic cells increase T-cell receptor sensitivity by pre-activating T-lymphocyte src kinases in the absence of antigen

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We have identified a novel mechanism that allows dendritic cells (DCs) to trigger unsurpassed numbers of antigen-specific T-cell receptors. This mechanism operates independently of conventional costimulation, the main established reason for superior T-cell stimulation by DCs. Instead, we observed that DCs, as compared with non-professional antigen-presenting cells (APCs), have an unsurpassed capacity to activate T-lymphocyte src kinases in the absence of antigen. Src kinases are switched on immediately after T-cell contact with unloaded DCs, which contrasts with the more delayed antigen-dependent src kinase activation exhibited by all types of APCs. Interfering pharmacologically with src kinase pre-activation blunts TCR triggering with DCs but not with non-professional APCs. Thus, DCs increase TCR sensitivity for antigenic ligands by pre-activating T-lymphocyte src kinases in an antigen-independent way. Detailed elucidation of the underlying mechanism will hopefully shed new light on the pathogenesis of T-cell-mediated diseases and pave the way for future therapeutic strategies.

P100

Agonists of proteinase-activated receptor-2 induce histamine release from human primary skin mast cells

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Proteinase-activated receptor-2 (PAR-2) belongs to a new G-protein-coupled receptor subfamily activated by serine proteinases. PAR2 has been demonstrated to play a role during inflammation and the immune response. Although PAR2 immunoreactivity has been demonstrated in human mast cells, its impact on skin mast cell function is still unclear. Therefore, we examined whether PAR2 is functionally expressed by human primary skin mast cell (HPMC) and the mast cell line HMC-1. Reverse transcription polymerase chain reaction and FACS analysis show expression of PAR2 both at the RNA and protein level. HPMCs also express PAR1, PAR3 and PAR4. Ca -mobilization studies reveal functional PAR2 expressed by human skin mast cells. PAR2 agonists induced histamine release from HPMC indicating a role of PAR2 in regulating inflammatory and immune responses by mast cells. Double-immunofluorescence staining reveals colocalization of PAR2 with tryptase in the majority of human skin mast cells. In conclusion, agonists of PAR2 induce up-regulation of mediators by human primary mast cells involved in inflammation and hypersensitivity. Thus, PAR2 may be an important regulator of skin mast cell function during inflammation and the immune response. Because tryptase activates PAR2 and induces inflammation and hypersensitivity reactions in many organs, one may speculate that tryptase may regulate human mast cell function in an autocrine manner.

P101

Presence of lytic proteins in myeloid and plasmacytoid dendritic cells

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Prevailing opinion holds that T cells are the major effector cells within the cellular infiltrate of eczematous skin diseases such as allergic contact dermatitis (ACD) or atopic dermatitis (AD). One of their functions is the induction of apoptosis in keratinocytes via the expression of death molecules such as FASL, TRAIL or perforin.

By investigating skin biopsies from 72-h epicutaneous patch test lesions (EPT) and AD, we detected molecules of the death machinery not only in infiltrating T cells but also in non-T cells.

A more thorough investigation of the non-T cells in these biopsies demonstrated that a substantial portion of these cells represent myeloid and plasma cytoid dendritic cells (DC). Interestingly, we demonstrate that in EPT as well as in AD lesions $CD123^+MHCII^+$ pDC are positive for granzyme B and TRAIL L. In contrast, $CD1c^+MHCII^+$ mDC display a broader variety of death molecules including granzyme B, perforin, TRAIL L, FAS L and the death receptors TRAIL R1 and FAS.

Further analysis conducted by flow cytometry from peripheral blood DC endorsed our finding that expression of lytic proteins on $CD123^+MHCII^+$ BDCA-2^+ pDC is limited to granzyme B and TRAIL L, whereas $CD11c^+MHCII^+CD14$-mDC exhibit a wide spectrum of death molecules intra- and extracellularly.

Taken together, these results indicate that DC utilize molecules of the death machinery to regulate T-cell-mediated allergic inflammation.

P102

'Skin conditioning' as a means to enhance dendritic cell migration for adoptive immunotherapy

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Different clinical trials pointed out recently that the effect of adoptive immunotherapy with tumor-specific dendritic cells (DCs) is severely limited by an ineffective migration of injected cells.

Therefore, we established a mouse model, adapted to human vaccination protocols, that offers a tool to improve DC migration from skin to lymph node.

Congenic (CD45.1) DCs were injected into the skin, and numbers and phenotype of DCs arriving in the lymph nodes were evaluated. First, results with applications of inflammatory cytokines had shown that a pretreatment of the injection site (conditioning) 24 h before DC injection is crucial for enhanced cell migration.

On the basis of these data, we investigated the immunomodulatory potency of diverse Toll-like receptors (TLR) ligands [e.g. Poly(I : C), CpG, Imiquimod] for skin conditioning prior to DC injection. Treatment with TLR ligands caused an increase of DCs arriving in the lymph node and, furthermore, improved antigen-specific T-cell responses.

This mouse model will allow us to optimize adoptive therapy with DCs via the skin.

P103

Function of Langerhans cell-like dendritic cells in antiviral immunity of the skin

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Langerhans cells (LCs), a subset of antigen-presenting dendritic cells (DC) found in the epidermis, play an important role in innate immunity. Many bacteria and viruses invade through the skin, and LCs, as a first line defense mechanism, are able to initiate appropriate immune responses to the pathogen.

We generated LC-like DCs from $CD34^+$ cells isolated from umbilical vein blood and compared their Toll-like receptor (TLR) expression and function to monocyte-derived DCs (Mo-DC). Expression of TLRs 1–9 could be demonstrated for the LC-like DCs, with the highest expression being of TLR-2, followed by TLR-4, TLR-7 and TLR-5. Functionally, LC-like DCs and Mo-DCs secreted high quantities of proinflammatory cytokines and chemokines after stimulation with the TLR-2 ligand peptidoglycan or the TLR-7 ligand R-848 (Resiquimod). After stimulation with the TLR-3 agonist, Poly(I : C) LC-like DCs produced significantly higher amounts of the interferon (IFN)-γnducible chemokines MIG

and I-TAC compared with Mo-DCs, whereas Mo-DCs secreted more IP-10. The ability of LC-like DCs to directly modulate pathogen replication was assessed using a viral infection model with either vesicular stomatitis virus (VSV) or murine γ-herpes virus (MHV-68). Human fibroblasts were pre-incubated with supernatants from LC-like DCs stimulated with Poly(I:C) or control and then infected with either VSV or MHV-68. Upon pre-incubation with Poly(I:C)-stimulated supernatants, we observed a marked decrease in the replication of both VSV or MHV-68, similar to pre-incubation of the cells with media containing IFN-α.

All together, these observations suggest that LCs contribute to the innate immune response against viral infection by the production of a specific pattern of cytokines and IFN-inducible chemokines as well as inducing a direct antiviral activity.

P104

Melanoma-associated chondroitin sulfate proteoglycan: A promising target antigen for cancer immunotherapy?

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The identification of tumor antigens recognized by T cells on human tumor cells (TCs) has opened new avenues in cancer immunotherapy. Unfortunately, clinical responses in most studies have been observed only in the minority of vaccinated patients. There are several possible reasons for these disappointing results. First, TCs can down-regulate antigen expression, as most antigens identified so far do not play an essential functional role for their malignant phenotype. Second, TCs can down-regulate or loose the expression of human leucocyte antigen molecules and thus escape recognition by T cells. Third, tolerance avoids the induction of potent and high-affinity T-cell responses, as most antigens used in tumor immunotherapy represent self antigens. An ideal target antigen therefore would be tumor-specific and functionally relevant for the tumor. Melanoma-associated chondroitin sulfate proteoglycan (MCSP) represents a transmembrane glycoprotein–proteoglycan complex, consisting of an N-linked glycoprotein of about 250 kDa and a >450 kDa proteoglycan component and is expressed in >90% of human melanoma tissues. MCSP was found to be involved in signal cascades concerning adhesion and extravasation of TCs and therefore thought to play an important role in determining the invasive and metastatic potential of melanoma cells. However, MCSP has been shown to be expressed in some normal tissues, and thus tolerance or autoimmunity (when breaking tolerance) could be a major drawback also with this antigen.

Surprisingly, we could rapidly generate MCSP-specific T-cell clones from healthy blood donors by the use of antigen-loaded DCs and identify several T-cell epitopes within the core protein of MCSP. The isolated T cells directly recognized MCSP-expressing melanoma cells. Furthermore, we could demonstrate by ELISPOT analysis a strong MCSP-specific T-cell immunity in the peripheral blood of the majority of healthy donors tested, and these donors showed no clinical signs of autoimmunity. Melanoma patients also showed MCSP-specific T-cell reactivity but clearly to a lesser extent than normal donors. This T-cell reactivity seemed to decrease.

P105 (V19)

Localized inflammatory skin disease following inducible ablation of I kappa B kinase 2 in murine epidermis

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In previous studies, we have shown that epidermis-specific deletion of I κB kinase 2 (IKK2) in the epidermis of newborn mice results in a T-cell independent inflammatory skin disease with dramatic invasion of macrophages and granulocytes into the skin. To investigate the consequences of IKK2 deletion in adult murine skin, we generated mice with inducible epidermal deletion of IKK2(K14-Cre-ERT2IKK2fl/fl). For that, we used a transgenic mouse line expressing tamoxifen-inducible Cre recombinase under the control of the human keratin 14 (K14) promoter. K14-Cre-ERT2IKK2fl/fl mice were born normally and did not show any signs of a skin disease for at least 6 months. Three weeks after local treatment of back skin with tamoxifen, a circumscribed skin disease started to develop. K14-Cre-ERT2IKK2fl/fl mice, older than 6 months, that were not treated with tamoxifen also developed signs of a skin disease at their neck and chest. Histological analysis of sections of diseased skin revealed increased cell density in the dermis as a result of invasion of inflammatory cells, hair follicle disruption, and pseudoepitheliomatous hyperplasia of the epidermis, but no tumor formation. The hyperplastic epithelium showed strong activation of the STAT3 and ERK1/2-signaling pathways that are known to be active in hyperproliferative and inflammatory skin diseases. Our results identify a primary role for IKK2 in the development of skin inflammation and confirm its requirement for the maintenance of skin homeostasis.

P106

High expression levels of the inflammatory cytokine interleukin-31 in patients with atopic dermatitis and allergic contact dermatitis, but not psoriasis

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Cytokine-regulated inflammatory processes play a major role in the development of many skin diseases. It is well established that T lymphocytes crucially contribute to the patho-genesis of diseases like atopic dermatitis or psoriasis. Many studies throughout the last decade have highlighted the role of several cytokines in triggering T-cell-mediated responses, particularly of interleukin (IL)-12 and interferon-γ for a T-helper type 1 (Th1) response and IL-4, -5 and -13 for a Th2 response. Recently, a novel cytokine, IL-31, was identified which is produced by activated T lymphocytes, preferentially by Th2 cells and to a minor extent by Th1 cells. Transgenic mice overexpressing IL-31 develop a phenotype closely resembling atopic dermatitis in humans. To evaluate the potential importance of this novel cytokine in the pathogenesis of T-cell-mediated skin diseases, we analyzed skin biopsies from patients with atopic dermatitis, allergic contact dermatitis and psoriasis. Confirming the results so far obtained only in transgenic mice, we find statistically elevated mRNA levels of IL-31 in biopsies taken from patients with atopic dermatitis. However, no increased transcription of IL-31 can be detected in biopsies taken from psoriatic plaques. Surprisingly, we detect strongly increased mRNA levels of IL-31 in many biopsies taken from patients with allergic contact dermatitis, which has so far been mainly recognized as

a Th1-mediated disease. A comparison of the mRNA levels of IL-31 with well-known Th1 or Th2 cytokines demonstrates a close correlation of the expression of IL-31 with IL-4 and IL-13, but not with IFN-γ. Therefore, IL-31 seems to play a crucial role in Th2- rather than Th1-mediated chronic skin diseases.

P107

Number and function of $CD4^+CD25^+$ regulatory T cells in patients with bullous pemphigoid

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Naturally occurring $CD4^+CD25^+$ regulatory T cells (Treg) are essential for the maintenance of immunological self tolerance. A defect in the Treg compartment seems to play a role in the pathogenesis of several human autoimmune diseases. Bullous pemphigoid (BP) is a subepidermal blistering disease associated with T-cell reactivity and autoantibodies to components of hemidesmosomes, particularly collagen XVII (BP180). In this study, we addressed the question whether regulatory T cells in the peripheral blood of patients with BP are reduced and/or functionally impaired thereby contributing to the loss of tolerance to self antigens.

Preliminary studies in seven patients with untreated BP revealed no significant difference in the frequency of $CD25^+$ and CD25 high expressing $CD4^+$ T cells compared with normal volunteers (mean 19.5 and 4% vs. 17.8 and 4%, respectively). Because suppression of autoimmune responses in BP is expected to take place primarily in the skin, we estimated the skin-homing potential of Tregs by analysing their expression of CLA. CLA expression was increased among $CD4^+CD25^+$ as compared with $CD4^+CD25^-$ T cells but was comparable between BP patients and controls (37.5 vs. 44.8%). The percentage of $CD25^+$ T cells among $CD4^+CLA^+$ T cells was also almost identical (34.3 vs. 34.5%). MACS-purified Treg of BP patients were functionally intact as assessed by their ability to suppress allogeneic T-cell proliferation. Thus, there appears to be no obvious defect in the overall number, migratory property or function of regulatory T cells in BP patients. Future studies will address the role of Treg in lesional skin and in the regulation of collagen XVII-specific T-cell responses.

P108

Human leukocyte antigen-A2-restricted peptides derived from the tumor stroma-associated antigen fibroblast activation protein-α induce specific T-cell responses in melanoma patients

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Fibroblast activation protein α (FAP-α, seprase) is a cell-surface protein with dual serine protease and dipeptidyl-peptidase activity. It is not expressed on normal adult tissue. However, FAP-α expression is up-regulated in the tumor micro milieu where it is mainly found on fibroblasts but can also be detected on tumor cells. Although the role of FAP-α in tumor progression is still controversially discussed, due to its strong induction in tumors, FAP-α is a promising target for cancer immunotherapy. By reverse immunology, we have identified several human leukocyte antigen (HLA)-A2-restricted peptides derived from FAP-α that induce a human T-cell response, measured by IFN-γ ELISPOT. Exchange of anchor amino acids of such peptides enhances their binding to HLA-A2 antigens, thereby rendering them more immunogenic. Moreover, improved peptide/major histocompatibility complex affinity allowed the construction of recombinant HLA-A2/FAP-α peptide complexes. Indeed, using such HLA-A2/FAP-α dextramers, FAP-α-specific T cells can be visualized in circulating blood of melanoma patients. Currently, we analyse the immunogenicity of the modified FAP-α peptides *in vivo* using HLA-A2/kb transgenic mice. This approach also serves to exclude major side effects of induced anti-FAP-α immune responses in a pre-clinical setting. The peptides with the highest potential in inducing stroma-specific immune responses will then be applied in immunotherapeutic studies in cancer patients.

P109

Ultraviolet-A light and singlet oxygen quantum yield of endogenous photosensitizers determined directly by its luminescence

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The ultraviolet-A (UVA) component of solar radiation has been shown to produce deleterious biological effects in which singlet oxygen plays a major role. In tissue, the UVA light is only weakly absorbed by a limited number of molecules, which may act then as photosensitizer. After UVA light absorption, the photosensitizer molecules cross over to its triplet state and transfer energy to generate singlet oxygen. To provide doubtless evidence for a correlation of UVA damage in tissue and singlet oxygen, it must be shown that these endogenous photosensitizers generate singlet oxygen to a sufficient extent. Comparable with exogenous photosensitizers, the efficacy of singlet oxygen generation (quantum yield) must be determined. In the present experiments, flavins, NAD/NADP, urocanic acid or different fatty acids were investigated.

These endogenous photosensitizers were excited in the range of UVA light using and :YAG laser at 355 nm. Singlet oxygen was detected directly by its time-resolved luminescence at 1270 nm. The respective decay rates and rate constants of singlet oxygen were determined, in particular at different oxygen concentrations. The singlet oxygen quantum yield ($\Phi\Delta$) could be calculated, e.g. for riboflavin in fully aerated solution of H_2O, a singlet oxygen quantum yield of $\Phi\Delta = 0.54 \pm 0.07$ was determined. That value is comparable with exogenous photosensitizers used in photodynamic therapy (Photofrin $\Phi\Delta = 0.33$). The singlet oxygen quantum yield depends critically on the oxygen concentration, i.e. the oxygen partial pressure (pO_2) in the respective experimental setup. That is important when comparing experiments of *in vitro* (pO_2 approximately 150 mmHg) and conditions *in vivo* such as the skin ($pO_2 < 20$ mmHg). The results show a decrease of $\Phi\Delta$ with decreasing oxygen concentration.

Our investigations provide clear evidence that UVA light at 355 nm generates singlet oxygen in endogenous sensitizers such as flavins, urocanic acid or fatty acids.

P110

Sweet-like bullous skin infiltrations in a chronic myeloic leucemia patient treated with tyrosine kinase inhibitors

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The occurrence of sweet-like cutaneous lesions with chronic myeloic leucemia (CML) has been recently observed especially in patients treated with tyrosine kinase inhibitors. We present a 67-year-old patient with a 6 years history of CML. AMN 107 tyrosine kinase inhibitor treatment kept the patient in the chronic phase of CML over the last months at the cost of neutro- and

thrombocytopenia. Under this treatment, he developed a pneumonia and sepsis with temperatures up to 40 °C. After 1 week, bullous skin infiltrations on both upper arms occurred as well as a monstrous and painful bullous swelling of the right neck with loss of contours. Serology revealed no indication of a viral infection (varicella zoster virus, herpes simplex virus, EBV, CMV, and HAV), blood cultures were negative, and lesional skin biopsy cultures revealed no viral, bacterial, or fungal pathogen. Histology of a skin biopsy from the right neck showed massive infiltrations of the whole dermis with neutrophile granulocytes as well as with monozytoid histiozytic cells also spreading into the spongiotic destroyed epidermis. In addition, atypical monozytoid and binuclear cells usually not found in classical sweet syndrome were present. After cessation of AMN 107 and initiation of an Augmentan/Avalox antibiotic treatment, the addition of prednisolone 1 mg/kg once daily led to rapid resolution of the sweet-like cutaneous bullous infiltrations. We conclude that the development of sweet-like bullous dermatoses in septic CML patients may be precipitated by tyrosine kinase inhibitors.

P111

Presence of cannabinoid receptors on cutaneous sensory nerve fibers and their role in pruritus therapy

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Chronic pruritus of any origin is difficult to treat and requires evaluation of new therapeutic strategies which were offered by recent neurophysiologic findings. Cannabinoid receptors mediate analgetic and psychopharmacological actions and have been localized in the central and peripheral nervous system as well as on cells of the immune system. Up to now, two cannabinoid receptors (CB1 and CB2) have been cloned, and recent studies on animal tissue gave evidence for their presence in the skin. In an immunohistochemical investigation, we investigated the precise cutaneous localization of CB1 and CB2 receptors. CB1 and CB2 were found on mast cells and cutaneous nerve fiber bundles. Immunoreactivity was found in large myelinated nerve fiber bundles as well as small unmyelinated nerves of the papillary dermis, at the dermal–epidermal junction and sporadically within the epidermis. Staining for CB1 and CB2 was also observed in single small nerve fibers that were associated with hair follicles. Double immunostaining with an anti-CGRP antibody suggested the presence of cannabinoid receptors on small sensory nerves. Recent neurophysiological studies showed that after activation of the cannabinoid receptors on sensory nerve fibers and mast cells, mast cell degranulation, neuropeptide release, and itch transmission is inhibited. Accordingly, in a surveillance study with a topical cannabinoid agonist, a significant antipruritic effect was achieved in 14 of 22 patients with chronic pruritus and prurigo. The mean pruritus reduction was 86.4%. These results suggest topical cannabinoid agonists as a new tool in the treatment of chronic pruritus by acting on several cutaneous molecular levels.

P112

IL-18 and CRP but not IL-8 are sensitive course indicators for acute clinical manifestations in Adamantiades–Behet's disease

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Adamantiades–Behet's disease (ABD) is an inflammatory multisystemic disorder characterized by periodic exacerbations of manifestations including oral aphthosis, genital ulcera, different types of cutaneous lesions and uveitis. T-helper type 1 (Th1) polarization of the immune response and expression of proinflammatory cytokines seem to occupy an important position in the pathogenesis of ABD. Whereas quantitative abnormalities of $CD4^+$, $CD8^+$ and γδ T cells and enhanced serum levels of some Th1 cytokines were found in ABD patients compared with healthy controls, no circulating individual course-indicator for the acuity of the illness could yet be identified among cytokines. Aim of this study was to investigate such a predictive course-parameter in patients' sera. We investigated interleukin (IL)-18, IL-8 and CRP serum concentrations and blood sedimentation rate (BSR) in 18 ABD patients, of whom 11 presented with disease exacerbations during the last 2 years, and healthy controls ($n = 16$). Active disease was defined by aphthous stomatitis (more than one aphthous ulceration) with or without other muco-cutaneous lesions such as erythema nodosum or by uveitis. Inactive disease was defined by no symptoms at all. Cytokine measurements were performed during active phase as well as inactive phase. IL-18 showed an increase in active and a decrease in inactive periods in the individual course of each investigated ABD patient ($n = 11$; $P = 0.009$). Comparably, CRP was a useful course-indicator ($P = 0.024$), whereas the CXC-chemokine IL-8 or BSR showed no reliable correlation with disease activity ($P > 0.5$). Besides that, in our ABD patients, IL-18 was not relevantly elevated compared with healthy controls (347 vs. 267 pg/ml; $P = 0.25$). In conclusion, we demonstrated the role of IL-18 as a sensitive parameter correlating with disease activity in ABD. IL-18 was elevated even in the presence of only few oral aphthae or mild uveitis. Further investigations will elucidate whether regular IL-18 determinations should be recommended revealing imminent worse systemic complications.

P113

Juvenile-onset Adamantiades–Behet's disease in Germany

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Adamantiades–Behet's disease (ABD) is characterized by skin lesions (oral and genital ulcers, pathergy) and ocular inflammation; it may also involve other organ systems, mainly joints, central nervous system and gastrointestinal tract. Symptoms usually start in the third decade of life, whereas the disease is uncommon in children. We aimed to enlighten characteristics of juvenile onset disease (JOD; first symptoms <16 years) in comparison with adult onset disease (AOD) by evaluating data from the German Registry of ABD. Of 590 patients of the German Registry [among them 227 of German (G) and 267 of Turkish (T) origin], 75 patients (13%; G: 34, T: 31) exhibited the onset of the disease and 23 of them (4%) the complete symptom complex at a juvenile age. First symptoms of patients with JOD were mainly oral aphthous lesions in 85% (vs. 81% in AOD). The most frequent second symptom was genital ulcers in 39% of patients (vs. 33%; n.s.). Ocular involvement was present in 57% of patients with JOD vs. 65% in AOD. JOD was characterized by an increase in familial cases (24 vs. 7% in AOD, $P < 0.0001$). Data concerning other major clinical features and the delayed course of the disease in JOD will be presented. The present data of the German registry confirm our former observation on 28 patients with JOD [Treudler et al. Dermatology 1999: 199: 15–9], showing that the major clinical features of JOD and AOD in Germany are comparable, but in

JOD, there is a higher rate of familial occurrence and the course of the disease is less severe.

P114

Epidemiology of Adamantiades–Behet's disease in Germany

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The German Registry of Adamantiades–Behet's disease (ABD), which was founded in 1990 and has received in 2000 the status of a charity organization provides data on the epidemiology and the clinical manifestations of ABD in Germany. A total number of 590 patients, including 227 German (G) and 267 Turkish (T) patients, were reported to the registry until 2005. Median age of onset was 26 years (range 0–72 years). The complete clinical picture according to the criteria of the 'International Study Group for Behet's disease' or the 'Classification-and-Regression-Tree' developed in 3 months (median). Interval between onset symptom and diagnosis was 36 months, being significantly longer than the duration of development of the complete clinical picture ($P < 0.001$). ABD was diagnosed later in G (52 months) than in T patients (27 months, $P < 0.005$). While G patients presented a 0.9:1 male-to-female ratio, male predominance was shown in T patients (M : F 1.9:1, $P < 0.001$). Familial occurrence was detected in 2.3% of the G and 14.2% of the T patients ($P < 0.001$). The frequencies of major clinical manifestations were oral ulcers 99%, skin lesions 81%, genital ulcers 64%, ocular manifestations 58%, arthritis 53%, and positive pathergy test 34%. Oral ulcers were with 84% the most common onset sign. G patients presented more often prostatitis/epididymitis (12.2 vs. 6.4%, $P = 0.045$) and lethal outcome ($n = 5$, 3.2% vs. none, $P = 0.016$) than T patients, whereas there was a trend for more common blindness in Turks (9.2 vs. 4.5% in Germans, $P = 0.064$). Among G patients, males presented more often than females systemic thrombosis (32.5 vs. 7.3%, $P < 0.001$), superficial thrombophlebitis (24.1 vs. 3.2%, $P < 0.001$), ocular lesions (60.2 vs. 42.0%, $P = 0.023$), cardiac (6.8% vs. none) and lung involvement (9.7 vs. 1.9%, $P = 0.027$) and HLA-B5 positivity (45.5 vs. 28.6%, $P = 0.03$), while females presented more often genital ulcers (73.9 vs. 57.4%, $P = 0.02$). Severe course developed in 12% of the patients: blindness 9%, meningoencephalitis 5%, lethal outcome 1%, hemoptysis 1%, and bowel perforation 0.5%. HLA-B5 was confirmed as marker of severe prognosis.

P115

Malignant atrophic papulosis Köhlmeier–Degos: first epidemiological and prognostic data

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Malignant atrophic papulosis, described by Köhlmeier and Degos in 1941, remains until today an unexplained entity. The disease can be lethal in more than 30% of the cases. The characteristic papular skin lesions with central porcelain-white atrophy and surrounding teleangiectatic rim are almost pathognomonic. Histology shows a wedge-shaped connective tissue necrosis in the deep corium due to a thrombotic occlusion of the small arteries. Various hypotheses have interpreted the disease as vasculitis, coagulopathy or primary disfunction of the endothelial cells. Statistical analysis of 81 patients (21 of the Support Network for Degos Disease and 60 from the bibliography) showed a mean age of manifestation of 35.3 years. The severity in prognosis is mainly determined by the presence of extracutaneous involvement, which occurs in 44% of the patients (central nervous system 8%, gastrointestinal tract 22%, and multiple organ involvement 67%). In about 85% of the cases with extracutaneous involvement, symptoms appear within the first 4 years of illness, whereas patients with positive family history exhibit a better prognosis.

P116

Identification and characterization of 18 patients simultaneously infected with varizella zoster and herpes simplex virus

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Herpes simplex as well as zoster/varicella infections are very common diseases. The pathogenes of both infections are DNA viruses from the same family. Recently, it has been shown that herpes simplex virus (HSV) and varicella zoster virus (VZV) can be found in the same ganglion of the central nerve system. However, there have been no clinical reports on simultaneous infections with HSV and VZV.

We identified 18 of 5289 (0.34%) patients between 2001 and 2004 who presented with infections with HSV and VZV detected by polymerase chain reaction (PCR) analysis of material obtained by sterile swabs from the skin lesions.

The mean age of the 18 patients (7 male and 11 female) was 53 years (range 2–83). The clinical diagnosis was zoster in 61% (11/18), varicella in 11% (2/18), herpes simplex in 22% (4/18) and erythema multiforme in 5% (1/18). In 61% (11/18), the infection was localized to the trigeminus region (9/18 with zoster infection), in 33% (6/18) to the trunk and in 5.5% (1/18) to the genital region. In 63.6% (7/11) of the patients with trigeminal zoster (mean age 66 years), the ophthalmic nerve was affected. In 11 of 1365 (0.81%) patients with the clinical diagnosis of zoster or varicella, coinfection with herpes virus was found additionally using routine laboratory analysis. Of those, the facial region was most commonly involved (9/13). In the majority of cases, one dermatome was affected suggesting that the viruses were reactivated in the same ganglion.

In one patient, impetigo at the cheek was diagnosed and treated with antibiotics without effect. Instead, erythema multiforme developed on hands, feet and mucous membrane. PCR analysis of the facial lesion showed coinfection with HSV and VZV. It remains to be speculated whether the biopsy-proven erythema multiforme was induced by drug intake or HSV infection.

This is the first report about dual infections with HSV and VZV in a cohort of 18 patients. It is a relatively rare event but should be considered in unusual cases and can be confirmed using PCR analysis.

P117

Immunohistochemical characterization of epidermal stem cells

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The epidermis is a very dynamic epithelium, which renews itself within 60 days. To realize such a frequent and rapid renewal, the epidermis has a pool of adult stem cells, which guarantees constant

availability and supply of differentiated cells. The decisive characteristics of these stem cells are, on the one hand, the capability of self-renewal and, on the other hand, formation of daughter cells, whose developmental path culminates in the terminal differentiation.

Efforts to identify epidermal stem cells in histological slides and to characterize various pathological skin changes require suitable markers which enable differentiation of stem cells from other cells. β1-Integrin has become established as a useful stem-cell marker. β1-Integrin is expressed in all basal keratinocytes. The expression of β1-integrin is only reduced when the keratinocytes leave the basal layer. These expression dynamics make it possible to identify the epidermal stem cells on the basis of their elevated β1-integrin level in the histological slide.

Further investigations are addressed to the use of new immunohistological marker systems. The transferrin-receptor and the melanoma-associated chondroitin sulfate proteoglycan are plausible but currently still involve methodological problems. The combination of various markers should enable unequivocal identification of epidermal stem cells such that assessment of pathological changes can be made by a specific marker pattern.

P118

Development and characterization of an *in vitro* assay for the study of human chemotherapy-induced hair follicle dystrophy

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Chemotherapy-induced alopecia (CIA) is a common side effect and one of the major unresolved problems of clinical oncology. Although the molecular mechanisms of CIA are becoming increasingly defined in the murine system, for which a well-established and instructive CIA model (=CIA induction using cyclophosphamide in C57BL/6 mice) exists, they are virtually unknown in the human system. Largely, this is due to the lack of adequate research models for human CIA. Therefore, we have explored whether microdissected, organ-cultured bulbi of human scalp hair follicles in anagen VI (hHF) can be exploited as such a model. Here, we show that hHF respond by the typical morphological changes of chemotherapy-induced follicle dystrophy when the cyclophosphamide metabolite, 4-hydroperoxycyclophosphamide (4-HCs), which spontaneously converts to highly toxic metabolites: melanin clumping and incontinence, shrinkage of the hair matrix, premature catagen entry, down-regulation of proliferation, ectopic and massively up-regulated apoptosis. These effects are dose-dependent. We are currently investigating how major regulators of intrafollicular apoptosis (p53, Bcl-2, Fas/FasL) are affected by 4-HC application on fibroblasts, melanocytes and epithelial cells which cell typesbesides hair matrix keratinocytes respond with increased apoptosis to 4-HC administration. This novel, pragmatic and clinically highly relevant *in vitro* assay for the study of human CIA can now be employed (a) to dissect the underlying molecular controls and (b) to explore the efficacy of agents that we and others have already reported to modulate rodent CIA (e.g. estrogens, calcitriols, glucocorticosteroids, cyclosporine A and CDK inhibitors).

P119

Evaluation of diagnostic criteria in Adamantiades–Behet's disease patients in Germany

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Diagnosis of Adamantiades–Behet's disease (ABD) is made on the basis of a clinical sign constellation. There exist several diagnosis criteria guidelines, but manifestations differ throughout the world. The classification criteria of the 'International Study Group for Behet's disease' (ISGBD) require one obligatory (oralaphthosis) and at least further two signs. The 'Classification-and-Regression-Tree' (CART method) demands less clinical features (2) for diagnosis. Recent data of the German Registry of ABD revealed that sensitivity of the criteria of the ISGBD was only 71% and of the CART method 87.6% in all 590 ABD patients included. ABD patients [12.4% ($n = 73$)] suffering from rare manifestations could not be classified by any of these criteria. In the following study, we looked for criteria, which enable the diagnosis by clinical means. Therefore, we investigated 70 cases of clearly diagnosed ABD patients as well as 30 non-ABD patients who were referred to our departments due to suspicion of suffering from ABD or who were mistakenly diagnosed having symptoms mimicking ABD, e.g. mucous bullous pemphigoid (BP). Special investigations (herpes simplex virus-polymerase chain reaction, BP autoantibodies) were necessary in questionable cases. We elaborated a list of signs for each patient, considering oral and genital aphthous-like lesions, folliculitis, erythema nodosum, skin ulceration, ocular lesions, peripheral/central neurological manifestations, thrombosis, superficial phlebitis, epididymitis, and pathergy test. Subsequently, we investigated how many signs are needed to get a 'cut-off point' associated with the highest sensitivity and specificity for ABD diagnosis. Using an ROC criterion test, sensitivity + specificity = maximum was reached by a constellation of three signs (sensitivity 95%; specificity 90%), whereas diagnosis by at least four signs enhanced false-negative results (sensitivity 61.4%), and diagnosis by only two symptoms led to more false-positive results (specificity 70%). In conclusion, we favor the three-sign constellation for ABD diagnosis, but we include diagnosis by only two symptoms if all other possible diagnoses are excluded.

P120

Influence of green and black tea, epigallocatechin-3-gallate and theaflavin on prostanoid synthesis *in vitro* and *in vivo* using microdialysis

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Introduction: Ultraviolet-B (UVB) irradiation is a potent inductor of oxidative stress and inflammation. The role of Epigallocatechin-3-gallate (EGCG), the major catechin in green tea, as well as Theaflavin (TF), the major catechin in black tea, in the inflammatory pathway is still controversial. We investigated the influence of EGCG and TF, as well as green (GTE) and black tea extract (BTE) on isoprostane and prostaglandin synthesis.

Methods: HaCaT cells were irradiated with 100 mJ/cm^2 of UVB and incubated with increasing doses of EGCG and GTE (containing 1, 10, 100 μM EGCG) as well as TF and BTE (containing 1, 10, 100 μM TF) for 22.5 h, 1.5 h after irradiation. For cutaneous microdialysis, areas of 2 cm^2 on the volar forearm of three volunteers were exposed to UVB irradiation (30–40 mJ/cm^2) and treated with compresses saturated with GTE or BTE, or

a microemulsion formulation with 5% of EGCG or TF for 1 h microdialysate samples were collected 22.5 h after irradiation at 30-min intervals upto 4 h. The lipid mediators F(2)-isoprostane 8-iso-PGF(2α) and prostaglandins 9α, 11α-PGF(2α)and PGE-2 in cells, supernatants and microdialysate samples were measured using sensitive gas chromatography-mass spectrometry/negative ion chemical ionization.
Results: GTE and black tea extract (BTE) but not EGCG and TF strongly decreased synthesis of all mediators in irradiated HaCaT cells and supernatants dose dependently. In contrast, high doses of EGCG and TF (100 μM) increased prostaglandin synthesis of UVB-exposed HaCaT cells. Microdialysate samples of all four treated spots showed decreased levels of F(2)-isoprostane 8-iso-PGF(2α) and 9α, 11α-PGF(2α) compared with UVB-irradiated spot. However, lower levels f F(2)-isoprostane 8-iso-PGF(2α) were obtained after application of GTE and BTE compared with EGCG or TF in microemulsion.
Conclusion: GTE and BTE seem to be potent inhibitors of UVB-induced prostaglandin and isoprostane synthesis *in vitro* and *in vivo*. Whereas antioxidative effects of EGCG and TF were observed *in vivo*, higher doses of EGCG and TF seem to be prooxidative *in vitro*.

P121

Effect of irradiation on the binding capacity of bovine collagen for inflammatory cytokines

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Introduction: The exudates of chronic wounds contain elevated levels of inflammatory cytokines such as interleukin (IL)-1β and IL-6. Therefore, the binding of these inflammatory mediators to reduce the overall concentration seems a suitable way to support the healing process. The aim of the present study was to investigate the binding capacity of the native as well as γ- or β-irradiated (maximum doses of 20 kGy) wound dressing Suprasorb® C-containing bovine collagen for the IL-1β and IL-6.
Materials and methods: The wound dressing samples were cut into equal pieces. Each specimen was taken in a final volume of 1 ml of IL solution, and the samples were incubated up to 24 h at 37 °C on a plate mixer. The concentrations of unbound ILs in the supernatants were determined using specific enzyme-linked immunosorbent assays (Milena Biotec, Bad Nauheim, Germany).
Results: The native bovine collagen from Suprasorb® C is able to decrease ($P < 0.01$) the concentration of IL-1β in solution significantly. γ- or β-Irradiated collagen can also bind significant amounts of IL-1β over a period of 24 h. Moreover, Suprasorb® C possesses binding capacity for IL-6. Already, after 8 h, a significant decrease ($P < 0.05$)of unbound IL-6 in solution was detectable. The γ- or β-irradiated wound dressings were also capable of reducing the concentration of IL-6 in solution.
Conclusions: Suprasorb® C possesses a high binding capacity for different ILs. The decrease of excessive inflammatory IL concentrations by Suprasorb® C in chronic wounds should improve the healing outcome. Treatment with γ- as well as β-irradiation is commonly used as sterilization method but can severely alter the properties of biomolecules such as collagen fibres. In this study was shown that γ- or β-irradiation of bovine collagen (maximum dose 20 kGy) does not change the binding affinity for IL-1β and IL-6 significantly.

P122

Influence of irradiation on the binding capacity of bovine collagen for inflammatory proteases

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Introduction: In contrast to physiological wound healing, chronic wounds are very often characterized by elevated levels of proteolytic enzymes like matrix metalloproteinases (MMPs) and neutrophile elastase. Therefore, the reduction of these protease concentrations seems to be a suitable way to promote normal wound healing. The aim of this study was to investigate the binding capacity of the native as well as γ- or β-irradiated (maximum dosis of 20 kGy) wound-dressing Suprasorb® C containing bovine collagen for neutrophil elastase, MMP-2 and MMP-13.
Materials and methods: The wound-dressing samples were cut into equal pieces. Each specimen was taken in a final volume of 1 ml of protease solution, and the samples were incubated up to 24 h at 37 °C on a plate mixer. The concentrations of unbound proteases in the supernatants were determined using specific enzyme-linked immunosorbent assays (ELISAs) (neutrophil elastase ELISA from Milena Biotec, Bad Nauheim, Germany, and Quantikine Immunoassays for pro-MMP-13 and MMP-2 from R&D Systems, Minneapolis, MN, USA, respectively).
Results: The native bovine collagen from Suprasorb® C is able to bind neutrophile lastase. Already, after 1 h, a significant ($P < 0.05$) decrease of the elastase concentration was observed. The wound dressings treated with γ- or β-irradiation were also able to bind significant ($P < 0.01$) amounts of elastase over the examined period. Similarly, a significant ($P < 0.01$) decrease of the MMP-2 concentration by native and irradiated bovine collagen was observed.
Conclusions: Suprasorb® C is able to bind proteases at different rates. In particular, it has a considerable binding capacity for neutrophil elastase and MMP-2. Therefore, it should be able to establish a physiological environment in chronic wounds and promote healing. Irradiation is used to sterilize materials but can influence the characteristics of biomolecules such as collagen fibres. In this study was shown that γ- or β-irradiation of bovine collagen up to a maximum of 20 kGy has no influence on the binding affinity for neutrophil elastase and MMP-2.

P123

Quantitative and positional analysis of DNA methylation in circulating DNA of melanoma patients

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DNA methylation is a well-known epigenetic phenomenon that occurs in higher order eukaryotes. Changes in the status of DNA methylation of tumor suppressor genes are one of the common molecular alterations in many cancers. Several studies have demonstrated tumor-specific alterations in DNA recovered from plasma or serum of patients with various malignancies, a finding with great potential for molecular diagnosis and prognosis estimation. The purpose of this study was to investigate the methylation status of five different genes [suppressor of cytokine signaling 1 (SOCS1), SOCS2, Ras association domain family protein 1A (RASSF1a), D-type p16INK4a cyclin-dependent kinase inhibitor (CDKN), and O6-methylguanine DNA-methyltransferase (MGMT)] involved in tumor suppression, cell cycle, and DNA repair. Serum before therapeutic intervention of 41 melanoma patients (stage I = 13; stage II = 15; stage III/IV = 13) was collected, cell-free DNA was isolated, and sodium bisulfite conversion of genomic DNA was performed. The methylation status of CpG islands in the promotor regions of SOCS1 and SOCS2; RASSF1a; CDKN; and MGMT were analyzed using methylation specific-polymerization chain reaction (MSP). For comparison, sera from healthy controls ($n = 20$) and patients with other skin tumors (nine basal cell cancers, five Kaposi's sarcoma) as well as different metastasized cancers (five breast cancers, five colon cancers) were also analyzed. Moreover, the expression of these genes was investigated in cell lines (BLM, A375, MV3, and M13). In addition, we examined whether methylation was involved in silencing of these genes in 20 fresh melanoma specimens and confirmed the hypermethylation status of

SOCS2 using positional methylation analysis (pyrosequencing). Interestingly, both identified CpG sites in the SOCS2 promoter were consistently methylated with site 2 being methylated in up to 25% (primary melanoma) and up to 38% (metastatic melanoma), respectively. Analysis of sera from cutaneous melanoma patients demonstrated circulating hypermethylated SOCS1 in 75%, SOCS2 in 43%, RASSF1a in 64%, CDKN2a in 75% and in MGMT in 64% with mRNA transcripts of these genes being down-regulated, except for MGMT. Detailed studies on larger number of patients are needed to reveal the potential usefulness of circulating cell-free DNA as a surrogate marker for melanoma.

P124

Interleukin-22 regulates the expression of genes responsible for antimicrobial defense, cellular differentiation, and mobility in keratinocytes: a potential role in psoriasis

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Interleukin (IL)-22 is a recently discovered mediator of the interferon (IFN)–IL-10 cytokine family. Similar to IFN-γ, IL-22 is produced preferentially by activated T-helper type 1 and NK cells. Here, we show that the expression of IL-22 is strongly elevated in diseased skin and blood plasma from psoriasis patients in comparison with healthy donors. As epithelial cells are known to be the major targets of IL-22, we performed a broad search for IL-22 effects on primary human keratinocytes. As deduced from gene chip-based analyses, IL-22, in contrast to IFN-γ, was surprisingly found to regulate the expression of only few genes in these cells. This may be due to the activation of different signal transduction pathways. Most importantly, IL-22 led to activation of STAT3, whereas IFN-γ primarily activated STAT1. The gene expressions identified to be regulated using IL-22 should enhance the antimicrobial defense (S100A7, S100A8, S100A9), inhibit cellular differentiation (e.g. FLG, KRT1, KRT10, CALML5), and increase cellular mobility (e.g. MMP1, MMP3, DSC1). In contrast, IFN-γ favored the expression of various molecules from the major histocompatibility complex pathways, as well as adhesion molecules, cytokines, chemokines, and their receptors. The IL-22 effects were transcriptionally regulated and either independent of protein synthesis and secretion or mediated by a secreted autocrine factor. Inflammatory conditions but not the keratinocyte differentiation state amplified the IL-22 effects. Interestingly, the high levels of IL-22 were associated with strongly up-regulated S100A7, S100A8, S100A9, and MMP1 expression in skin from psoriasis patients. Furthermore, IL-22 application in mice enhanced skin MMP1 expression. These data show that, despite similarities, IFN-γ primarily amplifies inflammation, whereas IL-22 may play a role in the innate immunity and the reorganization of epithelia, and simultaneous action of both cytokines may be responsible for psoriatic skin alterations.

P125

Eotaxin-3 is abundantly expressed on vascular endothelial cells and mononuclear infiltrate cells in bullous pemphigoid

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Lesional skin infiltrates in bullous pemphigoid (BP) are rich in eosinophils. Eosinophil-derived proteases, such as ECP or maltose-binding protein, are elevated in serum during blistering, and flame figures can be demonstrated in BP lesions. Once released, these proteases may persist in the dermis for up to 6 weeks and may be associated with pruritus. Tissue numbers of eosinophils depend on generation of mature cells in bone marrow, homing and apoptosis. Our previous data addressed eotaxin in BP, which can be demonstrated in serum, but only in low levels in the skin. Therefore, we studied eotaxin-2 and -3 in serum and skin.

Sera ($n = 25$) and perilesional skin ($n = 10$) were obtained from untreated BP patients. Eotaxin, eotaxin-2 and eotaxin-3 were quantified employing ELISA systems based on specific antibodies from R&D (Minneapolis, MN, USA). As controls, we employed sera from pemphigus vulgaris patients ($n = 10$) and healthy donors ($n = 30$). Skin biopsies were stained with antibodies specific for eotaxin, eotaxin-2 and eotaxin-3 using immunohistochemistry. For comparison, we stained sections from psoriatic skin lesions.

Serum levels for eotaxin and eotaxin-3 differed significantly between BP patients and pemphigus vulgaris (PV) as well as normal controls ($P < 0.01$ vs. $P < 0.05$), whereas eotaxin-2 was present in equal levels in sera from BP, PV and normals. Antibodies to eotaxin weakly stained keratinocytes and mononuclear infiltrate cells in BP lesional skin. Antibodies to eotaxin-3, in contrast, strongly stained vascular endothelial cells and mononuclear infiltrate cells.

As eotaxin-3 is strongly present on vascular endothelial cells, probably bound to the endothelial glykocalix, it can be functionally important to direct eosinophil influx into lesional skin in BP.

P126

Community-acquired *Staphylococcus aureus* in dermatology: characteristics and clinical significance of methicillin resistance and Panton–Valentine leukocidin

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Among community-acquired *Staphylococcus aureus* are strains associated with deep skin infections, carrying the genes for the toxin Panton–Valentine leukocidin. Additionally, methicillin-resistant *S. aureus* (MRSA) emerged as a community-based pathogen (C-MRSA) during the past 2 years, showing different characteristics when compared with hospital-acquired MRSA. The aim of this prospective study was to isolate *S. aureus* strains from dermatology outpatients and investigate them for antibiotic resistance, the presence of the lukS-lukF determinant of Panton–Valentine leukocidin and to further characterize strains found to contain the genes. Two hundred and thirty-eight outpatients in the Dermatology Department of Heidelberg University with inflammatory skin diseases, leg ulcers and deep skin infections were included. A standardized questionnaire documented potential risk factors for MRSA colonization/infection. The isolates collected from the lesional skin and partly from nostrils were investigated for lukS-lukF using polymerase chain reaction (PCR). If the presence of lukS-lukF was demonstrated, strains were further characterized using molecular typing [determination of SmaI pattern as well as spa sequence and multilocus sequence typing (MLST) of selected isolates], PCR demonstration of resistance genes, and characterization of the SCCmec element. One hundred twenty-five of two hundred thirty-eight carried *S. aureus*. Twenty-two of one hundred twenty-five *S. aureus* were lukS/lukF-positive. Seventeen of one hundred twenty-five were MRSA, 11 of them belonging to the Rhine–Hessen epidemic strain, 1/17 to sequence type (ST) 8, and one MRSA could not be classified. Four of seventeen were C-MRSA containing lukS/lukF as an important trait of C-MRSA [two ST 80 which contain far-1 coding for fusidic acid (FUS) resistance and one FUS sensitive strain (ST152)]. One of the C-MRSA

corresponded to the C-MRSA of ST 1 from the USA being sensitive for FUS. This endemic North-American C-MRSA was detected in Germany for the first time. Only 11/33 patients with topical use of FUS carried FUS-resistant *S. aureus*. The results demonstrate that C-MRSA may be detected in dermatology outpatients and that the risk factors for the acquisition of MRSA do not necessarily apply for C-MRSA. From these data, topical use of FUS does not select for FUS-resistant MRSA.

P127

Antifungal activity of chitosan flake 1130 against *Candida albicans* – comparative measurement of cultural growth with nephelometry and fluorescence (Alamar Blue™)

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Introduction: Chitosan is a water insoluble polyaminosaccharide with antimicrobial activity. It is obtained by alkaline deacetylation from chitin. Therefore, more than 50% of β-(1,4)-linked 2-acetamido-2-desoxy-β-D-glucopyranose of chitin has to be deacetylated into 2-amino-2-desoxy-β-D-glucopyranose.

Chitosan Flake 1130 (ChiPro GmbH) has a molecular weight of 120 kDa. The degree of deacetylation amounts 85.2%.

Methods: Different solutions of Chitosan Flake 1130 (1, 0.5, 0.25, 0.1, 0.05, 0.025, 0.01, 0.005 and 0.0025%) were prepared, and their influence on *Candida albicans* DSM 11225 has been investigated.

Method 1: The inoculum is adjusted to achieve a final concentration of 3×10^5 cells/ml. Yeasts were incubated with Sabouraud-Glucose medium at 30 °C. Measurements were done with a laser-based microplate nephelometer. (NEPHELOstar, BMG LABTECH GmbH, Offenburg, Germany) over period of 24 h.

Method 2: C. albicans was incubated according to NCCLS M27-A2 procedure for 48 h in the microplate nephelometer. Thereafter, the clouding of the suspension was measured using the light scattering in the nephelometer. High scattering values correspond to increased fungal growth.

Method 3: C. albicans was incubated according to NCCLS M27-A2 procedure. After 48 h, the fluorescence was measured using a microplate fluorometer (FLUOstar Galaxy, BMG LABTECH GmbH, Offenburg, Germany). Alamar Blue™ was used as fluorescence indicator. High fluorescence values correspond to increased fungal growth.

Results: There was an effective inhibition of *C. albicans*' growth between 0.05 and 1% (*Method 1*) as well as 0.5 and 1% (*Methods 2 and 3*), respectively.

Conclusion: Antifungal activity of Chitosan Flake 1130 could be verified for all three methods. However, there is a significant difference between NCCLS and non-NCCLS results. Moreover, it could be shown that the successful use of the microplate nephelometry as a simple and inexpensive method for monitoring of fungal or growth of other microorganisms.

P128

TAT-LACK-transduced dendritic cells induce antigen-specific $CD8^+$ T cells and efficiently vaccinate against murine cutaneous leishmaniasis

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In murine cutaneous leishmaniasis, protection against progressive disease is dependent on interferon (IFN)-γ-producing $CD4^+$ and $CD8^+$ T cells. An efficacious vaccine should thus induce T-helper type 1 and Tc1 cells. For this purpose, we generated a fusion protein comprised of the protein transduction domain (PTD) of HIV-1 TAT protein and the Leishmania-antigen LACK. PTD-TAT-LACK preferentially accumulates in the cytosol of dendritic cells (DC) and facilitates major histocompatibility complex class I-dependent antigen presentation. Previously, we showed that vaccination with TAT-LACK (TL)-transduced DC mediates better protection against progressive disease as compared with LACK(L)-pulsed DC. The aim of this study was to analyze the mechanisms responsible for the efficacy of TL compared with L as vaccine. First, fusion protein-transduced DC induced significantly higher proliferation of *ex vivo*-isolated, CFSE-labeled, Leishmania-primed $CD8^+$ T cells compared with L-pulsed DC (8 ± 2 vs. 2 ± 1% proliferating cells, $P \leq 0.05$). To determine which T cell mediates protection *in vivo*, we vaccinated naive BALB/c mice. Administration of TL-transduced DC resulted in enhanced priming of $CD8^+$ T cells as compared with injection of DC + L (16 ± 4 vs. 5 ± 2% proliferating cells, $P \leq 0.05$). The induction of antigen-specific $CD4^+$ T cells was comparable in both groups (7 ± 2 vs. 6 ± 3%). $CD4^+$ and $CD8^+$ T cells were subsequently depleted in BALB/c mice during vaccination and low-dose infections with *Leishmania major* were initiated 3 weeks later after complete restoration of the T-cell compartment. In DC + TL-vaccinated groups, depletion of either CD4 and CD8 T cells resulted in increased lesion volumes (anti-CD4: 79 ± 10, anti-CD8: 79 ± 10 mmE3) as compared with isotype-treated mice (46 ± 6 mmE3, $P \leq 0.05$, week 8). DC + L vaccination did not induce significant protection against progressive disease as compared with control mice. In this instance, lesion sizes were similarly independent of which T cell was depleted during vaccination (anti-CD4: 67 ± 15, anti-CD8: 77 ± 11 as compared with isotype control-treated mice: 94 ± 12 mmE3, week 8). In summary, our data indicate that TL-mediated protection is dependent on both $CD8^+$ and $CD4^+$ T cells and that TAT fusion proteins are superior in activating cytotoxic T cells compared with proteins lacking the PTD.

P129 (V10)

Elucidation of the role of interleukin-17 in murine cutaneous leishmaniasis

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Interleukin (IL)-17 is a proinflammatory cytokine secreted by activated T cells (CD4 > CD8). Protective immunity against murine experimental cutaneous leishmaniasis is critically dependent on the efficient development of interferon (IFN)-γggr;-producing T-helper type 1 (Th1)/Tc1 cells, as observed in genetically resistant C57BL/6 mice. BALB/c mice, in contrast, succumb to infection due to uncontrolled Th2 development. We sought to determine the role of IL-17 in the pathogenesis of leishmaniasis. During the course of infection with *Leishmania major*, we determined that dramatically increased amounts of IL-17 were detected in lymph node cultures from wildtype (wt) BALB/c mice as compared with C57BL/6 mice. We subsequently used IL-17 BALB/c knockout mice to study the role of IL-17 in L. major infections in more detail. BALB/c IL-$17^{-/-}$ mice were infected with both standard high dose (2×10E5) and physiologically relevant low dose (10E3) inocula of L. major, and lesion development was monitored over the course of >3 months. Lesion sizes in IL-17-deficient mice were smaller from week 6 on postinfection

as compared with those in control BALB/c mice (e.g. 27 ± 3 vs. $72 \pm 12\,mm^3$ in week 9, $n = 12$, $P \leq 0.002$). Control BALB/c mice had to be euthanized at week 12 due to uncontrolled lesion progression, whereas lesions in IL-17$^{-/-}$ mice remained stable for an additional 2 months. In parallel, ears of IL-17$^{-/-}$-infected mice contained significantly fewer parasites ($2.2 \times 10E5$) as compared with control ears ($2 \times 10E6$, week 3, $n = 12$, $P \leq 0.05$). Finally, similar amounts of antigen-specific IFN-γ were detected in lymph node cultures from infected IL-17$^{-/-}$ as compared with control mice. Importantly, the amounts of the Th2 cytokines IL-10 and IL-4 were only slightly lower in IL-17$^{-/-}$ mice, suggesting that the regulation of Th1/Th2 cytokines does not play an important role in the increased resistance of IL-17-deficient BALB/c mice. Neutrophil recruitment into lesional tissue was markedly decreased in IL-17$^{-/-}$ mice, however. Our study appears to exclude an important role for IL-17 in T-cell activation in L. major-infected BALB/c mice and strongly suggest that increased IL-17-dependent neutrophil recruitment in BALB/c mice may be involved in enhanced disease susceptibility.

P130

Pseudolysin, an elastolytic protease from *Pseudomonas aeruginosa*, induces proinflammatory mediators and antibiotic peptides in keratinocytes by stimulation of protease-activating receptors

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The gram-negative bacterium *Pseudomonas aeruginosa* secretes several proteases of which four have been characterized. Because of their ability to degrade structure proteins and cytokines, these proteases are often seen as factors of pathogenicity. Purified fractions with proteolytic activity derived from crude supernatants of the bacterium *P. aeruginosa* are capable to induce calcium-influx in HaCaT keratinocytes as well as in primary human keratinocytes, but not in fibroblasts. Further investigations revealed that the calcium-influx-inducing activity in keratinocytes refers to a casein-specific proteolytic activity. Studies with various protease inhibitors suggested that these activities are caused by the *P. aeruginosa* protease pseudolysin. Whereas HaCaT keratinocytes and primary keratinocytes are inducible by PAR1, PAR2, PAR3 and PAR4 agonists, fibroblasts can only be stimulated by agonists of the PAR1 and PAR2. Specific desensitation experiments to inhibit the calcium-stimulating activity of pseudolysin in keratinocytes by pre-stimulation with specific agonists of PAR1–4 with subsequent stimulation with pseudolysin or vice versa resulted in no or incomplete mutual inhibition. Inhibition of the calcium-mobilizing activity of pseudolysin was achieved by pre-stimulation with plasmin, subtilisin A or the combination of PAR1-, PAR3- and PAR4-activating peptides. Pre-stimulation with pseudolysin inhibited the calcium-influx inducin activity of plasmin, subtilisin A and single PAR1-, PAR3- and PAR4-activating peptides. Stimulation of HaCaT keratinocytes and primary keratinocytes with pseudolysin resulted in increased mRNA expression of the proinflammatory cytokines tumor necrosis factor-α and IL-8 as well as the antimicrobial peptide hBD-2. This shows the induction of a signal-transduction pathway in keratinocytes by a synergistic activation of three protease-activated receptors. The utilization of this pathway by a microbial protease leads to the induction of epidermal defense mechanisms such as cytokine and antimicrobial peptide expression in human keratinocytes. This mechanism might be involved in the high resistance of intact epidermis against colonization with *P. aeruginosa*, whereas fibroblasts exposed to the surface like in leg ulcers are frequently colonized by this organism.

P131

Viral chemokine antagonist vMIP-II blocks T helper-1-cell migration

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Introduction: Chemokines exhibit elementary functions in immune cell homeostasis and migration. A specific inflammatory subset of these small cytokines recruit mainly immune cells like T helper-1 (Th1) cells to the site of inflammation. One promising strategy of preventing immune cell infiltration is the blockade of chemokine receptors using specific chemokine receptor antagonists. Here, we investigate the effects of the chemokine receptor antagonist vMIP-II on the interactions of infiltrating T cells and endothelial cells while transendothelial migration.

Methods: Here, we show that the viral chemokine receptor antagonist vMIP-II, which blocks CCR1, CCR2, CCR5 and CXCR4 and is therefore defined as broad-spectrum antagonist, blocks the MCP-1 and RANTES-induced Th1 chemotaxis in a static migration assay up to 70%. Using intravital microscopy of mice ears, we could show that a local depot of murine RANTES increased rolling of Th1 cells up to 50%.

Results: Pre-incubation of T cells with vMIP-II resulted in a 39% reduction of the RANTES-induced rolling. We could show that this increased rolling is mainly P-selectin dependent, as P-selectin-deficient mice showed no increased rolling after RANTES treatment. In a cutaneous hypersensitivity response, vMIP-II significantly reduced the DNFB-induced ear swelling in C57Bl/6 mice.

Conclusion: Viral MIP-II is a promising tool for the specific blockade of T-cell migration and infiltration even under physiological conditions of relevant *in vivo* disease models.

P132

Growth factors and sex steroids at age-specific levels regulate the biological activity of human sebocytes

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Menopause in females, which is characterized by a sudden decline of sex-specific hormones, has been associated with a rapid worsening of skin structure and functions, whereas patients with isolated GH deficiency (IGHD) as well as primary IGF-I deficiency also present signs of early skin aging. To test the effects of sex steroids and growth factors on the skin-aging process, we treated human sebocytes with GH, IGF-I, 17β-estradiol, DHEA, and progesterone as single agents in concentrations corresponding to those circulating in young (f20) and postmenopausal women (f60). Cell proliferation and lipid synthesis were measured using the 4-methylumbelliferyl heptanoate fluorescence assay and Nile-Red microassay/fluorescence microscopy, respectively. While progesterone and DHEA showed no effect on lipid synthesis, after treatment with IGF-I for 48 h at f20 and f60 concentrations, a significant dose-dependent increase of neutral ($P < 0.001$) and polar lipids ($P < 0.001$) was observed at both concentrations tested. Cell size was also increased. GH at f20 and f60 enhanced significantly the production of neutral ($P < 0.01$ and $P < 0.05$, accordingly) and polar lipids ($P < 0.05$ and $P < 0.01$, accordingly) in sebocytes but not in such an extent as IGF-I. 17β-Estradiol only induced polar lipid synthesis ($P < 0.01$) and cell size. No treatment altered sebocyte proliferation. In conclusion, among all hormones tested, IGF-I and GH were the most potent regulators of lipid synthesis in human sebocytes, suggesting that the GH/IGF-I axis is essential for the maintenance of skin moisture with age.

P133 (V34)

The HET-CAM as an *in vivo* model to objectify pharmacological effects on lymphangiogenesis

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In the development of drugs which affect lymphangiogenesis, an *in vivo* model is needed for screening of various active substances and substance classes which enables reproducible and reliable statements on the lymphangiogenetic efficacy.

Hemo- and/or lymphangiogenesis can be induced in the HET-CAM model by application of vascular endothelial growth factor (VEGF) and VEGF-C. Thanks to optimization of the stimulation and application times, as well as the morphological and immunohistological evaluation of the CAM, a suitable model appears to have been found for such investigations.

The results available thus far permit the conclusion that especially morphological changes, such as loop formation on the external surface of the CAM, are reliable parameters for lymphangiogenesis. Whether the available immunohistochemical markers (LYVE-1, D2-40, Prox-1, Podoplanin) are suitable for use in the chicken egg model cannot yet be definitively proven.

P134

Imiquimod interacts with adenosine receptor signaling in a TLR-7- and TLR-8-independent fashion – a novel mode of action

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Imiquimod is a topical small-molecule immune response modifier of the imidazoquinoline family that has shown profound antitumoral and antiviral efficacy, both *in vitro* and in clinical applications *in vivo*. It has been demonstrated recently that this activity is mediated through the Toll-like receptor (TLR)-7- and TLR-8-signaling cascade resulting in secretion of pro-inflammatory cytokines and, consecutively, induction of a tumor-directed cellular immune response. In addition, at higher concentrations, imiquimod exerts a direct pro-apoptotic activity in tumor cells.

We demonstrate here that imiquimod is able to induce activation of the transcription factor NF-κB and the downstream production of pro-inflammatory cytokines in the absence of TLR-7 and TLR-8. Using stable transfectants of adenosine receptors, we then show in radioligand-binding competition experiments that imiquimod binds to adenosine receptors at concentrations relevant in clinical settings, with highest affinities to the A1 and A2A subtypes. In competition experiments, using known agonists and antagonists of adenosine receptors, we demonstrate that imiquimod acts as an adenosine receptor antagonist. In addition, imiquimod had some direct inhibitory effect on adenylyl cyclase activity. Finally, using transformed human keratinocytes, we provide experimental evidence that imiquimod and A2A adenosine receptor-specific compounds similarly induce pro-inflammatory cytokines in the absence of immune cells. Given that adenosine receptor signaling is involved in inflammatory cascades, there appears to be an imiquimod-induced regulatory loop involving adenosine receptors that augment the inflammatory responses observed *in vivo*.

Thus, imiquimod appears to antagonize an important adenosine receptor-dependent suppressive feedback mechanism of inflammation. This novel mechanism presumably acts synergistically with the positive induction of pro-inflammatory cytokines and can, at least in part, explain the profound inflammation observed *in vivo*.

P135

Characterization of cytochrome P450 expression in organotipic skin models

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Our long-term goal is the development and optimization of an *in vitro* model for the study of drug metabolism in human skin. Cytochrom P450 (CYP) enzymes are crucial for phase-one drug metabolism. Little is known about expression or inducibility of CYP's in organotypic skin models. The only data that exist were obtained from human skin samples or mono-layer cell culture experiments with keratinocytes and fibroblasts. We constructed an organotypic skin model by embedding fibroblasts from neonatal foreskins in a collagen matrix from calf skin and by seeding keratinocytes on top. For comparison we bought commercialized skin models from different companies. To induce enzyme activity, we treated models with liquor carbonis detergens or pix lithanthracis. RNA was isolated using phenol chloroform extraction and purified. Gene expression profiles were studied by cDNA microarray analysis. Microarray data were confirmed using real-time polymerase chain reaction analysis. For quality control of the models and to detect and localize enzyme expression, immunofluorescence staining was performed with antibodies against CYP's and structural proteins. The immunofluorescence staining demonstrated the regular structure of our models. We could prove that CYP 1A1, 1B1, 2C, 2E1, 2J2, 3A5 and 4B1 are expressed in organotypic skin models. The expression of CYP 1A1 and 1B1 was highly inducible by treatment with liquor carbonis detergens. The proof of CYP expression in organotypic skin models is an essential step in the development of an *in vitro* model for the study of drug metabolism in human skin, because these enzymes are indispensable for drug metabolism.

P136

***In vitro* and *in vivo* comparison of two different light sources for topical photodynamic therapy**

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For the treatment of actinic keratosis (AK), photodynamic therapy (PDT) with 5-aminolevulinic acid (ALA) is an effective and safe treatment option. Matching the absorption maxima of ALA, incoherent lamps are often used.

To evaluate the efficacy of recently developed light-emitting diodes (LEDs), we performed a comparative trial.

Human epidermal keratinocytes (HEKs) were incubated for 24 h with ALA (100, 200,300, 400 or 500 μmol/l) and irradiated consecutively using either an incoherent halogen lamp ($\lambda em = 580–700$ nm, 24 J/cm^2, 40 mW/cm^2) or an LED system ($\lambda em = 633 \pm 3$ nm; 3, 6, 12 or 24 J/cm^2, 40 mW/cm^2). Topical ALA-PDT was performed on 40 patients with AK ($n = 584$) in a symmetrical dissemination suitable for two-side comparison. After incubation with ALA (20% in cream base), irradiation was performed with the incoherent lamp (160 mW/cm^2; 100 J/cm^2) on one side and the LED system (80 mW/cm^2; 40 J/cm^2) on the opposite side followed by re-evaluation up to 6 months.

No significant differences between the LED system (3, 6, 12 or 24 J/cm^2) vs. the incoherent light source (24 J/cm^2) regarding cytotoxicity could be shown *in vitro*. The complete remission rate yielded in the *in vivo* investigation was also not significantly different at 6 weeks ($P = 0.95$), 3 months ($P = 0.75$) and 6 months ($P = 0.61$) following therapy. Six weeks following therapy, complete remission rates of 84.3% (LED system) and 82.8% (incoherent lamp) were achieved. There was also no significant difference between both light sources regarding pain during light treatment ($P = 0.68$) and patient satisfaction ($P = 1.0$) as well as cosmesis ($P = 1.0$) following therapy.

These results show the efficacy of an LED system for ALA-PDT both *in vitro* and *in vivo*. ALA-PDT with the LED system showed a non-inferiority regarding the clinical outcome treating AK as compared with the incoherent lamp.

P137

Enhanced photocarcinogenesis in interleukin-12-deficient mice

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Ultraviolet-B (UVB)-induced DNA damage is the basis for the development of UV-induced skin cancer, because reduction of DNA damage reduces the risk to develop UV-induced skin tumors in mice. The immunomodulatory cytokine interleukin (IL)-12 was recently shown to exert the capacity to reduce UV-induced DNA damage, presumably via the induction of nucleotide excision repair. Because IL-12 is also produced in the skin, we were interested to study whether endogenous IL-12 might protect from photocarcinogenesis. Thus, we utilized knock-out mice (IL-12$^{-/-}$ mice) which lack the IL-12-p40 chain and thus do not secrete biologically active IL-12. We postulated that endogenous IL-12 should protect and that IL-12$^{-/-}$ mice should have an increased risk for skin cancer. IL-12$^{-/-}$ and wildtype (wt) mice were exposed $\times 3$/week to UVB starting with 5 kJ/m^2, which was subsequently increased up to 20 kJ/m^2. Examination of skin biopsies obtained after 6 weeks revealed significantly increased numbers of sunburn cells in IL-12$^{-/-}$ mice. Staining of epidermal sheets with an antibody directed against the tumor suppressor gene p53 revealed a higher number of p53-mutated patches in the skin of IL-12$^{-/-}$ mice. In addition, the size of the clones was significantly higher in the IL-12$^{-/-}$ mice. After around 200 days first skin tumors developed. Kaplan–Meier analysis indicated a significantly increased probability of tumor development in the IL-12$^{-/-}$ mice. In addition, the number of tumors developing in the individual mice was significantly higher in the IL-12$^{-/-}$ than in the wt mice. Tumors in IL-12$^{-/-}$ mice were more poorly differentiated and revealed a more malignant phenotype, because they grew much faster than those obtained from wt mice upon inoculation into immunodeficient nu/nμ mice. This was also confirmed in an electrophysiological assay evaluating the intrinsic invasive potency of tumor cells (TCs). In this assay, breakdown of the transepithelial electrical resistance across a renal cell monolayer indicates opening of tight junctions induced by TC invasion. Transepithelial electrical resistance was stronger reduced upon seeding in tumor cells obtained from IL-12$^{-/-}$ than from wt mice. Together, these data indicate that IL-12 deficiency is associated with an increased susceptibility to develop UV-induced skin cancer, implying that endogenous IL-12 may protect from photocarcinogenesis.

P138 (V17)

Interleukin-18 reduces ultraviolet-induced DNA damage and thereby affects photoimmunosuppression

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Ultraviolet (UV)-induced DNA damage has been recognized as the major molecular trigger for photoimmunosuppression. Interleukin (IL)-12 prevents UV-induced immunosuppression via its recently discovered capacity to reduce DNA damage presumably via induction of the nucleotide excision repair (NER). Because the proinflammatory cytokine IL-18 shares some biological activities with IL-12, we studied the effect of IL-18 on UV-induced DNA damage and immunosuppression. IL-18 significantly reduced UV-induced apoptosis of keratinocytes and supported the long-term survival of cells upon UV exposure. Injection of IL-18 into mice, which were exposed to UV, significantly lowered the number of sunburn cells. Accordingly, immunohistochemistry revealed reduced amounts of cyclobutane pyrimidine dimers in epidermal cells upon injection of IL-18. These effects were not observed in NER-deficient (Xpa$^{-/-}$) mice, indicating that IL-18-like IL-12 reduces DNA damage via DNA repair. Accordingly, UV-mediated suppression of the induction of contact hypersensitivity, which is known to be primarily triggered by DNA damage, was prevented upon injection of IL-18 into mice before UV exposure. However, IL-18 was not able to prevent UV-induced immunosuppression in NER-deficient Xpa$^{-/-}$ mice. This indicates that the preventive effect of IL-18 on photoimmunosuppression is due to its effect on DNA repair. In contrast to IL-12, IL-18 was not able to break either in wildtype or in Xpa$^{-/-}$ mice established UV-induced immunotolerance which is clearly not mediated via DNA damage but via regulatory T cells. This indicates that IL-12 is still unique in its capacity to restore immune responses because of its effect on regulatory T cells and thus represents a very potent immunostimulatory cytokine. Together, these data identify IL-18 as a further cytokine which exhibits the capacity to affect DNA repair. Although being primarily a proinflammatory cytokine through this capacity, IL-18 can also foster an immune response which is suppressed by UV radiation.

P139

Senescence of human fibroblasts after psoralen photoactivation is mediated by ATR kinase and persistent DNA damage foci at telomeres

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PUVA [psoralen + ultraviolet A (UVA)] therapy is a common treatment for different malignant and inflammatory skin diseases. However, long-term PUVA-treated patients show accelerated aging of irradiated skin and an increased incidence of various skin cancers.

Cellular senescence is a phenotype that is linked with aging. It is causally involved in the altered tissue composition of aged skin and in the promotion of growth of adjacent pre-malignant and malignant epithelial cells.

Using different psoralen derivatives and wavelengths, we could demonstrate that DNA interstrand crosslinks (ICL) induced by psoralen photoactivation cause cellular senescence of human dermal fibroblasts. We now used this model to analyze the underlying DNA damage response.

Using kinase assays, we now demonstrate that Ataxia teleangiectasia-mutated (ATM) and Rad3-related kinase (ATR), and not its relative ATM, is activated in response to ICL induced by psoralen photoactivation in different human skin cells including fibroblasts. Ablation of ATR expression by smart pools iRNA in human fibroblasts prior to psoralen photoactivation prevented cellular senescence and resulted in nuclear fragmentation and cell death, indicating the importance of ATR for the manifestation of the senescent phenotype. After psoralen photoactivation, ATR phosphorylates and colocalizes with the histone H2AX (then termed γ-H2AX) in the vicinity of DNA damage.

Quantification of ATR/γ-H2AX-containing DNA damage foci by confocal microscopy revealed an initially diffuse nuclear colocalization which is consistent with random nuclear damage. Several days later, only fewer foci persist that predominantly colocalize with telomeric DNA and telomere-bound proteins. Accordingly, telomere chromatin immunoprecipitation analysis revealed an ATR accumulation at telomeric DNA in PUVA-senesced fibroblasts.

In summary, we identify ATR kinase as a novel mediator of telomere-dependent senescence in response to ICL induced by

photoactivated psoralens. As the human telomeric repeats contain a sequence that can be crosslinked by 8-methoxy psoralen, and because repair kinetics of ICL at telomeres are unknown, it will be interesting to determine whether indeed persistent psoralen DNA ICL at telomeric DNA represent the underlying damage signal.

P140

Investigation of photodynamic inactivation of bacteria using the detection of singlet oxygen luminescence

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In view of the increasing resistance of bacteria to antibiotics, photodynamic inactivation of bacteria is a promising new technique. The objective was to evaluate localization of the photosensitizer Photofrin in Gram(+) *Staphylococcus aureus* and Gram(–) *Escherichia coli* by detection of singlet oxygen time-resolved by its luminescence at 1270 nm directly. Singlet oxygen was generated by energy transfer from the photoexcited Photofrin, dissolved in aqua dest. After incubation of *S. aureus* or *E. coli* with Photofrin and subsequent irradiation, the viability of *S. aureus* decreased yielding 99.9% dead bacteria, whereas the viability of *E. coli* was hardly affected. Sodium azide, quencher of singlet oxygen, inhibited the killing of *S. aureus*. Fluorescence microscopy showed an uptake of Photofrin by *S. aureus* but not by *E. coli*. Due to the limited resolution of the microscope, the subcellular localization of Photofrin in bacteria failed, and therefore, a detailed insight into the mechanisms of action was not possible. However, the localization of Photofrin is correlated to the localization of singlet oxygen, which is correlated to luminescence decay time of singlet oxygen measured. The resolution of this method is given by the diffusion length of singlet oxygen, which is very short in abiological environment. When incubating *E. coli* with 300 µg/ml Photofrin for 90 min, no singlet oxygen luminescence was detected confirming the results of cell viability experiment. When incubating *S. aureus* with Photofrin, a singlet oxygen luminescence decay time of 6 ± 2 µs was measured. Adding the quencher sodiumazide, we shortened the luminescence decay time (3 ± 1 µs). Obviously, the decay time of luminescence is an intermediate time of singlet oxygen decaying in phospholipids (14 ± 2 µs) of membranes and in the surrounding water (3.5 ± 0.5 µs). Thus, singlet oxygen seems to decay in outer cell wall areas of *S. aureus*, which is then the subcellular localization of Photofrin. The luminescence decay time in large agglomerates of bacteria was much longer (40 ± 16 µs) than in the suspension with single bacteria. This is the time that singlet oxygen was measured by its luminescence inside living bacteria.

P141

The common R683W rather than a new fs670→693ter mutation in the *XPD* gene determines the xeroderma pigmentosum phenotype in a 15-year-old boy

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We examined the clinical, molecular, and genetic features of a newly diagnosed 15-year-old boy (XP2GO) with signs of xeroderma pigmentosum (XP) and progressive neurologic symptoms. The parents are not consanguineous. Increased sun sensitivity led to the diagnosis of XP at 2 years of age, and a strict ultraviolet (UV) protection scheme was implemented. Besides recurrent conjunctivitis and bilateral pterygium, only mild freckling is present on his lips until today. He shows reduced deep tendon reflexes, progressive deafness, and progressive mental retardation. Enhanced MRI shows diffuse frontal cerebral atrophy. Typical symptoms of trichothiodystrophy (TTD)-like brittle hair with a tiger-tail pattern and of Cockayne syndrome (CS)-like cachectic dwarfism, cataracts, pigmentary retinopathy, and spasticity are absent. XP2GO fibroblasts exhibited a reduced post-UVC cell survival ($D10 = 7.4\,J/m^2$) and a reduced nucleotide excision repair capability as reflected by reduced host cell reactivation of a UV-treated luciferase gene-containing reporter plasmid. Cotransfection of different wildtype XP cDNA containing plasmids assigned XP2GO to the XP complementation group D. Mutational analysis of the *XPD* gene in XP2GO revealed two different mutations: a common R683W amino acid change (C2125T) known to be associated with XP and a novel frameshift mutation fs670→693ter (del2084G). The latter mutation potentially behaves as a null allele. Thus, the R683W mutation predominately determines the clinical XP with neurologic abnormalities phenotype, as mutations in the *XPD* gene may result in six distinct clinical phenotypes, XP, XP with neurologic abnormalities, TTD, and XP/CS complex, XP/TTD, or COFS syndrome.0

P142 (V03)

Differential regulation of the hyaluronan metabolism in the epidermal and dermal compartment by ultraviolet B irradiation of human skin

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Understanding of extracellular matrix (ECM) metabolism and its influence by environmental factors is necessary for devising therapeutic strategies aiming at local control of ECM–cell interactions in regeneration. Hyaluronan, a major component of cutaneous ECM, is involved in tissue repair processes. Human skin is directly exposed to the environmental hazard of UVB irradiation. This study aimed to investigate the regulation of HA metabolism in human skin during acute UVB-induced inflammation. Acute erythematic UVB irradiation caused a marked increase in the gene expression of HA-synthesizing enzymes HAS-1, HAS-2 and HAS-3 in HaCaT and human skin 24 h after UVB irradiation as evaluated using quantitative real-time polymerase chain reaction. Net HA production was increased in the cell culture media of HaCaT as well as epidermal HA immunohistochemical staining. Intriguingly, HA staining was decreased in the dermal compartment. Moreover, HA was also decreased in fibroblasts culture media 24 h after UVB irradiation. mRNA of HA-degrading enzymes HYAL-2 and HYAL-3 were not markedly up-regulated in HaCaT but in fibroblasts 24 h after UVB irradiation. Up-regulation of HAS-1, HYAL-2 and HYAL-3 mRNA could be induced in fibroblasts by humoral factors in culture media of UVB-irradiated HaCaT cells. Dermal microdialysis fluid revealed increased HA of molecular weight lower than 100 kDa 24 h after UVB irradiation as measured using enzyme-linked immunosorbent assay. However, neither in fibroblast culture media nor in microdialysis fluid hyaluronan oligomers could be detected by FACE analysis 24 h after UVB irradiation. Three hours after UVB irradiation HaCaT and fibroblasts HA synthesis was decreased. A subsequent differential time course of mRNA regulation of HA-metabolizing enzymes in HaCaT and fibroblasts appear to account for differential epidermal vs. dermal regulation of HA.

P143

In vitro and _in vivo_ evidence for senescence-associated mutations of mitochondrial DNA as an early event in transformation from nevi to malignant melanoma

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It has recently been reported that senescence-associated growth control represents an important protection from tumors including malignant melanoma. We and others have previously shown that aging associated mutations of mtDNA can be induced by ultraviolet (UV)-A radiation *in vitro* and *in vivo* and that these mutations persist or even increase in resting cells while they disappear in proliferating cells. However, the question whether UV-A-induced mtDNA mutations play a role in the pathogenesis of malignant melanoma has not been addressed. In the present study, we could show *in vitro* by real-time polymerase chain reaction (PCR) that, compared with normal melanocytes, melanoma cells are highly susceptible to generation of mtDNA mutations by low-dose repetitive UV-A irradiation. These mutations are functionally relevant, because they are associated with a decrease of mitochondrial oxygen consumption (clark-type electrode), mitochondrial membrane potential (FACS-measured JC-1 fluorescence) as well as up-regulation of matrix metalloproteinase-1 (real-time RT-PCR). Expression of senescence marker β-galactosidase was increased in oxidatively stressed cells, indicating an association of mtDNA mutations with senescence. *In vivo* in laser capture microdissected samples from normal human nevi, we detected background levels of mtDNA mutations. However, in samples from nevi showing clinical and histological signs of dysplasia, these mutations were increased 2.5 fold. Interestingly, in malignant melanoma, initially high mutation levels later decreased from small to large tumor thickness (range 0.25–25.0 mm). In addition to known mutations, an unreported mtDNA deletion of 4866 bp in length was detected (real-time PCR, sequence analysis) in tumor samples and UV-A-irradiated melanoma cells. These results indicate a high susceptibility of melanoma cells to the induction of functionally relevant UV-A-mediated mtDNA mutations, and this loss of senescence-associated growth control may represent an early event in the malignant transformation from inert nevi to proliferative and invasive malignant melanoma.

P144

Fox-P3+ regulatory T cells decrease during medium dose UVA1 therapy in the skin of generalized atopic eczema patients

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We have treated five patients with generalized atopic eczema using medium dose (750 J/cm^2) UVA1 therapy and have analyzed the skin-infiltrating T-cellular subsets before and after therapy using immunohistochemistry and real-time polymerase chain reaction (PCR). As we saw in all investigated patients a good clinical response to UVA1 treatment, we hypothesized that changes in TH1, TH2 and/or T-reg cells might be responsible for this effect. Skin biopsies were split for immunohistochemistry and real-time PCR and analysed for CD3, CD4, Fox-P3, GATA-3 and T-bet transcription as well as for CD3, CD4, CD152, Fox-P3 and GITR staining. As previously described, the number of lesional T cells dropped after irradiation. Within the population of T cells, no specific difference in the kinetics of Fox-P3-positive cells and Fox-P3-specific mRNA was noted as compared with GATA-3-positive T cells. These results were paralleled using reverse transcriptase (RT)-PCR for IL-10 and staining for CD152 and GITR, surface molecules which have been described for regulatory T cells. Interestingly, this was not the case in peripheral blood mononuclear cells from the UVA1-treated AE patients, in which we found an increase in mRNA for FoxP3 and IL-10. In conclusion, the clinical benefit of UVA1 treatment in AE patients does not seem to be due to a preferential survival/proliferation of Treg cells in the skin but rather due to the broad decrease of lesional T cells in these patients.

P145

Transplant recipients and other patients that have to protect themselves against solar ultraviolet radiation are at risk to develop vitamin D deficiency

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Ultraviolet (UV) exposition is the main reason for the development of non-melanoma skin cancer. For immunosuppressive therapy markedly increases the risk to develop UV-induced skin cancer, it is of high importance these patients protect themselves against UV light. Additionally, sun protection is of particular importance in patients suffering from genetically determined defects in UV-induced DNA damage repair as in the genodermatoses xeroderma pigmentosum (XP) and basal cell nevus syndrome (BCNS). However, 90% of all requisite vitamin D is formed within the skin through the action of the sun – a serious dilemma, for a connection between vitamin D deficiency and severe health problems including various types of cancer (e.g. colon-, prostate- and breast cancer), autoimmune diseases including type 1 diabetes, bone diseases, as well as an increased risk for hypertension and cardiovascular disease, has been shown in a large number of studies. We tested the hypothesis whether patients who protect themselves consequently against UV light, including renal, heart, lung, liver and hematopoietic stem cell transplant recipients, patients under immunosuppressive therapy (e.g. patients with systemic lupus erythematodes) and patients with genodermatoses XP and BCNS, are at risk of developing vitamin D deficiency. We found that patients in all these different risk groups are at high risk to become vitamin D deficient. It has been shown that a single oral dose of 50 000 IU vitamin D once a week for 8 weeks is efficient and safe to treat vitamin D deficiency. Another means of guaranteeing vitamin D sufficiency is to give 50 000 IU of vitamin D once a month. If we detect and treat vitamin D deficiency in risk groups, these measures will protect transplant recipients and other patients sufficiently against the serious health problems of vitamin D deficiency without increasing the risk to develop UV-induced skin cancer.

P146

Development of an _in vivo_-like skin model for studies of cooperation between keratinocytes and fibroblasts with respect to the cutaneous vitamin D3 pathway

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Human skin is qualified for autonomous synthesis of hormonally active 1α, 25-dihydroxyvitamin D3 (calcitriol). Epidermal keratinocytes essentially contribute to the synthesis of calcitriol by ultraviolet B (UVB)-induced transformation of 7-dehydrocholesterol via pre-vitamin D3, vitamin D3 and 25-hydroxyvitamin D3 (25-OHD3). Dermal fibroblasts, however, synthesize only intermediates of calcitriol (vitamin D3 and 25-OHD3), which may contribute to an amplified vitamin D3 metabolism in keratinocytes in a paracrine fashion. Presently, it is not clear whether vitamin D3 intermediates generated in fibroblasts result in elevated serum levels of vitamin D3 and

25-OHD3. Both cell types are separated by the basal membrane *in vivo* which excludes a direct cell contact; however, this includes a paracrine exchange of growth factors and cytokines, which may interact with the synthesis and action of calcitriol. It is the aim of this study to clarify the cooperative effects of dermal fibroblasts with epidermal keratinocytes. An organ-typical cell culture model consisting of keratinocytes and fibroblasts separated by a permeable collagen I membrane (thickness: 20 µm in dry conditions; size of exclusion: ≤ 30 kDa) was used. Keratinocytes were cultured on top of this collagen membrane, and fibroblasts were seeded on the lower surface of this diaphragm under submerged conditions. In a preliminary study, we investigated the effect of UVB radiation (wavelength: 300 nm, dose: 12.5 mJ/cm^2) on cell number and viability of cells in monoculture and coculture. After UVB irradiation of the coculture, both cell types displayed a changed proliferative behavior in comparison with irradiated monoculture. This organo-typical skin model promises to be a useful and trend-setting tool for cooperative studies between dermal and epidermal cells.

P147

Infrared-mediated hyperthermia is effective in the treatment of scleroderma-associated Raynaud's phenomenon

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Scleroderma is a multiorgan disease affecting various organs in addition to the skin, Raynaud's phenomenon (RP) being almost universal. Here, we report on the results of low-intensity hyperthermia (mean body temperature elevation by 1.3 °C), induced by repeated whole body near-infrared (IRA) irradiation, on the severity of RP. We performed a prospective study, consisting of a 5-week treatment phase (two irradiations per week), followed by a 6-week observational phase. In addition to RP, we also examined the effect of IRA-mediated hyperthermia on skin thickness, arthralgia, pulmonary function, and general disease activity. Outcome variables were fingertip rewarming in response to cold challenge, an RP-visual analogue scale (VAS-RP), the modified Rodnan skin score (MRSS), the DAS28 arthralgia score, the health assessment questionnaire (HAQ-DI), as well as lung diffusion capacity (DLCO). The study cohort consisted of 58 patients (31 with limited, 27 with diffuse scleroderma). Fingertip rewarming upon cold challenge (time to regain 63% of pre-cooling temperature) decreased from $8{:}39 \pm 4{:}54$ to $4{:}53 \pm 4{:}18$ (min : s; $P < 0.001$); VAS-RP decreased from 1.17 ± 0.71 to 0.83 ± 0.58 (29.1% reduction, $P < 0.001$). Mean MRSS in patients with diffuse scleroderma decreased from 18.7 ± 9.4 to 13.6 ± 6.6 ($P < 0.001$). Mean DAS28 in patients positive for rheumatoid factor decreased from 4.8 ± 1.3 to 4.2 ± 1.0 ($P < 0.003$). HAQ decreased from 0.40 ± 0.34 to 0.31 ± 0.31 ($P < 0.001$). In patients exhibiting less than 75% of predicted baseline DLCO, lung diffusion improved from 61 ± 11 to 66 ± 15% DLCO ($P = 0.005$). Intriguingly, most of the changes persisted for several weeks after the end of treatment. Our data document the effectiveness of IRA-mediated hyperthermia for the treatment of scleroderma-associated RP and suggest that it may be effective for several other disease manifestations.

P148

Respiratory burst is decreased in scleroderma patients and normalized by near-infrared-mediated hyperthermia

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The production of reactive oxygen species (ROS) by fibroblasts contributes to the pathogenesis of scleroderma. In contrast, the contribution of neutrophils and monocytes to respiratory burst activity in scleroderma is unclear. Furthermore, we have recently shown that near-infrared-mediated mild hyperthermia (IRA treatment) has a beneficial therapeutic effect on scleroderma-associated Raynaud's phenomenon, skin thickness, and arthralgia, while cellular effects of IRA treatment have not been studied. To test whether IRA treatment might alter the respiratory burst in scleroderma, we determined respiratory burst at baseline, as well as upon high-level stimulation by phorbol myristyl-acetate and low-level stimulation by non-opsonized zymosan in neutrophils and monocytes from scleroderma patients ($n = 22$), age- and sex-matched control probands ($n = 22$), as well as patients with psoriasis ($n = 20$). Furthermore, patients with systemic sclerosis were analyzed at different time points during IRA treatment. There was no increase, but instead a slight but statistically significant reduction in baseline- as well as zymosan-stimulated respiratory burst in scleroderma neutrophils ($P < 0.001$) and monocytes ($P < 0.005$). Decreased respiratory burst was also observed in psoriasis patients, indicating a non-specific phenomenon. IRA treatment induced a cell-type specific normalization of respiratory burst detectable only in neutrophils but not in monocytes. Intriguingly, this neutrophil-specific burst elevation persisted for 6 weeks after the end of treatment, thus paralleling the previously noted clinical responses effected by IRA treatment. Our data rule out a significant cell-autonomous overproduction of ROS in scleroderma by neutrophils and monocytes, thereby indirectly implicating fibroblasts as most likely source for clinically relevant ROS accumulation. Furthermore, they define a neutrophil-specific cellular effect of IRA-mediated hyperthermia.

P149 (V23)

A semi-synthetic β 1-3 glucansulfate reduces blood borne metastasis of B16-melanoma cells

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According to recent clinical trials, treatment with LMW heparins improves the survival of tumor patients, whereby their main mode of antimetastatic action is still not completely understood. As heparins have several disadvantages (e.g. animal origin, complex composition, batch variability, and high anticoagulant activity), heparin-like, structurally defined alternatives with an improved action profile may be an interesting option. We have therefore developed the glucansulfate PS3, which was shown to exhibit anti-inflammatory and antiangiogenic effects. The aim of the present study was to investigate the effects of PS3 and heparin (UFH) on mechanisms involved in tumor cell (TC) metastasis and to compare their antimetastatic activity *in vivo*. The selectin-mediated TC (U937, LS180) adhesion was examined in a static microplate (MP) cell-adhesion assay, a flow chamber model and by intravital microscopy. As a model for the interactions of TC with the basement membrane, the adhesion of TC (MDA-MB231) to laminin was used. The influence on the TC-mediated proteolysis was investigated measuring the inhibition of elastin degradation by TC (MCF-7). The inhibition of the extracellular matrix-degrading hyaluronidase was evaluated by the colorimetric Morgan–Elson reaction. Finally, the antimetastatic activity was examined in the B16.F10 melanoma lung metastasis model in mice. PS3 showed to interfere with several processes involved in metastasis. In all the assays, PS3 proved to be superior to UFH. It blocks the P-(IC50 5 µg/ml) and L-selectin-(IC50 10 µg/ml)-mediated TC adhesion and thus an initial step of their extravasation. It inhibits the TC adhesion to laminin

(IC50 5 μg/ml) and thus impairs the interactions of migrating TC with the basement membrane. In contrast to UFH, PS3 concentration dependently inhibits the elastinolytic activity of TC. In addition, it inhibits hyaluronidase (IC504.5 μg/ml), which is produced by certain TC. In addition, PS3 inhibited lung metastasis formation *in vivo* using the B16.F10 melanoma cell line. Like heparins, these misynthetic glucansulfate PS3 represents a multivalent biomodulator suggesting that not a single, but rather the concert of several effects is responsible for the antimetastatic activity.

P150

Expression of integrin-α10 is induced in malignant melanoma

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Recently, integrin-α10 was described as a collagen type II-binding integrin expressed mainly in chondrocytes. However, we detected integrin-α10 to be up-regulated in malignant melanoma compared with primary melanocytes. Consequent studies on additional melanoma cell lines and melanoma tumor samples confirmed this finding.

Expression of Integrin-α10 was shown to be controlled by AP-2 and Ets-1, two transcription factors known to be involved in melanoma development and progression. By down-regulation of integrin-α10 via stable antisense transfection, we analysed the functional relevance of integrin-α10 expression. Proliferation assays and colony-forming assays revealed no changes comparing the antisense integrin-α10 cell clones to the controls and the wildtype melanoma cell line. However, migration assays showed a reduced migratory potential of the antisense integrin-α10 cell clones suggesting a role of integrin-α10 in melanoma migration.

Our studies thus indicate that up-regulation of the integrin-α10 expression contributes to melanoma progression.

P151

A delta opioid receptor agonist desensitizes CXCR4 chemokine receptor and suppresses pulmonary metastasis of murine B16.F10 melanoma cells

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Tumor metastasis is the leading cause of mortality in most malignant cancers. Clinicians and pathologists have long known that this process is not random, as metastasis occurs in certain organs more than others depending on the origin of the primary tumor. Melanoma shows preferential sites of metastasis to lung, liver and brain, whereas prostate cancer cells tend to metastasize to bone. Metastasis formation requires a sequence of several essential steps, one of which is the migration and adhesion of blood-born tumor cells (TCs) to sites of metastasis.

The chemokine receptor CXCR4 has been shown to play a critical role in cancer progression by promoting the directional migration of TCs in the circulation to lung and liver, where its ligand CXCL12 is overexpressed. Indeed, CXCR4 antagonists such as AMD 3100 have been shown to significantly reduce pulmonary metastasis in several murine models. In this study, we show that an opioid receptor agonist is able to desensitize the chemokine receptor CXCR4 and impairs both CXCR4-mediated cell migration and adhesion and rolling of murine B16.F10 melanoma cells. Because of this, the opioid agonist interferes with metastasis progression and greatly reduces the number of metastasis nodules in mouse models of metastasis progression. These findings open new perspectives in cancer therapy especially in melanoma treatment.

P152

Paucity of Foxp3+ cells in skin and peripheral blood distinguishes Sézary syndrome from other cutaneous T-cell lymphomas

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Cutaneous T-cell lymphomas (CTCL) are mainly comprised of two variants: mycosis fungoides (MF) with $CD4^+$ tumor cells (TCs) confined to the skin and the leukemic Sézary syndrome (SS) with TC spread to the blood. A recent study suggested that CTCL is a malignant proliferation of regulatory T cells (Treg), because under certain circumstances *in vitro* CTCL TCs could become FOXP3+, the currently best identified marker of Treg. However, it remains unclear whether FOXP3+ CTCL cells exist *in vivo* and whether FOXP3 might serve as a CTCL TC marker. Therefore, we investigated FOXP3 expression in 30 CTCL patients and 16 control patients with chronic inflammatory skin diseases (eczema and psoriasis). Immunohistochemical analysis revealed significantly lower numbers of $CD4^+$ FOXP3+ cells within the dermal lymphomononuclear infiltrate of Sézary patients (16% FOXP3+ cells of $CD4^+$ cells) in contrast to MF [43% ($P < 0.05$)] and rare types of CTCL [45% ($P < 0.05$)] and inflammatory skin diseases (38%). Furthermore, $CD4^+CD25^+$ FOXP3+ T cells were also markedly reduced in peripheral blood of Sézary patients compared with controls as determined using FACS, quantitative polymerase chain reaction, and functional analysis of Treg characteristics. Our results support the conclusion that the neoplastic cells in CTCL do not express the Treg marker FOXP3. Our data also identify SS as the first reported disease with a clear reduction in Treg numbers within the $CD4^+$ population. This lack of Treg might account for the more aggressive nature of SS. Therapeutically, this loss of Treg could possibly be counteracted by extracorporeal photopheresis which was recently shown to induce antigen-specificTreg.

P153

Crossreactivity of mimotopes with a monoclonal antibody against the high molecular weight-melanoma-associated antigen does not predict crossreactive immunogenicity

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The high molecular weight-melanoma-associated antigen (HMW-MAA) is highly expressed in advanced primary and metastatic melanoma. An epitope of the core protein of the HMW-MAA is recognized by the murine monoclonal antibody 225.28S. In this study, we aimed to characterize peptides that antigenically mimic this epitope and to determine their efficacy as components of an HMW-MAA-based anti-melanoma vaccine. Therefore, we screened a constrained 10 mer phage display peptide library against the mAb 225.28S. Selected phage

displayed peptides were then tested for their specificity for the antibody's antigen-binding site. DNA sequences coding for specific peptide ligands were determined. Binding of the mAb 225.28S to the HMW-MAA was inhibited in a dose-dependent manner by phage-displayed peptides from 51 to 83% and by synthetic peptides from 38 to 87%. Subsequently, immunogenicity of the five mimotopes with the highest inhibition capacity was examined in rabbits. Immunizations with synthetic mimotopes conjugated to tetanus toxoid resulted in peptide specific antibodies, but none of the highly antigenic mimotopes induced HMW-MAA crossreactive antibodies. This report describes an example of disparity between antigenicity and crossreactive immunogenicity complicating the selection of potential vaccine candidates.

P154

Influence of the cytoplasmic domain of E-cadherin on endogenous N-cadherin expression in malignant melanoma

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Malignant transformation of melanocytes frequently attend with loss of E-cadherin expression and induction of N-cadherin expression. The switch of the cadherin class is an interesting phenomenon of melanoma cells and in epithelial-mesenchymal transition (EMT) in general. Therefore, we analyzed the capacity of E-cadherin to regulate expression of N-cadherin in melanocytic cells.

Our experiments revealed that melanoma cells down-regulate endogenous N-cadherin expression after transient transfection of full length E-cadherin but also of the cytoplasmic domain of E-!cadherin. Therefore, we concluded that the extracellular domain of E-cadherin and cell–cell contacts are not necessary for negative regulation of N-cadherin. Melanoma cells re-expressing full length or cytoplasmatic E-cadherin have reduced nuclear factor (NF)κB activity in comparison with mock-transfected cells. Down-regulation of NFκB activity, either directly or by re-expression of E-cadherin led to a suppression of N-cadherin promoter activity and expression. Vice-versa the up-regulation of NFκB activity in melanocytes and melanoma cells leads to an up-regulation of N-cadherin expression in these cells. Consequently, an NFκB-binding site in the N-cadherin promoter was characterized.

In summary, our results suggest that N-cadherin is directly regulated by the E-cadherin. Loss of E-cadherin induces NFκB activity and N-cadherin expression in tumorigenic EMT.

P155

Differential prognostic impact of the mutational status of B-RAF and N-RAS in melanoma

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Mutations in B-RAF and N-RAS genes are reported to be crucial for tumor initiation and maintenance in melanoma. Mutations in both genes are hypothesized to result in the same cellular phenotype and exert a similar impact on the clinical course of disease. This study aimed at the investigation of the mutational profile of B-RAF and N-RAS in both tumor tissue samples and corresponding cell lines from melanoma patients, and its putative impact on survival. We screened 77 tumor tissue samples and 85 cell lines from 89 patients for mutations in exons 11 and 15 of the B-RAF gene and exons 1 and 2 of the N-RAS gene using polymerase chain reaction single strand conformation polymorphism and direct DNA sequencing. Mutations were correlated with survival data obtained within a median follow-up time of 36 months. B-RAF mutations were detected in 54% cell lines and 56% tissues, N-RAS mutations in 27% cell lines and 25% tissues, respectively. Mutations in B-RAF and N-RAS revealed a differential impact on prognosis. Patients carrying a B-RAF mutation in tumor tissue showed an impaired median survival (8.0 vs. 11.9 months, $P = 0.041$), whereas patients whose tumor cell lines presented N-RAS mutations revealed a favorable prognosis (median survival 15.4 vs. 6.2 months, $P = 0.002$), each in comparison with the wildtype gene status. These findings indicate substantial differences between the effects of B-RAF and N-RAS mutations on the clinical outcome of melanoma patients that should be considered in future clinical trials using these molecules as therapeutical targets.

P156

Antisense melanoma inhibitory activity cell clones as a model system for restored transforming growth factor-β signaling in malignant melanoma

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Malignant melanomas are able to escape transforming growth factor (TGF)-β-mediated growth inhibition and anti-proliferative effects by expressing the smad inhibitors ski and sno. In our model system, we demonstrate that melanoma inhibitory activity (MIA)-deficient melanoma cell clones do not express the smad inhibitors ski and sno and exhibit a restored TGF-β-signaling cascade.

Transfection of the parental cell line and the MIA-deficient cell clones with a smad responsive luciferase construct (CAGA9-Luc) revealed an induction of luciferase activity in the MIA-deficient cells HMB2-MIA5 and HMB2-MIA8 after TGF-β1 treatment but not in the MIA-expressing control cells. Due to this finding, we analyzed the expression pattern of TGF-β downstream target genes. A strong induction of JunB and Id-1 expression was detectable in the MIA-deficient cell clones after TGF-β1 treatment.

Analysis of the model cell clones regarding components of the TGF-β-signaling cascade revealed that Ski and Sno are not expressed in HMB2-MIA5 and HMB2-MIA8 but in the parental cell line HMB2 and the transfection control HMB2 lacZ. Further investigation showed that Ski and Sno expression might be dependent on an active mitogen-activated protein kinase (MAPK)-Erk-signaling pathway, because MIA-deficient cell clones do not have a constitutively active Erk2 kinase. Treatment of HMB2 cells with a MEK1 and 2 inhibitor revealed a reduction in Ski and Sno expression levels, which leads to the conclusion that Ski and Sno expression is regulated, at least in part, via MAPK-Erk signaling.

P157

High frequency of FGFR3β mutations in adenoid seborrheic keratoses

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FGFR3 germline mutations cause autosomal dominant skeletal disorders including achondroplasia, thanatophoric dysplasia, SADDAN, Crouzon, and Muenke syndrome. Somatic mutations of FGFR3 have been identified in several cancers like multiple myeloma and bladder carcinoma. More recently, FGFR3 mutations have also been detected in 39% of seborrhoic keratoses of the hyperkeratotic and acanthotic subtype, which are very common benign skin tumors. In a mouse model, the expression of a mutant FGFR3 (S249C) in the basal epidermis using the keratin 5 promoter caused verrucous skin tumors similar to seborrhoic keratoses.

We investigated a series of 26 seborrhoic keratoses of the adenoid subtype using a multiplex SnaPshot assay covering nine activating FGFR3 mutations. In 21 of 26 (81%) adenoid seborrhoic keratoses activating FGFR3 mutations were detected. In two lesions the A393E mutation was found, which has not been described in acanthotic and hyperkeratotic seborrhoic keratoses yet. Furthermore, two lesions showed the presence of two simultaneous activating FGFR3 mutations.

Adenoid seborrhoic keratoses seem to be characterized by a higher frequency of FGFR3 mutations than the other two histological subtypes. The mechanism for the somatic mutations in these benign skin tumors remains elusive.

P158

Vorkommen und bedeutung von antineuronalen antikörpern bei patienten mitmalignem melanom

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Nahezu alle Tumorentitäten können mit paraneoplastischen Phänomeneneinhergehen. Bestimmte Tumoren führen überdurchschnittlich häufig zuParaneoplasien, die das periphere oder zentrale Nervensystem betreffen. KlassischeBeispiele hierfür sind das Opsoclonus-Myoclonus-Syndrom oder dasLambert-Eaton-Syndrom bei kleinzelligem Bronchialkarzinom. In seltenen Fällenwurden auch bei Patienten mit malignem Melanom Paraneoplasien, die auf derBildung antineuronaler Antikörper beruhten gesehen. Bis heute wurden nachunserem Kenntisstand keine Screeninguntersuchungen in einem größeren Kollektivvon Melanompatienten auf das Vorhandensein von antineuronalen Antikörpern imSerum durchgeführt.

Im Rahmen dieser Untersuchung wurden Serumproben von 41 Melanompatienten inder verschiedenen Stadien der Erkrankung (Stad. II: $n=3$; Stad. III: $n=3$; Stad. IV:$n=35$. Tumorklassifikation nach AJCC 2001) mittels indirekter Immunfluoreszenz aufkommerziell erhältlichen Chip-Assays der Firma Euroimmun untersucht.Hauptaugenmerk der Suche galt folgenden antineuronalen Antikörpern:ANNA-1 = anti-Hu, PCA-1 = anti-Yo und ANNA-2 = anti-Ri. 4 der 41 untersuchtenSerumproben zeigten in der Immunfluo- reszenzuntesuchung Bindungsmuster, die aufdas Vorliegen von anti-Yo-Antikörpern verdächtig waren. EineBestätigunguntersuchung mittels Western-blot-Technik konnte diese Vermutung nichtbestätigen, aber bei 3 der 4 Proben zeigte sich eine auffällige granuläre Zytoplasma- fluoreszenz der Purkinjezellen, in einer Serumprobe gepaart mit demNachweis von hochtitrigen Antikörpern gegen Golgiapparat. Zwei dieser drei Probenzeigten im Western-Blot identische Banden auf einer Höhe von 40 kDa und 85 kDa,deren Definition noch unklar ist. Ferner fanden sich bei 32% der untersuchtenSerumproben anti-Myelin-Antikörper, bei 29% ANAs, bei 7,3% Endothel-Antikörperund bei 4,8% Antikörper gegen Becherzellen. Das Vorhandensein vonAutoantikörpern gegen neuronale Strukturen und anderes körpereigenes Gewebescheint bei Patienten mit malignem Melanom häufiger zu sein als bisherangenommen. Ob diese Antikörper prognostische Bedeutung besitzen oder lediglichein reines Epiphänomen darstellen müssen weitere Untersuchungen zeigen.

P159

Re-expression of the retinoblastoma-binding protein 2-homolog 1 reveals tumor suppressive functions in highly metastatic melanoma cells

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The loss of cell cycle control in malignant melanomas is thought to be due to a lack of retinoblastoma protein (pRb)-activity. We have recently reported a progressive deficiency of the retinoblastoma-binding protein 2-homolog 1 (RBP2-H1) in advanced and metastatic melanomas *in vivo* suggesting a role of RBP2-H1 in loss of pRb-mediated control. Therefore, in this study, we re-established the pRb-modulating function of RBP2-H1 in highly metastatic A375-SM melanoma cells by re-expressing its C-term (cRBP2-H1). As we have previously shown, the corresponding domains comprise a pRb-binding region (non-T/E1A-pRb-binding domain). As a result, we detected pRb hypophosphorylation selectively at Ser795 but not at Ser780 and Ser807/811 throughout the G1 phase of the cell cycle. As a further consequence, a block in G1/S transition was observed accompanied by a significant decrease of cellular proliferation. As demonstrated by cDNA microarrays of cRBP2-H1-transduced cells and confirmed by quantitative TaqMan TM reverse transcription-polymerase chain reaction, a differential expression of further melanoma progression-related genes was observed, among them BMP2, FST, transforming growth factor (TGF)A, HGF 4, TCF4 and MITF. Conclusively, these data suggest that RBP2-H1 exerts a broad tumor-suppressive function partially mediated by pRb modulation. Therefore, re-establishing of RBP2-H1 could evolve as an interesting novel approach in developing experimental treatments for metastatic melanomas.

P160

Oligonucleotide microarray-based screening for molecular markers in melanoma progression revealed two independent novel predictors

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The molecular and genetic events that contribute to the genesis and progression of cutaneous malignant melanoma, a complex and aggressive disease with a high propensity for metastasis, are poorly understood. High-throughput oligonucleotide microarray (human U133A, Affymetrix Inc., Santa Clara, CA, USA)-based profiling ($n=27$) with the objective of identifying key molecular regulators in this process revealed several significantly regulated candidate genes, which were subsequently confirmed using real-time RT-PCR. Two potential independent predictors, activator of S-phase kinase (ASK/HuDbf4) and tumor-potentiating region (Tpr), were significantly overexpressed in cutaneous melanoma metastases, primary melanomas and metastatic melanoma cell lines (BLM, MV3, M13) as opposed to congenital nevi and normal human melanocytes (NHM). Approximately 86% melanoma metastases overexpressed ASK/HuDbf4 and Tpr in comparison with other potential markers for detection of melanoma progression/metastasis namely CD146/MUC18 (13%) and c-Met (53%). Furthermore, as genomic methylation patterns are frequently altered in tumor cells with global hypomethylation accompanying region-specific hypermethylation events, we examined possible epigenetic regulation of ASK and Tpr. In silico promoter analysis of ASK

and Tpr revealed the presence of CpG islands within the promoter regions. Subsequently, treatment of NHM with 5-aza-2-deoxycytidine restored their expression to oncogenic levels. Taken together, these data suggest demethylation as a mechanism for overexpression of these two genes during transformation. Earlier studies have also reported Tpr-met hybrid RNA and protein resulting from a chromosomal translocation event in several human tumors including melanoma. The fusion contains the constitutive promoter and first 424-coding nucleotides (142 amino acids) of Tpr and the tyrosine kinase domain of the c-met proto-oncogene. By real-time quantitative PCR, we confirmed the presence of exon 21 which is specific only for Tpr and not Tpr-met hybrids suggesting that the overexpression observed is of full length Tpr. Our findings facilitate the understanding of mechanisms by which human melanoma progresses to malignancy and expediting the development of efficacious therapeutic modalities to constrain melanoma progression.

P161 (V35)

The proapoptotic Bcl-2-related protein Nbk/Bik induces apoptosis in human melanoma cells by a caspase-independent pathway that affects the lysosomes

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Proteins of the Bcl-2 superfamily located at the outer mitochondrial membrane are central regulators of apoptosis and include both antiapoptotic as well as proapoptotic factors. The proapoptotic BH3-only protein Nbk/Bik has been described to inhibit Bcl-2 and Bcl-xL, thereby supporting the death-promoting ability of Bax. To evaluate its function in melanoma, we investigated the response after Nbk/Bik overexpression in cultured human melanoma cells and in a melanoma mouse model.

Untransfected melanoma cell lines expressed Nbk/Bik only weakly at the mRNA and protein level. The conditional expression of Nbk/Bik, using an inducible tetracycline-responsive expression system, triggered apoptosis and enhanced sensitivity to proapoptotic stimuli such as agonistic CD95 activation or the chemotherapeutics etoposide, doxorubicin and pamidronate. For investigating the effects of Nbk/Bik *in vivo*, stably transfected melanoma cells were subcutaneously injected into nude mice. Significantly delayed tumor growth was observed when mice received doxycycline for induction of Nbk/Bik expression.

By investigating the mechanism of Nbk/Bik-induced cell death, we saw after induction typical hallmarks of apoptosis such as DNA fragmentation and cleavage of the death substrate DFF45/ICAD. On the contrary, common characteristics of apoptosis such as cytochrome c release and caspase activation were not detected. Interestingly, inhibition of lysosomal cathepsins significantly decreased apoptosis induction, whereas selective caspase inhibition remained without effect. Moreover, acridine orange staining revealed changes in lysosomal pH value after Nbk/Bik induction.

These data indicate the high potential of Nbk/Bik in regulating apoptosis in melanoma by a new, lysosome-dependent pathway and may corroborate the potency of novel antimelanoma strategies based on activation of BH3-only proteins such as Nbk/Bik.

P162 (V25)

Identification of important cell cycle regulators as down-stream targets for tumor progression factor osteopontin using a combination of RNA interference and proteome technology

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In a large-scale gene expression study of 40 primary melanomas and metastases, osteopontin (OPN) was identified as a new tumor progression factor for malignant melanoma. However, as yet little is known about its role in tumor progression. Toidentify down-stream targets of OPN and thereby characterize its putative functions, OPN expression was down-modulated in melanoma cells using short interfering RNA (siRNA). Subsequently, total protein extracts from melanoma cells treated with siRNA or non-specific scrambled RNA were subjected to two-dimensional gel electrophoresis and differentially expressed proteins were identified using Maldi-ToF and Q-ToF mass spectrometry. By this means, a series of new OPN-dependent proteins, e.g. p21Waf1/Cip1 and retinoblastoma protein, was identified. The latter both are central molecules in cell cycle regulation. These data were further confirmed using Western blot analysis, which included also other cell cycle molecules such as cell cycle-dependent kinases. In accordance with these findings, functional assays (BrDU uptake) showed that OPN is involved in cellular proliferation and cell-cycle progression. The studies on functional down-stream targets of OPN were then extended by analyses of up-stream gene regulatory elements. It could be demonstrated using mobility shift assay and chromatin immunoprecipitation that c-Myb is centrally involved in OPN regulation. Taken together, the presented report demonstrated that a combination of siRNA technique and proteome analysis identified functionally relevant down-stream targets of tumor progression factor OPN. Interfering with OPN itself via siRNA or up-stream regulatory elements opens interesting perspectives for future innovative treatment approaches for malignant melanoma.

P163

Absence of classical MAP kinase pathway signalling in merkel cell carcinoma

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Merkel cell carcinoma (MCC) is a highly metastatic skin tumor that poses a considerable therapeutic challenge. The genetic and molecular mechanisms involved in development and progression of this cancer are not well defined. Because alterations in the Ras/Raf/MEK/MAP kinase pathway are found in the majority of cancers, we assessed the relevance of the MAP kinase pathway in MCC. For this purpose, we analyzed for activating B-Raf mutations, the presence of the Raf kinase inhibitor protein (RKIP) and the phosphorylation status of ERK. To this end, all MCC samples analyzed were negative for the B-RafV600E mutation, as determined by a newly developed very rapid and sensitive real-time polymerase chain reaction assay. Remarkably, RKIP, which was shown to interfere with the activation of MEK by Raf, was highly expressed in primary as well as in metastatic MCC. Immunohistochemical analysis of the phosphorylation status of ERK in MCC tissues revealed in more than 90% of the samples a complete lack of activated ERK in tumor cells (TCs); in the few positive cases the phospho-ERK was restricted to a minor fraction

of the TCs. Thus, our data unequivocally demonstrate the inactivity of the classical MAP kinase signal transduction pathway in MCC which seems to be due to a lack of activation as well as an active deactivation. These findings should be accounted for in future therapeutic approaches for this tumor.

P164

Influence of hyaluronan and ultraviolet-B on melanoma cell motility

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Several autocrine factors, such as Hyaluronan (HA), interleukin (IL)-8 or IL-1β are able to induce melanoma cell motility. HA is the most abundant extracellular matrix molecule of the skin and plays an important role in the tumorigenesis of different cancers; amongst others, it can promote cell migration in melanoma cells. Ultraviolet-B (UVB) irradiation is known to be an important factor in the pathogenesis of malignant melanoma (MM) and recent work suggests a modulation of the HA metabolism by UVB. Therefore, the purpose of this study was to investigate the influence of autocrine soluble factors on melanoma cell motility and the influence of UVB on the HA metabolism of melanoma cells.

Cell motility was measured using a TILL Photonics video microscopy system with tracking extension. We found that soluble factors from stroma and melanoma cells are able to modulate tumor-cell motility. In agreement with other studies, HA is able to increase cell motility but only at high doses. Direct UVB irradiation does not influence melanoma cell motility 24 h after irradiation. Quantitative reverse transcription-polymerase chain reaction revealed a modulation of the HA metabolism by UVB, resulting in an increase in HA production 48 h after irradiation, as determined using enzyme-linked immunosorbent assay. This increased HA is not able to enhance melanoma cell motility. Other factors are more important regarding cell motility. Further experiments will have to identify them.

P165

Casein kinase 1α is repressed during melanoma progression and regulates β-catenin signaling in melanoma cell lines

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The identification of differentially expressed genes which are critical in homeostasis and disease is a central task in cancer biology. Using a polymerase chain reaction-based subtractive hybridization approach comparing mRNA from metastatic and primary melanoma cells, we identified the casein kinase 1α among others as being differentially expressed. The casein kinase 1α is a Ser/Thr kinase which participates in the degradation of cytoplasmic β-catenin by phosphorylation at specific residues thereby inhibiting the β-catenin-signaling pathway. Expression analysis on RNA and protein level with melanoma cell lines representing different progression stages indicated that during melanoma progression and invasion the casein kinase 1α is down-regulated. Consistent with the lower protein expression of casein kinase 1α in several metastatic melanoma cell lines, a higher level of β-catenin and nuclear translocation was found. Functional studies indicated that the casein kinase 1α plays a critical role in melanoma cell survival. Overexpression of casein kinase 1α in metastatic melanoma cell lines with a low endogenous level of this Ser/Thr kinase caused a diminished cellular growth *in vitro* indicating a role for casein kinase 1α in melanoma cell proliferation.

Nuclear β-catenin acts as a transcription factor in combination with DNA-binding proteins such as Tcf/Lef-1 and activates the transcription of oncogenic genes like c-myc, cyclin D1, survivin, matrix metalloproteinase 7 and many others. Our data suggest that casein kinase 1α may function as an important negative regulator of the Wnt-signaling pathway in melanocytes and that through its down-regulation during melanoma progression a high cellular level of active β-catenin is generated.

P166

Small fragments of hyaluronic acid induce metalloproteinase and cytokine up-regulation in human melanoma cells – possible role of Toll-like receptor-4 and CD44

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Hyaluronic acid (HA) is a major component of the extracellular matrix (ECM) and has been shown to play a role in progression and metastasis of malignant melanoma via interaction with its receptor CD44. Furthermore, HA is known to be cleaved into small molecular fragments (sHA) at sites of inflammation and tumor environment. These fragments can induce cytokines and metalloproteinases in dendritic and endothelial cells.

This prompted us to investigate whether sHA induces cytokine/metalloproteinase up-regulation also in human melanoma cells.

By measuring the transcriptional response of different human melanoma cell lines with quantitative real-time polymerase chain reaction, we show that addition of sHA to these cells leads to increased expression of matrix metalloproteinase (MMP)-2, interleukin (IL)-1β, IL-8 and Basigin (CD147), a known inductor of MMP-2, in a time-dependent manner. All these factors can contribute to melanoma progression by inducing angiogenesis, tumor invasiveness, or metastasis.

Next, we tested the involvement of Toll-like receptor (TLR)-4 as a receptor for sHA, similar to effects shown in dendritic and endothelial cells. We could show the constitutive expression of TLR-4 on the surface of melanoma cells *in vitro* and *in vivo* using FACS analysis and immunohistochemical staining. Furthermore, stimulation with sHA leads to a profound up-regulation of both TLR-4 and, to a lesser extent, CD44 expression on the surface of melanoma cells. In addition, sHA stimulation leads to an increase of detectable bioactive NFκB (subunit p65), a key component of the TLR pathway, as shown by intracellular staining.

When using a melanoma cell line deficient for CD44, the increase of TLR-4 on the cell surface following sHA stimulation could also be detected, as well as the transcriptional signal for IL-8, but not for IL-1β, MMP-2 and Basigin.

Therefore, we favour the hypothesis that TLR-4 might at least partly be involved in the sHA-signalling pathway in human melanoma cells and that sHA might serve as an endogenous ligand for TLR-4, but the role of CD44 or possible other receptors in this pathway has also to be considered.

P167 (V18)

Down-regulation of cFLIP sensitizes melanoma cells to TRAIL- or CD95L-mediated apoptosis

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The prognosis of metastasized melanoma is poor and novel treatment modalities are urgently needed. Induction of apoptosis by death ligands such as TRAIL or CD95L may represent an attractive novel therapeutic option. However, detailed knowledge of stage-specific death receptor sensitivity in melanoma is missing. In this study, we therefore analyzed those signalling pathways in human melanoma. CD95-R and

TRAIL-R1, but not TRAIL-R2 surface expression, correlated with apoptosis sensitivity in melanoma cell lines of all progression stages. In contrast, expression of proteins of the apical apoptosis-signalling cascade (FADD, the initiator caspases 8 and 10 and their inhibitor cFLIP) did not correlate with apoptosis sensitivity, indicating that death ligand sensitivity is controlled at different levels. Because both TRAIL-R1 and TRAIL-R2 are able to activate the apoptotic program, we asked whether cFLIP, highly expressed in several of the cell lines tested is sufficient for resistance to TRAIL-R2-mediated apoptosis. We, thus stably transduced cFLIP-expressing IGR cells (a highly TRAIL-resistant melanoma cell line expressing only TRAIL-R2) with a cFLIP-specific retroviral siRNA. Successful stable down-regulation of cFLIP was associated with a dramatic increase in TRAIL sensitivity and concomitant increased activation of caspase 8 and 3. Moreover, increased recruitment of caspase 8 to and release from the death-inducing signalling complex was observed, demonstrating the functional relevance of cFLIP.

Our results indicate that death receptor sensitivity is regulated at the intra- and extracellular level in melanoma. We demonstrate that down-regulation of cFLIP is sufficient to sensitize melanoma cells expressing only TRAIL-R2, indicating that TRAIL-R1-negative tumor cells have all constituents necessary for death receptor-mediated apoptosis. Therefore, modulation of cFLIP may represent another potential candidate of therapeutic strategies including TRAIL receptor agonists for melanoma.

P168

Increased colloid osmotic pressure enhances the uptake of macromolecules in a xenograft tumor model

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It is a common observation that many solid tumors show an increased interstitial fluid pressure which causes a barrier to transcapillary transport. Clinically, a high tumor interstitial fluid pressure (TIFP) may result in reduced delivery and uptake of anticancer drugs and thus lack of therapeutic effects. In the present study, the impact of an increased colloid osmotic pressure (COP) on the TIFP was investigated. COP was increased by intravenous (i.v.) injection of human serum albumin (HSA). Furthermore, the intra-tumoral distribution of macromolecules in control and COP-increased animals was shown by i.v. injected fluorescent-coupled dextrans.

A431 squamous epidermoid vulva carcinoma cells were subcutaneously implanted into the back of anaesthetized NMRI nude mice or Rowett nude rats. During tumor growth, TIFP was measured three times per week using the wick-in-needle technique to measure TIFP directly in the central region of solid tumors. The animals were injected with hyperosmotic HSA solutions i.v. to increase the COP. During HSA injection, the micropuncture method was used to measure the TIFP in peripheral parts of the tumors, and blood pressure was measured invasively in the arteriacarotis. In our experimental set-up, we could demonstrate that the increase of COP reduces the TIFP significantly. At different time-points after the HSA injection fluorescent-dextrans were i.v. applied. The distribution of dextrans in tumor tissue with reduced TIFP in contrast to untreated control tumors was investigated using fluorescence microscopy of cryosectioned tumor tissue.

These data demonstrate for the first time that an increased COP can lower TIFP. Furthermore, our studies suggest that a reduced TIFP can augment the delivery of macromolecules to tumor tissue. Therefore, lowering of TIFP could enhance the efficacy and efficiency of antitumor therapies utilizing chemotherapeutics.

P169

GBP5 expression and their putative interaction partners in cutaneous T-cell lymphoma

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Cutaneous T-cell lymphoma (CTCL) is a neoplasm of the immune system with malignant proliferation of CD4T lymphocytes and primary manifestation in the skin. GBP5ta was identified by screening a CTCL cDNA library with patients' sera and belongs to a protein family with five different genes in humans (gbp1-5). GBP5ta is a splicing variant of gbp5 and lacks its C-terminal isoprenylation motif. Guanylate-binding proteins (GBP) are part of the GTPases superfamily including Ras which is important in cell signaling and proliferation. Using real-time polymerase chain reaction analysis, we found only GBP4 and GBP5, but not GBP1, 2 or 3 in CTCL cell lines. Western blotting with newly generated polyclonal antibodies against GBP5ta revealed GBP5ta and the longer version GBP5a/b in normal CD4 and CD8 T cells and in CTCL cell lines, while tumor tissues were solely positive for GBP5ta. Within subcellular fraction of CTCL lines, we found the main portion of GBP5ta protein in the nuclear fraction and only a small part in the cytosol and the crude membrane fraction. A perinuclear localization of GBP5 proteins was supported using immunocytochemistry. GFP–GBP5ta fusion proteins are presently used for further analysis of the subcellular localization. In the first pull-down assays, we detected Ras in the GBP5ta complex, which points towards a role in regulation of protein transport between the nucleus and the cytoplasm. In conclusion, the lack of the C-terminal end with its isoprenylation site seems to be responsible for the altered subcellular localization of GBP5ta. Further studies will reveal whether GBP5ta might exert an anti-inhibitory function in proliferation by competing with GBP5a/b.

P170

Nachweis eines identischen T-zell klones in der haut und im peripheren blutbei patienten mit lymphomatoider papulose

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Kutane Lymphome sind die zweithäufigste Gruppe der extranodalen Lymphome. Ihrejährliche Inzidenz liegt bei etwa 0.5–1/100.000. Gerade die lymphomatoide Papulose (LyP) stellt eine besondere Entität unter den niedrigmalignen CTCL dar.

Sie ist in erster Linie durch ihr chronisch- rezidivierendes Krankheitsbildgekennzeichnet. Besonders auffällig sind dabei die papulonodulärenHautveränderungen, die in einem Zeitraum von Monaten bis Jahren zu spontanen Remissionen neigen. Meist wird jedoch das Ausheilen der Hautveränderungen vomgleichzeitigen Auftreten neuer Läsionen begleitet. In 10–20% der Fälle kommt es imVerlauf der Erkrankung zur Entwicklung anderer maligner Lymphome (z.B. Mycosisfungoides (MF), $CD30^+$ großzelliges Lymphom, M. Hodgkin (HD)).

Dieser eher untypische Verlauf einer malignen Erkrankung wirft insbesondere dieFrage nach der Pathogenese dieser Entität auf, und ob den rezidivierendenHautveränderungen ein zirkulierender Klon im peripheren Blut zugrunde liegt, dersich immer wieder neu in der Haut manifestieren kann und sogar Grundlage einesspäteren nodalen Lymphoms (z.B. M. Hodgkin) ist.

Zur Klärung dieser Frage wurden Haut- und Blutproben von 27 Patienten untersucht(davon 16 LyP, 3 LyP/MF, 1 LyP/HD, 7 cALCL). Die DNA der Proben wurde mittels PCR amplifiziert. Hierzu wurde das Biomed II TCR-gamma Protokoll sowie Primersetverwendet. Zur Verifizierung des Amplifikats wurde eine Gelelektrophorese auf 2%ethidiumbromid- Agarosegel durchgeführt. Anschließend wurde das PCR-Produktmittels Genescan im ABI 310 PRISM auf Klonalität untersucht.

Bei 4 von den 27 untersuchten Patienten ließ sich ein identischer Klon in der Hautsowie im peripheren Blut nachweisen (2 LyP/MF, 1 cALCL, 1 LyP). In der Mehrzahlder Fälle (23 Patienten) konnte kein Zusammenhang zwischen einer Klonalität imperipheren Blut und in der Haut erbracht werden.

In weiteren Studien sollte geklärt werden, ob der Klon lediglich in der Haut persisiert,oder ob durch weitere Untersuchungen höherer Sensitivität (klonspezifische PCR) ein eindeutiger Nachweis eines identischen Klons im Blut und in den Hautläsionengelingt.

P171

Recover in and others: cancer-retina antigens in melanoma

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Recoverin is a Ca^{2+}-binding protein of vertebrate photoreceptors involved in the visual cascade and can function as a paraneoplastic antigen in lung cancer. It has been shown for lung cancer patients that its aberrant expression can lead to the development of paraneoplastic retina degeneration. In this study, we have addressed the expression of recoverin in the context of malignant melanoma, a tumor originating from neuroectodermal melanocytes. We showed that recoverin mRNA and protein are frequently expressed in melanoma tissues and cell lines, and serum autoantibodies against recoverin can be detected in melanoma patients. In contrast, four patients suffering from melanoma-associated retinopathy did not show serum antibodies against recoverin. Using two mouse melanoma models, we confirmed that recoverin can be expressed in spontaneous developing melanoma and can induce an autoantibody response. Besides this, we could show for the first time that recoverin is expressed in healthy skin. This expression was detected in melanocytes but not in keratinocytes and merely at mRNA levels, but not at protein level. Thus, we suggest recoverin as the first member of a new class of tumor antigens called cancer-retina antigens. Furthermore, we hypothesize a molecular transition of recoverin expression from mRNA in melanocytes to protein in melanoma.

We have previously shown, that the photoreceptor genes rhodopsin and arrestin can be expressed in melanoma cells, as well, and can be recognized by serum antibodies from melanoma patients (Int. J Cancer, 2005). It remains to be elucidated, what further proteins belong to this group, what controls the expression of cancer-retina antigens, and whether there is a functional impact of cancer-retina antigens on melanoma cancerogenesis, a disease clearly linked to UV-exposure.

P172 (V28)

Identification and characterization of mouse Survivin peptides *in vitro* and *in vivo*

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Therapeutic vaccinations have used peptides derived from tumor-associated antigens for a long time. However, these peptides are often restricted to certain kinds of tumors and can be lost during further cancer development.

Survivin, a universal tumor antigen is expressed in a large variety of cancers and in tumor-associated endothelium. In humans, Survivin-derived peptides are currently used for vaccinations in the treatment of melanoma, pancreatic, colon and cervical cancer. However, Kb-binding peptides for melanoma models in mice do not exist.

We have identified two peptides derived from murine Survivin by searching for potential binding peptides. *In vitro*, these peptides bind equally well to Kb as compared with a strong binding peptide derived from mouse MAGE. To analyse the ability to induce CTLs *in vivo*, we vaccinated mice twice with peptide-loaded dendritic cells (DCs). After subcutaneous challenge with living syngeneic melanoma cells, control mice receiving DCs loaded with an irrelevant peptide developed fast growing tumors. However, tumors of mice vaccinated with MAGE-peptide-loaded DC showed a significant reduction of growth. To analyse specific CTL activity of vaccinated mice *in vitro*, we used restimulated bulk CTL in a cytotoxicity assay. Mice vaccinated with Survivin-peptide-loaded DC showed specific lysis of melanoma cells while syngeneic fibroblasts were not killed.

In summary, we describe two peptides derived from mouse Survivin protein binding to Kb which can be used to induce antitumor immunity and therefore are a valuable tool to improve vaccination strategies *in vivo*.

P173

The disintegrin-cystein domain of a disintegrin and metalloproteinase-9 is cell adhesive and induces cell signalling

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The family of A disintegrin and metalloproteinase (ADAM) consists of proteins composed of metalloproteinase and disintegrin-cysteine domains, therefore displaying simultaneously enzymatic and adhesive functions. The multifunctional aspects of these proteases have suggested a pivotal role for these proteins in a variety of physiological and pathological processes. Between the ADAM members, we have previously shown that ADAM-9 is strongly expressed by several melanoma cells of various invasive abilities. *In vivo*, in human melanoma specimen ADAM-9, expression is strong at the tumor-stroma border where direct or indirect interactions between tumor cells and fibroblasts occur. As the role of ADAM-9 in the peritumoral areas is unclear, we have used an *in vitro* approach to gain more insight into the adhesive function of this protein. To this end, we have produced the recombinant disintegrin-cysteine domain of this protein in eukaryotic cells. Melanoma cells of various invasive abilities and human primary fibroblasts from different donors adhered to immobilized recombinant protein. The adhesion was dependent on the presence of Mn^{2+} thus suggesting an integrin-mediated process. Inhibition studies with either inhibitory antibodies or cyclic peptides showed that adhesion was mediated by β1 integrin receptors and independent

of the RGD recognition motif. Cell recognition of this recombinantly expressed domain occurred not only when the protein was immobilized to an underlying surface but also when supplied in soluble form. Interestingly, binding of soluble recombinant disintegrin-cysteine domain to high but not low invasive melanoma cells resulted in an enhanced *de novo* synthesis of pro-MMP-2, whereas in human dermal fibroblasts, in addition to MMP-2 also a moderate induction of MMP-1 was observed.

In summary, these results indicate that ADAM-9 synthesis in melanoma is likely to contribute to increased MMP production and might be involved in increased proteolysis of extracellular matrix during tumor invasion of malignant melanoma.

P174

B-RafV600E mutation in malignant melanoma is associated with increased expressions of brain and acute leukaemia, cytoplasmatic and FYN

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Activating mutations of the protooncogene B-Raf are present in approximately 50‰ of melanomas, and the majority of these mutations are of the V600E type. Although different downstream target genes of the V600E mutant have been identified, the contribution of activating B-Raf mutations to malignant transformation needs to be further clarified. Microarray gene analyses were performed for human melanoma cell lines harbouring B-RafV600E mutations in comparison with cell lines without mutations in this genomic section. Microarray gene analysis revealed a more than twofold down-regulation of 43 and an increase of 39 gene products in B-RafV600E mutated melanoma cell lines. Brain and acute leukaemia, cytoplasmatic (BAALC) and FYN, were constantly up-regulated in mutated cell lines by a mean of 11.45 and 2.25-fold, respectively. Confirmation assays using real-time polymerase chain reaction analyses for these two newly identified, putative target genes, confirmed their up-regulation in cell lines characterized by a constitutive activation of B-Raf ($n = 28$). *Ex vivo* analysis of melanoma specimens further confirmed the B-Raf activation dependent up-regulation of BAALC. Yet, bulk analyses of tumor specimens did not reveal a significant correlation of B-Raf activation and FYN mRNA expression. Detailed analysis discriminating tumor and stromal cells, however, indicated a strong expression of Fyn protein in tumors harbouring B-RafV600E mutations.

In conclusion, we identified two genes, BAALC and FYN, up-regulated by activating B-Raf mutations, which have been associated with cell dedifferentiation and cell migration; thus, they may function as downstream effectors during melanoma genesis.

P175

Effects of melanoma growth in mice on numbers and functions of Treg

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In different kinds of cancer, it has been shown that the number of $CD4^+CD25^+$ regulatory T cells is enhanced in the peripheral blood as well as in the tumor itself. Their suppressive capacity was demonstrated *in vitro*; however, their *in vivo* function as well as their contribution to tumor growth is largely unclear.

To investigate whether spontaneously developing melanomas lead to expansion or activation of regulatory T cells, we analysed the $CD4^+CD25^+$ regulatory T cells in spleens, lymph nodes and tumors from melanoma-bearing mice.

Therefore, C57BL/6 mice were challenged subcutaneously with $\times 1$ 105 B16 cells and were killed when the medium size of the tumor reached approximately 5 mm^3. Tumor tissue, spleen and draining LN's were removed and either used for immunofluorescence staining on cryosections or for FACS analysis on the isolated T-cell subpopulations. Analysis of the spleen, the LN and the tumors of the B16-treated mice showed that Treg were found in all organs examined using the characteristic markers for regulatory T cells, i.e. CD4, CD25 and Foxp3. However, when the number of tissue-resident Treg was determined, no differences in Treg frequencies were detectable in tumor-bearing animals compared with control mice. Moreover, Treg derived from spleen as well as from lymph node suppressed allo-MLRs equally well, indicating no significant functional difference in Treg derived from tumor-bearing mice vs. control mice. To further expand our studies on natural-occurring tumors, we used the RET-mouse strain that spontaneously develops melanomas. Similar to transplanted B16 tumors, we could not detect different numbers of $CD4^+CD25^+$ Treg in either spleen, lymph node or tumor tissue in tumor-bearing mice vs. controls. Thus, this data indicate that early developing melanomas have no effects on Treg distribution or homing to lymphoid organs.

P176 (V01)

Mutant CDK4 and aberrant hepatocyte growth factor signalling synergistically promote rapid induction of carcinogen-induced metastatic melanoma

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Background/aim: Novel mouse models of melanoma are being generated which recapitulate genetic changes observed in the human disease. Mice carrying a mutant CDK4 as a dominant oncogene, which has been found in rare cases of familial and sporadic melanoma, develop slowly growing melanocytic tumors in the dermis following carcinogen treatment with DMBA which do not metastasize. Here, we investigated whether CDK4 mutant mice additionally overexpressing the hepatocyte growth factor (HGF), which supports an autocrine c-met-signalling loop characteristic for metastatic melanoma, would develop more rapidly growing melanocytic neoplasms with the ability to metastasize.

Methods: Newborn HGF-tg C57BL/6 mice with wildtype CDK4 or with heterozygous or homozygous mutant CDK4 were treated with 0.2 mg DMBA at day 4 after birth followed by twice weekly TPA beginning 3 weeks later. The appearance and size of tumors developing in the skin were recorded macroscopically over time and subsequently analyzed using histopathology.

Results: Carcinogen treatment of HGF-tg mice induced multiple slowly growing melanocytic nevi and around 10 melanomas within 6 months. HGF-tg mice heterozygous for mutant CDK4 showed around 30 melanomas and lived until around week 16. HGF-tg mice homozygous for mutant CDK4 showed on average around 50 rapidly growing melanomas within the first few weeks of life and had to be sacrificed at the age of 12 weeks. Importantly, melanomas in HGF-tg CDK4-mutant mice spontaneously metastasized to the lymph nodes and the lung. Histopathological examinations revealed striking similarities with human metastatic melanomas. Apart from very few papillomas in the skin, tumors of other histology were not observed.

Discussion: Our results show that loss of cell cycle control due to mutant oncogenic CDK4 and aberrant signal transduction due to overexpression of HGF synergistically promote the rapid development of wide-spread carcinogen-induced metastasizing melanomas in C57BL/6 mice in the absence of other tumors. This new experimental mouse model can now be exploited to further study the pathogenesis of melanoma and evaluate new treatment modalities for this deadly disease.

P177

Expression of BRN2, osteopontin and nestin in melanoma and nevi. New markers of tumor progression?

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It has been shown recently that the POU domain transcription factor BRN2 is involved in neuronal differentiation of the developing nervous system and is overexpressed in melanoma cell lines. Due to the neuroectodermal origin of melanocytes, BRN2 may be implicated in the development of melanoma. However, there have been no reports on its expression in melanoma tissues. It could be shown that interactions between BRN2 and the intermediate-filament nestin regulate neural primordial cells and that nestin is also expressed in melanoma. Another possible target gene involved in BRN2-associated melanoma formation is osteopontin (OPN), a multifunctional extracellular matrix protein, which mediates cell adhesion, migration and chemotaxis and is highly expressed in different tumors including melanoma.

In this study, the protein expression of BRN2, OPN and nestin was analyzed in melanomas of different progression stages and in melanocytic nevi. The differential transcription levels of BRN2 and nestin were analyzed using quantitative real-time polymerase chain reaction (PCR) in melanoma cell lines and melanocytes.

Immunohistochemical analysis showed BRN2 protein expression in 34 of 48 (71%) primary melanomas and seven of 15 (47%) metastatic lesions. OPN protein was expressed in 26 of 34 (77%) primary and in seven of 14 (50%) metastatic melanomas. Nestin was expressed in 25 of 40 (63%) primary and in nine of 15 (60%) metastatic melanomas. Highest expression levels for BRN2 (90%), OPN (86%) and nestin (78%) were found in vertical growth phase melanomas as opposed to radial growth phase melanomas. In nevi, BRN2 expression was detected in 65% (15 of 23). OPN was found in six of 19 (32%) investigated nevi. Nestin staining was weak or absent in nevi (two of 18 (11%). There was no correlation between protein expression and dysplasia of nevi. Quantitative PCR analysis demonstrated high expression levels of BRN2 and nestin mRNA in melanoma cell lines.

In conclusion, BRN2, OPN and nestin are highly expressed in melanoma and seem to be associated with vertical growth. OPN and nestin might be associated with melanoma progression, because the levels in primary melanomas are increased when compared with melanocytic nevi.

P178

No discernible pattern of mitochondrial DNA deletions in basal and squamous cell carcinoma samples isolated by laser capture microdissection

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Mutations of mitochondrial (mt) DNA have been shown to be increased in a number of tumors including breast cancer, prostate cancer, colon carcinoma, as well as head and neck cancer. We have previously shown that mtDNA mutations are increased in chronically ultraviolet radiation (UV)-exposed skin. Therefore, we employed an established real-time polymerase chain reaction technique (PCR) to investigate whether mtDNA deletions are increased in histological samples from patients with basal (BCC) and squamous cell carcinoma (SCC) as compared with adjacent non-tumor but chronically UV-exposed skin of the same individual. Tumor and non-tumor samples were confirmed by an independent pathologist, hematoxylin eosin stained and then laser capture microdissected with a PixCell® IIe Laser-Capture Microdissection (LCM2105) System device to avoid non-tumor contamination. Subsequently, DNA extraction and real-time PCR for the 4977 bp common deletion, considered to be a marker for mtDNA deletions, was carried out in 24 BCC and 22 SCC. Of these samples, 19 showed a decrease, five an increase for BCC as well as 18 with a decrease and four with an increase for SCC with maximum and minimum 2-ΔΔ ct values. These values ranged from 0.013 to 7.396 for BCC and from 0.006 to 6.964 for SCC, respectively, where values below one indicate a decrease and values above one an increase compared with control samples. Furthermore, there was no influence of age and gender of the investigated patients. Thus, these findings indicate that there is no discernible pattern of large mtDNA deletions in BCCl and SCC.

P179

High-throughput tissue microarray analysis of methylthioadenosinephosphorylase expression in melanocytic skin tumors

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Purpose: To investigate whether protein expression of methylthioadenosinephosphorylase (MTAP) is associated with clinico-pathologic characteristics in benign and malignant melanocytic skin tumors.

Experimental design: Tissue microarrays were used to analyze MTAP expression and the Ki-67-labeling index immunohistochemically. Cytoplasmic MTAP expression was scored semiquantitatively (0 to 2+).

Results: MTAP protein expression of any intensity (1+ to 2+) was detected in 72% (227/315) of informative cases. MTAP expression was significantly reduced in malignant melanomas ($P < 0.001$) and melanoma metastases ($P < 0.001$) compared with benign nevi. Between melanomas and metastases no difference in MTAP expression could be shown. In primary melanomas, a low Ki-67-labeling index <5% was associated with MTAP protein expression ($P = 0.04$) suggesting that loss of MTAP expression is associated with proliferation. None of the other factors was significantly related to MTAP expression. Interestingly, lymph node metastases demonstrated a significantly higher MTAP expression compared with skin metastases ($P = 0.01$). If all patients were jointly analyzed, MTAP expression was not associated with prognosis. In an explorative subgroup analysis, considering 26 patients with MTAP positive (1+ to 2+) melanomas and recorded tumor recurrence, patients with interferon (IFN) therapy ($n = 18$) had a significant benefit ($P = 0.009$) compared with patients without IFN treatment ($n = 8$). This was not seen in the group of patients with MTAP-negative tumors.

Conclusions: MTAP protein expression could be a predictive marker of therapy resistance in melanoma patients with disease progression and treated with IFN.

P180

Simultaneous blockade of the mitogen-activated protein kinase- and AKT-signaling pathways abrogates invasive tumor growth in malignant melanoma

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Malignant melanoma is a highly aggressive tumor which is widely resistant to current medical treatments. Therefore, new strategies

for the medical treatment of melanoma have to be developed. We examined whether targeting the RAS/RAF/mitogen-activated protein kinase kinase/extracellular signal-regulated kinase (RAS/RAF/MEK/ERK) and the phosphatidyl 3′-kinase/AKT (PI3K/AKT)-signaling pathways would have therapeutic activity against melanoma. In particular, we examined the effects of the RAF kinase inhibitor BAY 43-9006 and the PI3K inhibitor wortmannin in monolayer and organotypic cultures of metastatic melanoma cells on the biological functions of invasive tumor growth, proliferation, and survival/apoptosis. Our results show that (1) the RAF kinase inhibitor BAY 43-9006 affects both melanoma cell proliferation and survival, reduces invasive tumor growth and down-regulates the expression of the adhesion molecule β3 integrin; (2) the PI3K inhibitor wortmannin does not affect proliferation and survival but enhances the proapoptotic effect of BAY 43-9006, decreases invasive tumor growth and down-regulates the expression of the adhesion molecule MelCAM; and (3) the combination of BRAF kinase inhibitor BAY 43-9006 and PI3K inhibitor wortmannin results in down-regulation of both the adhesion molecules β3 integrin and MelCAM and completely suppresses invasive melanoma growth with very few apoptotic melanoma cells scattered throughout the dermis. The data of these studies suggest that the simultaneous blockade of the RAS/RAF/MEK/ERK and the PI3K/AKT-signaling pathways is a promising strategy for the effective medical treatment of melanoma.

P181

New insights into the molecular basis of the antitumoral activity of resveratrol

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Given that the so-called 'french paradoxon' (i.e. the comparatively low incidence of cancer in french wine growers) has been attributed, at least in part, to resveratrol, a hydroxystilbene naturally occurring in grapes, this compound has received considerable attention in cancer research. It has been proposed previously that the profound pro-apoptotic activity of resveratrol towards various malignant tumors is dependent on its metabolization into piceatannol by CYP1B1, a cytochrome P450 isozyme that is almost exclusively expressed by some tumor cells.

In this study, we have investigated the mode of action of resveratrol in melanoma cells. To our surprise, it was found that the supposedly active metabolite piceatannol did not induce apoptosis in four different melanoma cell lines as assessed using DNA fragmentation as well as annexin V-staining. In contrast, exposure to resveratrol resulted in marked induction of apoptosis in melanoma cells. Interestingly, the susceptibility to resveratrol-induced apoptosis did not correspond to the expression of CYP1B1, suggesting CYP1B1-independent mechanisms of apoptosis in these cells.

Using an *in vitro* system with regenerating $NADPH/NADP^+$, we could show that not only CYP1B1 but also CYP1A1 and CYP3A4 have the capacity to metabolize resveratrol. As detected using MS/MS tandem mass spectrometry, the activity of the latter two isozymes was even markedly higher than that seen with CYP1B1.

We have then tested the hypothesis that resveratrol is metabolized into piceatannol via reactive intermediates. Indeed, when defined amounts of acetyl cystein (ACC) were added to the CYP assays, resveratrol adducts were readily detected using HPLCMS/MS indicating the formation of resveratrol-1,3-diketone as an extremely reactive intermediate.

Overall, these results suggest a novel mode of action of resveratrol inasmuch as reactive resveratrol intermediates may generate protein or nucleic acid adducts, thus resulting in the cytostatic or pro-apoptotic activity observed on the cellular level.

P182

Construction of a multifunctional tetracycline-regulated vector for gene transfer

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Cancer gene therapy using pro-apoptotic genes has been extensively tested in the pre-clinical setting. Although, a variety of techniques and vectors has been developed to target toxic genes to cancer cells, the inability of these vectors to control the expression of suicide genes makes them not viable for clinical application. Here, we report the construction of a novel shuttle vector for laboratory research and clinical application. We designed a multifunctional- and bicistronic single vector based on the tetracycline-regulated (tet-off) expression system. Specificity of gene expression was achieved using the human telomerase promoter. We analyzed the function of this vector in HeLa cells using fluorescence microscopy and reverse transcription-polymerase chain reaction. The detection of green fluorescent protein (GFP) expression in HeLa cells transiently transfected with the bicistronic GFP cultured in tetracycline-free medium suggested the ability of this bicistronic system to drive gene expression in eukaryotic cells. Furthermore, we could tightly regulate the induced expression of GFP by varying the tetracycline concentration in various cell lines including several melanoma cell lines. We also demonstrated that the GFP-induced expression by the bicistronic system is comparable with those induced by the respective binary system (cotransfection). Our data provide evidence for the ability of the bicistronic vector to drive and to regulate gene expression at a level comparable with that induced by the binary system. Therefore, this vector may become a useful gene delivery system for laboratory research and clinical application.

P183

Chemokine receptors in head and neck cancer: association with metastatic spread and regulation during chemotherapy

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Head and neck carcinomas are histologically and clinically heterogeneous. While squamous cell carcinomas (SCC) are characterized by lymphogenous spread, adenoid cystic carcinomas (ACC) disseminate preferentially hematogenously. To study cellular and molecular mechanisms of organ-specific

metastasis, we used SCC and ACC cell lines and tumor tissues obtained from patients with primary or metastatic disease. Comprehensive analysis at the mRNA and protein level of human chemokine receptors showed that SCC and ACC cells exhibited distinct and non-random expression profiles for these receptors. SCC predominantly expressed receptors for chemokines homeostatically expressed in lymph nodes, including CC chemokine receptor (CCR) 7. No difference in expression of chemokine receptors was seen in primary SCC and corresponding lymph node metastases. In contrast to SCC, ACC cells primarily expressed CXC chemokine receptor (CXCR) 4. In chemotaxis assays, ACC cells were responsive to CXCL12, the ligand for CXCR4. Exposure of ACC cells to cisplatin resulted in up-regulation of CXCR4 on the cell surface, which was repressed by the transcriptional inhibitor α-amanitin. Treatment of ACC cells with CXCL12 resulted in the activation of Akt and ERK1/2 pathways. Furthermore, CXCL12 suppressed the rate of apoptosis induced by cisplatin in ACC cells, suggesting that signaling via CXCR4 may be part of a tumor cell survival program. Discrimination of the chemokine receptor profile in SCC and ACC *in vitro* and in tissues provided insights into their distinct biologic and clinical characteristics as well as indications that chemokine receptors might serve as future therapeutic targets in these malignancies.

P184

CCR10 and CXCR4 regulate tumor progression and metastasis of malignant melanoma

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Background: Malignant melanoma is an aggressive malignant tumor, and its high tendency to metastasize represents the major survival-limiting factor.
Methods: To investigate the role of chemokine receptors in melanoma progression and metastasis, we analyzed cell lines as well as tissue samples of primary melanoma tumors and metastases using quantitative real-time-polymerase chain reaction, DNA microarrays, and immunohistochemistry. The regulation of chemokine receptors by factors of the local microenvironment was assessed and their *in vivo* functional role was determined in two melanoma xenograft models.
Results: Here, we show that melanoma cells preferentially express two chemokine receptors, CXCR4 and CCR10. CXCR4 expression directly correlates with the invasive stage of the primary tumor, is induced by transforming growth factor (TGF)-β1 and shows enhancement at the invasive tumor front. Notably, growth factors such as bFGF, EGF, and TGF-β1, or the proinflammatory cytokines TNF-α and IL-1fÒ induce chemokine receptor expression using melanoma cells. *In vivo*, neutralization of CXCL12/CXCR4 interaction results in significant suppression of the development of lung metastases. Clinically, melanoma is characterized by a high frequency of skin metastases which correlates with the abundant expression of CCR10, a receptor binding to the skin-specific chemokine CCL27. Cutaneous and subcutaneous melanoma metastases exhibit significantly increased levels of CCR10 suggesting the selection of CCR10 high-expressing melanoma cells. In addition to the induction of migration and invasion, CCL27 also enhances melanoma cell proliferation *in vitro* and *in vivo*.
Conclusion: Taken together, our findings strongly suggest that specific chemokine receptors are regulated by the tumor microenvironment and play critical roles in melanoma progression and metastasis.

P185

Decreased levels of proapoptotic Bcl-2-related multidomain proteins Bax and Bak in primary melanoma are associated with worse patient prognosis

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Prognosis of primary melanoma presently is based on morphological parameters such as tumor thickness. Immunohistology is applied to demarcate melanomas from other skin lesions. However, reliable prognostic markers are needed to allow a better stratification of patients with regard to therapeutic options. Resistance of cancer cells to therapy and to the immune response may result from defects in signalling cascades leading to apoptosis. High expression of antiapoptotic Bcl-2 proteins has been attributed to chemoresistance in melanoma, and we have shown that the ratio of Bax (a proapoptotic Bcl-2-related protein) to Bcl-2 was decisive for melanoma cell susceptibility to apoptotic signals. To clarify the suitability of pro- and antiapoptotic Bcl-2 proteins as prognostic markers for melanoma, we performed a retrospective study on patients with primary superficial-spreading melanoma (SSM, $n=24$) or nodular melanoma (NM, $n=16$) of 1.5–4 mm tumor thickness. Twenty patients had survived the follow-up of 10 years, whereas the other 20 patients developed metastases. Tumor sections were analysed by immunohistology for expression of Bcl-2-related proteins (antiapoptotic: Bcl-2, Mcl-1; proapoptotic: Bax, Bak, Bok). In parallel to the Bcl-2 proteins, we analysed components of the intrinsic or extrinsic proapoptotic pathways and two regulators of the cell cycle. Evaluation revealed a correlation between reduced expression of Bax and Bak in melanomas and worse patient prognosis. In SSM, high Bax was associated with 10-year survival rates of 73%, whereas low Bax resulted in only 38% survival. High Bak (SSM or NM) was associated with a 10-year survival of 62%, whereas low Bak resulted in only 18% survival. Down-regulation of Bax and Bak in melanomas with worse patient prognosis was confirmed in a second set of tumors. Regulators of apoptosis may be candidates for independent prognostic markers in melanoma. The study also underlines the role of the mitochondrial apoptosis pathway and of proapoptotic Bcl-2-related proteins for therapy resistance and melanoma progression.

P186

Bcl-xAK, a novel Bcl-x splice product without BH3 domain, triggers apoptosis in human melanoma cells

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Pro- and antiapoptotic proteins of the large Bcl-2 family are critical regulators of apoptosis via the mitochondrial pathway. Whereas antiapoptotic proteins of the family share all four Bcl-2 homology

domains (BH 1–4), proapoptotic members may lack some of these domains, but all so far described proapoptotic Bcl-2 proteins enclose BH3. The *bcl-x* gene gives rise to several alternative splice products resulting in proteins with distinct functions as the antiapoptotic Bcl-xL with all four BH domains and the proapoptotic Bcl-xS with only BH3 and BH4. In stably transfected human melanoma cell lines, we have shown previously that overexpression of Bcl-xS was sufficient to induce apoptosis, whereas overexpression of Bcl-xL had no proapoptotic effect. We have found a novel Bcl-x splice product of 138 amino acids termed Bcl-x atypical killer (AK) which encloses the Bcl-2 homology domains BH2 and BH4 as well as the transmembrane domain, but lacks BH1 and BH3. Weak endogenous expression of Bcl-x AK was seen in melanoma and other tumor cells. Interestingly, its overexpression by applying a tetracycline-inducible expression system resulted in significant induction of apoptosis in melanoma cells, which occurred in synergism with drug-induced apoptosis. After exogenous overexpression, Bcl-xAK was localized both in mitochondrial and in cytosolic cell fractions. By these findings, a completely new class of Bcl-2-related proteins is introduced which promotes apoptosis independently from the BH3 domain and implies additional, new mechanisms for apoptosis regulation in melanoma cells.

P187

The effects of arsenic trioxide on cutaneous T-cell lymphoma cells *in vitro* and on artificial Mycosis fungoides tumors *in vivo* in a mouse model

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Mycosis fungoides (MF) and its leukemic variant Sézary syndrome (SS) are the most frequent types of cutaneous T-cell lymphomas (CTCL). They progress from a patch/plaque stage to a tumor stage that often kills the patients. Until now there is no cure for these diseases. SS patients have been treated systematically with arsenic trioxide with little success, as too little arsenic trioxide reached the skin.

MF and SS cell lines undergo apoptosis when they are treated with arsenic trioxide. Using Western blotting and electrophoretic mobility shift assay, we found that arsenic trioxide suppresses the expression of the apoptosis preventing bcl-2, bcl-xL, and Mcl-1 genes. T also inhibited the DNA-binding activities of the NFκB and STAT5 transcription factors that increase the expression of apoptosis preventing genes including bcl-2.

When we injected artificial MF tumors that had been derived from MyLa 2059 cells injected into nude mice, we found that arsenic trioxide concentrations of 0.25 and 1 mM injected into the tumors caused partial remission in seven of eight mice and one complete remission.

Therefore, we conclude that arsenic trioxide induces apoptosis of MF and SS cells by the inhibition of the expression of apoptosis-preventing genes. This may occur by the inhibition of transcription factors that stimulate the expression of apoptosis-preventing genes. Arsenic trioxide in higher concentrations may be also suitable for the local treatment of MF tumors.

P188

A novel three-dimensional organ construct to study squamous cell carcinoma of the skin *in vitro*

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Squamous cell carcinoma (SCC) of the skin is a malignant neoplasm that occurs in all ethnic groups primarily because of chronic sun exposure. It constitutes a major health problem worldwide, and its incidence is increasing. There is currently no reliable human system for the *in vitro* study of the dynamics of SCC tumor growth and for assessing, e.g. the efficacy of innovative therapeutic strategies. For this reason, we have developed and characterized a novel three-dimensional human SCC construct with which to assess tumor growth and to evaluate the mechanism of action of a modified form of photodynamic therapy (PDT) for treating this type of malignant neoplasm.

SCC cells were seeded onto the surface of a three-dimensional skin equivalent. Tumor growth and invasion was studied histologically and by immunohistochemistry using different markers. After generation of a SCC-like construct *in vitro*, the therapeutic effects of a modified form of PDT using δ-aminolevulinic acid followed by exposure to a light source were studied.

Within 5 days an SCC-like lesion developed *in vitro*. Immunohistochemical characterization revealed loss of epidermal and dermal differentiation, an increased proliferation rate and, within tumor areas, the expression of markers specific for cutaneous SCC. Furthermore, typical signs resembling tumor invasion were observed, e.g. the destruction of the basement membrane. Utilization of a modified form of PDT revealed tumor regression with apoptosis and necrosis within 5 days.

In this study, we demonstrated the feasibility of creating a fully functional three-dimensional SCC-like construct of human skin suitable for the study of tumor development and dynamics *in vitro*. Histological and immunohistochemical characterization of our novel *in vitro* disease model showed features closely resembling human SCC *in vivo*. Using a modified form of PDT, we were able to demonstrate the utility and possibilities of our construct as a potential screening system for the development of new treatment strategies for this common type of epidermal neoplasm.

P189

Melanoma-derived matrix metalloproteinase-1 promotes thrombus formation and cell adhesion via endothelial PAR1

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As a hallmark of malignant tumors, cancer cells leave their original site and migrate via the vascular system to settle metastases. A critical step in this process is extravasation, the ability of circulating tumor cells (TCs) to adhere to and pass the endothelium as the physiological barrier between blood and tissue. This multistep process requires efficient communication between TCs and endothelium.

In the present study, we analyzed melanoma-derived soluble factors interacting with endothelial thrombin receptor (PAR1), the principal mediator of endothelial cell activation. We find that soluble matrix metalloproteinase (MMP)-1 canonically cleaves endothelial PAR1 receptors displayed by calcium fluxes and acute release of von Willebrand factor (+230%, $n = 12$), a core protein for the initiation of thrombus formation. We further observe that melanoma cell lines (A375, WM9, A7) secrete soluble agonists that induce an acute prothrombotic, proinflammatory and cell adhesive endothelial surface. By specific antagonism of endothelial PAR1 and melanoma-derived soluble MMP-1, we demonstrate a successful interference in the process of tumor-derived initiation of thrombosis and cell adhesion. These findings demonstrate a so far undescribed pathway of tumor-endothelial crosstalk via an intravascular MMP1/PAR1 axis and might serve as a future target for therapeutical prevention of tumor-derived thrombosis.

P190

Gene expression analysis of proteinase-activated receptor-2-deficient and wildtype mouse papillomas

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Proteinase-activated receptors (PARs) play an important role in the tumorigenesis of various tissues. PAR-2 is a G protein-coupled receptor which can be activated by specific proteolytic cleavage of the extracellular N-terminus. The truncated N-terminus then interacts with the second extracellular loop of the receptor, leading to receptor activation. PAR-2 is strongly expressed in epidermal keratinocytes and is known to inhibit keratinocyte proliferation *in vitro*. Moreover, PAR-2 formation is down-regulated in dedifferentiated epidermal skin tumors. We could show that in a chemically induced skin tumor model, PAR-2-deficient mice developed fivefold more tumors as compared with the wildtype. Histological analysis showed that these tumors were mainly benign papilloma-type tumors. At later stages, squamous cell carcinomas were also observed. However, no differences regarding keratinocyte differentiation and apoptosis could be observed in these papillomas. To further analyze potential reasons for the facilitated tumor growth in PAR-2-deficient animals, we studied differences in papilloma gene expression of both groups by quantitative reverse-transcription polymerase chain reaction. We found that the expression of certain chemotactic cytokines and IL-1α were up-regulated in PAR-2-deficient mouse papillomas. IL-1α is known to promote the expression of various growth factors and angiogenic factors. Together with the up-regulation of pro-inflammatory cytokine expression, this leads to the conclusion that in PAR-2-deficient mice, immune defense mechanisms of the skin, i.e. against papilloma viruses or transformed cells might be impaired, thus facilitating epidermal tumor formation and progression.

P191

Biology of human natural killer cells in melanoma patients

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Human natural killer (NK) cells comprise approximately 15% of all circulating lymphocytes and are defined phenotypically by their expression of CD56 and lack of expression of CD3. Thanks to their early production of cytokines and chemokines, and ability to lyse target cells without prior sensitization, NK cells are crucial components of the innate immune system.

Numerous reports show alterations in NK-cell cytotoxicity in advanced stages of different malignancies including melanoma as tested *ex vivo* against different cell lines.

Only few NK cells are found in melanoma metastases that may indicate an inefficient homing to malignant tissues.

We conducted a study to investigate potential deficiencies of NK cell functions in melanoma patients. Peripheral blood mononuclear cell of 12 melanoma stage IV patients without previous chemotherapy and six healthy donors were analyzed using flow cytometry for differences in expression of molecules involved in NK-cell activation (NKp46, NKp44, CD44), inhibition (CD85j, NKG2a, KIR receptors), homing (chemokine receptors, CD62L, LFA-1, CD2), killing (Perforine, Granzyme A, TNF-α family ligands) and apoptosis (TRAIL receptors).

We found elevated expressions of KIRs and NKG2a inhibitory receptors, suggesting a role of non-classical human leukocyte antigen (HLA)-I molecules such as HLA-G, ligand of KIR2DL4, in NK-cell inhibition and impairment of NK-killing machinery especially reduced expression of Perforine and Granzyme A in melanoma stage IV patients compared with the healthy donors.

The expression of TRAIL and CD40L was slightly and TRAIL-R3 receptor strongly increased. Expression of CXCR3 and LFA-1 that are involved in homing to peripheral tissues was reduced.

Our analyses show that NK cells in advanced melanoma patients are functionally impaired. Especially they lack the crucial effector molecules for inducing cytolysis and have reduced levels of tissue homing receptors which can explain their absence in the tumor.

Nevertheless, increased expression of CD40L, TRAIL and TRAIL-R3 suggests that they have been activated and may be resistant to apoptotic triggers.

P192

Cyclodextrin-induced apoptosis in HaCaT keratinocytes

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Introduction: Cyclodextrins (CDs) are cyclic oligosaccharides bound by α-(1,4) glycosidic bonds. α-CD are comprised of 6, β-CD of 7 and γ-CD of 8α-D(+)-glucopyranose units. CDs form stable rings with a hydrophobic interior, which allows the formation of inclusion complexes with a variety of chemical substances stabilized by hydrogen bonds and hydrophobic forces. In this way a lot of organic compounds can be included. Such kind of ligand-receptor complexes can be used for different applications, e.g. for transdermal therapeutic systems. Until now, there is only little known about cytotoxic effects of CDs on human skin cells. Here, we provide evidence that β-CD and methyl β-CD activate caspases (−8, −3/−7) and induce apoptosis in human keratinocytes (HaCaT).

Material and methods: CDs were purchased from the Wacker Chemie GmbH (Munich, Germany). HaCaT keratinocytes were grown in 75-cm^2 cell culture flasks and maintained in Dulbecco's modified Eagle's medium at 37 °C in a humidified atmosphere containing 5% CO_2. For experiments, cells were seeded in 96-well flat-bottomed microplates. After 48 h, the cell culture medium was replaced by either fresh DMEM (negative control) or CDs dissolved in DMEM. After 24 h, the activity of caspases was determined using the Caspase-Glo Assay kits specific for caspase 8 and caspase 3/7 (Promega GmbH, Mannheim, Germany).

Results: Incubation with 1% (w/v) β-CD leads to a 2.5-fold increase in caspase-8 activity compared with control cells. Also, methyl-β-CD activates caspase-8 significantly. Similar results were found for caspases 3/7. However, the enhancement of the enzyme activities was even more pronounced. Incubation with 1% (w/v) β-CD or methyl-β-CD stimulates nearly a 50-fold higher caspase 3/7 activity than the control medium. In contrast, α-CD, γ-CD and their chemically modified derivates were well tolerated by HaCaT cells. In cells which were pretreated with the caspase-8 inhibitor Ac-IETD-CHO (Biomol, Germany) prior to incubation with 1% (w/v) β-CD or methyl-β-CD, significantly reduced activities of caspase-8 as well as -3/-7 were observed. This indicates an involvement of caspase-8 in CD-induced caspase-3/-7 activation.

Conclusion: CDs and their derivatives were widely used in the pharmaceutical field. As the presented data show, β-CD and methyl-β-CD are able to trigger apoptotic cell death. The potential to induce cellular damage should be taken into consideration for applications of CDs in clinical dermatology.

P193

Evidence for expression of the melanocortin-1 receptor and an immunoregulatory role of α-melanocyte-stimulating hormone in human basophils

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The neuropeptide α-melanocyte-stimulating hormone (α-MSH) elicits its pleiotropic biological activity via binding to melanocortin receptors (MC-Rs) that exhibit a cell- and tissue-specific expression. We investigated whether human basophils, a cell type crucially involved in pathogenesis of the late-type tissue response in type I allergies are target cells for α-MSH. Using reverse transcription-polymerase chain reaction analysis and specific primers against all known MC-R subtypes, we show for the first time that the human basophil cell line KU812 constitutively expresses transcripts for MC-1R but not for any other MC-R subtype. MC-1R expression on the cell surface of these cells was confirmed by FACS analysis using an antibody directed against the amino acids 2–18 of the extracellular N-terminal domain of human MC-1R. Double staining and FACS analysis with an antibody against immunoglobulin E or the basophil-activation marker CD203c in combination with the anti-MC-1R demonstrated MC-1R surface immunoreactivity also in basophils of peripheral blood samples derived from healthy human volunteers. Interestingly, and in contrast to most cutaneous human cell types, KU812 cells lacked expression of proopiomelanocortin, the precursor for α-MSH. The MC-1Rs as detected in KU812 cells were functionally active as α-MSH treatment resulted in a significant and dose-dependent intracellular cAMP response. Moreover, α-MSH suppressed the expression of IL-4 mRNA induced by calcium ionophore A23187 in KU812 cells but not the expression of IL-5, IL-6 and IL-13 induced by the above stimulus or phorbol myristyl acetate. Our preliminary data highlight a novel biological activity of α-MSH in human basophils that may be of relevance for both pathogenesis and future therapeutic intervention of allergic diseases.

P194

A high ERK/p38 ratio as well as cisplatin-mediated activation of ERK protect cells from apoptosis in melanoma

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Activation of mitogen-activated protein kinase (MAPK) signal transduction is a common mechanism of many cellular processes with contrasting biological functions like cell survival, division and death. In tumor development and progression, there is a disturbed balance between expression of extracellular signal-regulated kinase (ERK) and p38 stress-activated protein kinase, which are particularly involved in cell proliferation and death. A high ERK/p38 ratio was reported to allow cell proliferation, while a high p38/ERK ratio induced dormancy and cell arrest. In the present study, we examined the ERK/p38 ratio in human melanoma metastases *ex situ* and the effect of epidermal growth factor (EGF) on this ratio and its modulation after treatment with cisplatin, a common antimelanoma drug, as well as in combination with the MAPK inhibitor U0126. We observed a high ERK/p38 ratio in eight of 13 samples of melanoma metastases and in all four investigated melanoma cell lines as well as in normal human melanocytes. Human recombinant EGF further increased the high ERK/p38 ratio in primary melanocytes that were shown to contain EGF receptors and melanoma cell lines. Furthermore, we tested whether *in vitro* treatment with cisplatin can revert the ERK/p38 ratio and induce apoptosis as a consequence of an altered ERK/p38 balance. In contrast to non-apoptotic melanoma cells of metastases, the cisplatin-treated melanoma cell lines A375 and BLM maintained a high ERK/p38 ratio despite significant apoptosis. Cisplatin-mediated apoptosis induced the cleavage of PARP and capase-3 in A375 and BLM cells. Inhibition of the ERK1/2 pathway by U0126 and treatment with cisplatin induced an earlier and stronger cleavage of PARP and caspase-3 in these cells. In A375 cells, cisplatin mediated the activation of ERK1/2 and not p38 that interacts with the tumor suppressor protein p53 as well as with the cell survival protein p90-RSK that became phosphorylated at serin position 6 (p53) and at threonin/serin positions 359/363 as evidenced using Western blot and immunoprecipitation assays. In BLM cells, ERK1/2 activated using cisplatin was shown to interact only with p-90-RSK and not with p53. Our study suggests that a high ERK/p38 ratio in malignant melanoma metastases mediates tumor growth, survival and progression.

P195 (V27)

Knockout of the respiratory chain in keratinocytes shows that functional mitochondria are essential for epidermal differentiation

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To investigate the role of the mitochondrial respiratory chain in epidermal differentiation *in vivo*, we have crossed mice having exons 6 and 7 of the Tfam gene flanked by loxP sites with mice carrying the Cre-recombinase transgene under control of the keratinocyte-specific human K14 promoter. This leads to an epidermis-specific deletion of the Tfam protein controlling transcription and replication of mitochondrial DNA (mtDNA), which contributes essential subunits for the respiratory chain. Tfam is detectable in the epidermis at birth, but older knockout animals show a progressive loss. At postembryonic day 6, the mtDNA-encoded subunit II of cytochrome oxidase is not detectable any more, showing complete loss of respiratory chain function. The epidermis gradually gets thinner, the basal layer is disordered, hair follicles fail to develop normally and there is no pelage at day 6. The knockout mice stop gaining weight at day 3 and die between day 4 and 7. The tongue epithelium is disordered, and papillae show a progressive degradation. The animals develop an ulceration at the back of the tongue, the resulting pain probably inhibiting food intake, leading to the observed weight loss and ultimately death. Considering the proposal by some authors that the epidermis is a physiologically anaerobic tissue, the drastic effect of ablating respiratory chain activity is even more striking. As for now, we are unsuccessful in our attempts to cultivate keratinocytes from these animals. A HeLa cell line without mtDNA used as a model system showed a profound proliferation defect, which is not simply due to energetic or biosynthetic problems, because levels of ATP, UTP, heme-containing proteins and FeS cluster enzymes were normal.

These results point to a hitherto unknown role of mitochondria in cellular proliferation and differentiation. An intact mitochondrial respiratory chain is essential for differentiation of epidermis and tongue epithelium.

P196

E-cadherin affects the functional activity of the Par3-Par6-aPKC polarity complex in keratinocytes

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In simple epithelial cells, there is a close relationship between the establishment of polarity and the formation of intercellular junctions. Much less is still known about this correlation in stratifying simple epithelial cells. Recently, we found that inactivation of E-cadherin specifically in the epidermis resulted in disturbed skin barrier function due to structural and functional alterations in tight junctions located in the granular layer of the epidermis. This correlated with the loss of active atypical protein kinase C (PKC) from the membrane.

In simple epithelia, atypical PKC forms a complex with the polarity proteins Par3 and Par6 and, as such, contributes to tight junction formation. To understand the role of this polarity complex in the skin, we have characterized the localization of the Par3-Par6-atypical PKC complex in normal skin during pathological conditions such as wounding and in human hyperproliferative skin diseases. At this, we found are distributed localization of this complex during wound healing. In addition, we analysed the expression and recruitment of the Par3-Par6-atypical PKC complex to the junctions during Ca^{2+} induced differentiation using both western blot and immunofluorescence analysis. To examine whether atypical PKC activity indeed plays a role in epidermal tight junction formation, we have inhibited aPKC in keratinocytes and found that the formation of epithelial resistance after Ca^{2+}-induced differentiation is substantially delayed. Additionally, we detected reduced expression of tight junctional molecules in E-cadherin-deficient keratinocytes, implicating atypical PKC in E-cadherin-mediated regulation of epidermal tight junction formation. Our results show that E-cadherin is essential for the epidermal barrier formation by regulating tight junctional architecture most probably via signalling molecules such as atypical PKC.

P197

Improved capacitation and acrosome reaction of human spermatozoa by magnetic activated cell sorting

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Objective: Capacitation and acrosome reaction (AR) of human spermatozoa are prerequisites for fertilization. Annexin-V-MACS is able to separate apoptotic from non-apoptotic sperm based on their externalization of phosphatidylserine (EPS). The non-apoptitic (EPSneg) fraction is characterized by lowest amounts of membrane alterations, caspase activation, disrupted mitochondrial potential and DNA fragmentation. The aim of our study was to investigate the separation effect of Annexin-V-MACS on capacitation and AR in non-apoptotic sperm.
Material and methods: Semen specimens from 10 healthy donors were separated into two samples each, one was left untreated (control) and the second was subjected to Annexin-V-MACS. Two aliquots of both the control as well as the EPS negative fraction after Annexin-V-MACS were incubated in BWW at 37 °C, 5% CO_2 for 3 h either with 3% BSA (capacitation) or without additives. Capacitation (CAP) was monitored using tyrosine phosphorylation (TyrP, Western blot). AR was determined by labeling with mab CD46-FITC before and after stimulation with calcium ionophore A23187, followed by flow cytometric evaluation of the percentage of $CD46^+$ sperm.
Results: Densitometric analyses of the 105 and 80-kDa bands of the TyrP Western blots demonstrated significantly higher TyrP in the capacitated aliquots compared with the non-capacitated aliquots. Furthermore, EPSneg samples presented with significantly more TyrP compared with the non-separated semen samples (TyrP : control 100%, control-CAP 136 ± 30%, EPSneg 115 ± 13% and EPSneg-CAP 165 ± 34%). There was no difference in spontaneous AR in all groups ($CD46^+$ sperm : control 3.5 ± 0.7%, control-CAP 4.8 ± 1.2%, EPSneg 3.4 ± 1.6% and EPSneg-CAP 4.9 ± 1.9%). In contrast, after induction of AR the capacitated as well as EPSneg aliquots showed significantly increased amounts of $CD46^+$ sperm. AR was best inducible in EPSneg sperm after CAP ($CD46^+$ sperm : control 25.9 ± 11.4%, control CAP 44.3 ± 9.3%, EPSneg 37.4 ± 8.0% and EPSneg CAP 55.7 ± 15.1%).
Conclusion: Non-apoptotic human spermatozoa with intact plasma membranes are characterized by superior ability to capacitate and consequently by maximum potential to perform AR after stimulation. Selection of EPSneg sperm may be of advantage for assistant reproduction to prepare the sperm subpopulation with the highest fertilizing potential.

P198

Transforming growth factor-β potentiates interleukin-1β/p38-mitogen-activated protein kinase-signaling pathways: consequence for c-fos and metalloproteinase gene expression in human epithelial cells

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The transcription factor c-fos is involved in the regulation of numerous AP-1-dependent genes, i.e. cytokine-induced metalloproteinase gene expression. High expression of these extracellular matrix (ECM)-degrading enzymes exerts deleterious consequences to connective tissue integrity in inflammatory disorders and is critical to convey the invasive phenotype of tumor cells. However, the precise mechanism how proinflammatory cytokines, i.e. interleukin (IL)-1β and profibrotic cytokines, i.e. transforming growth factor (TGF)-β, orchestrate the balance between ECM destruction, and neosynthesis remains unclear. Using human HaCaT keratinocytes, we determined that TGF-β enhances IL-1β-induced, mitogen-activated protein kinase-mediated, c-fos gene transcription as revealed using Northern blotting. We further provide evidence that TGF-β potentiated IL-1β-induced p38-MAPK phosphorylation by inhibiting mitogen-activated protein kinase phosphatase-1 expression on the mRNA and protein level. By contrast, IL-1β prevented TGF-β-induced SMAD-specific gene transactivation, whereas TGF-β-mediated SMAD phosphorylation and nuclear translocation remain unaffected. In addition, the inductive activity of IL-1β on the AP-1-dependent expression of various matrix metalloproteinase family members was significantly agonized by TGF-β as shown by quantitative real-time polymerase chain reaction. Together, these results identify TGF-β as a potent regulator of IL-1β-mediated gene expression in keratinocytes critical for cellular processes such as ECM degradation and epithelial tumor-cell invasion.

P199

A potential role for MColA in compensating matrix metalloproteinase-13 deficiency

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Extracellular matrix (ECM) remodelling during wound healing is required for cell migration, release of factors/bioactive peptides, and renewal of the extracellular environment. Matrix metalloproteinases (MMPs) have been shown to play a crucial

role in these processes. During granulation tissue formation, expression and activity of the interstitial collagenases MMP-13 and MMP-14 is increased in response to cytokines and growth factors, extracellular matrices, and cell–cell communication. Previously, we have shown that fibroblasts contacting fibrillar type I collagen, an *in vitro* model for latest phases of healing, exhibit *de novo* synthesis of MMP-13 and MMP-14, which are believed to represent the major interstitial collagenases in mice. To elucidate the specific role of individual MMPs in the cellular response to ECM components, we cultured MMP-13-deficient murine fibroblasts as monolayers and in contact to three-dimensional collagen type I for 48 h. Fibroblasts derived from MMP-13 flox/flox animals were used as controls. No differences between MMP-13-deficient and the flox/flox cells were observed regarding collagen gel contraction.

Expression of pro-MMP-13 and pro-MMP-14 was induced when the cells were grown in contact to type I collagen, and as a result of MMP-14 induction, pro-MMP-2 was activated. Surprisingly, analysis of cell supernatants showed a decreased activation of pro-MMP-2 in MMP-13-deficient as compared with control cells, even though, transcripts levels for MT1-MMP were unaltered in both cells. Interestingly, the transcripts of the recently described murine orthologue of human MMP-1, MColA, were found to be up-regulated in MMP-13-deficient fibroblasts grown as monolayers or in collagen gels.

In conclusion, our data indicate that fibroblasts from MMP-13-deficient mice respond to ECM stimuli similar to control cells. In addition, the function of MMP-13 in dermal fibroblasts is not replaced by MMP-14, but the data rather suggest a role for the collagenase MColA in compensatory mechanisms induced by MMP-13 deficiency.

P200

Cell synchronization effect on on-viral cutaneous transfection

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The skin is an attractive organ for the development of therapeutic and prophylactic genetic medicines. The tissue is readily accessed, has a significant regenerative capacity, and can easily be monitored by either direct observation or biopsy.

Current gene delivery systems can be divided into two categories: viral-based and non-viral-based systems. Effective non-viral gene transfer systems have been developed because delivery may be much safer and perhaps more pharmaceutically acceptable than using viral vectors. However, the efficiency of current non-viral-based systems is very poor. So the question does arise: Is there a combination of methods to increase the efficiency of non-viral gene transfer?

In the present study, we investigated the transfection efficiency of the transfection reagent FuGENE^{TM} 6 on human keratinocytes. Human keratinocytes were isolated from foreskin, cultured, synchronized by methotrexate treatment and transfected with the reporter gene plasmid coding for enhanced green fluorescent protein (EGFP). The number of cells producing EGFP was determined using flow cytometry. Our study demonstrates that FuGENE^{TM} 6 is an efficient transfection reagent for non-viral gene transfer into human keratinocytes. Cell synchronization with methotrexate does not increase the transfection rate.

P201

Calcium regulates human SZ95 sebocyte numbers and differentiation in an inverse manner compared with human keratinocytes

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Calcium (Ca^{2+}) and vitamin D are well-known major promoters of keratinocyte differentiation. In contrast and despite that Ca^{2+}-binding proteins are expressed in all epithelial skin cells, no data exist on the effects of Ca^{2+} and vitamin D on sebaceous glands. This study provides first knowledge in this field and indicates that human sebocytes respond to Ca^{2+} and vitamin D *in vitro* in a reverse manner compared with epidermal keratinocytes. The increase of Ca^{2+} concentration in culture medium from 0.05 to 1.4 mM in 24 h increased SZ95 sebocyte numbers up to $43 \pm 10\%$ in a gradual Ca^{2+} concentration-dependent manner, while Ca^{2+} decrease from 1.4 to 0.05 mM did not influence cell numbers. A Ca^{2+} concentration change between 0.05 and 0.2 mM in both directions was critical for lipids, because Ca^{2+} raise decreased significantly cellular neutral lipids and membrane polar lipids and Ca^{2+} reduction increased neutral and polar lipids in 24 h. Transmission electron microscopy showed ultrastructural features such as increased Golgi components and lipid droplets consistent with increased lipogenesis, following low Ca^{2+} switch. To corroborate these data, the Ca^{2+} chelator EGTA was added to SZ95 sebocytes cultured in 0.4 mM Ca^{2+}, whereas it did not influence SZ95 sebocyte numbers after 24 h of treatment but increased neutral ($40 \pm 5\%$) and polar ($26 \pm 6\%$) lipids at concentrations equimolar to Ca^{2+}. In addition, caspase 3/7 activity was increased 1.9- to 2.1-fold by Ca^{2+} removal as compared with 1.4 mM Ca^{2+} in medium, indicating enhanced apoptosis, which was also supported by ultrastructural observations. Calcitriol (1–100 nM) enhanced the numbers of SZ95 sebocytes maintained at slow growth conditions (up to $59 \pm 4\%$), while it reduced sebocyte lipids.

P202

Identification of a novel 54 kDa hepatocyte growth factor isoform during wound repair

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Hepatocyte growth factor (HGF) is a mesenchymal-derived 64-kDa cytokine that is proteolytically generated from a HGF pro-form (94 kDa). Binding its receptor MET leads to stimulation of proliferation, motility, and morphogenesis in different epithelial cell types, supports angiogenesis, modulates fibrinolysis and monocyte performance, thus suggesting an important function in tissue repair. Recently, a kallikrein-mediated 54-kDa HGF isoform of unknown biological significance was identified. However, so far, kallikrein-mediated processing of HGF *in vivo* has not been reported. Furthermore, the impact of the 54/64-kDa HGF/MET axis on cutaneous tissue repair remains elusive. Goal of the project is a functional analysis of HGF processing and ligand/receptor signaling in cutaneous wound repair. As revealed by immunohistochemistry of murine wounds, expression of HGF and MET was highly up-regulated during cutaneous repair. Whereas HGF expression was localized in the granulation tissue, epithelial wound edges and cells within the granulation tissue stained highly positive for MET. In wound fluid of healing and chronic non-healing human wounds, high levels of HGF protein were detected. As indicated by Western blot analysis, the HGF form present in wound fluid was predominantly detected by 54 kDa; the 64-kDa form was detected at low levels. Kallikrein activity was increased in wound fluid of healing and non-healing wounds. Our data indicate that, next to the known 64-kDa HGF protein, a

novel, alternative 54-kDa HGF isoform seems to influence HGF/MET signaling in cutaneous wound repair. Current studies investigate the biological significance of kallikrein-mediated processing of HGF in tissue repair.

P203 (V13)

The role of laminin 5 for keratinocyte polarity and its effect on epithelial cell morphology

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Laminin 5 is an important ligand in the process of keratinocyte migration and wound healing in skin. Polarized migration includes the formation of lamellipodia extending in the direction of the wound bed. Migrating keratinocytes produce a migration track containing components of the extracellular matrix that serve as a provisional basement membrane. To understand the role of laminin 5 in the migration behaviour and cell morphology, we used primary keratinocytes from a patient with a lethal variant of junctional epidermolysis bullosa that are deficient in laminin 5. Using the stroboscopic analysis of cell dynamics technique, we investigated the velocity and persistence of lamellipodia as well as the ruffle frequency and translocation velocity of the cells after stimulation with epidermal growth factor on different matrices. Whereas, on collagen I and collagen IV, an inefficient lamella dynamic was observed, this could be reversed by plating laminin 5-deficient keratinocytes on fibronectin and a matrix produced by the HaCaT cell line. Moreover, we observed a bipolar and tripolar fibroblastic shape of the deficient keratinocytes on collagen without directed migration. This phenotype could be rescued by plating the cells on fibronectin and HaCaT matrix. Inhibition experiments suggest that integrin-a2 is the main receptor for the deficient cells on collagen, whereas integrin-a5 is used on fibronectin. This implies that different signalling pathways may be triggered by different substrates. The ultrastructural analysis of the migration track revealed lack of spherical and elongated tubular structures that are thought to contain a6β4 integrin. Our findings suggest that laminin 5 is essential for the polarity and epithelial cell morphology of keratinocytes and is essential for an efficient lamellipodium activity and ruffle frequency in migrating keratinocytes.

P204

Influence of chitosan on wound healing is dependent on concentration and molecular weight

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Chitosan, one of the few natural cationic polymers, is derived by deacetylation of chitin. The various chitosan types differ in their origin, degree of deacetylation, molecular weight, and solubility. As reports concerning the ability of chitosan to improve wound healing are contradictory, we investigated whether the influence of chitosan on wound healing is dependent on concentration and/or molecular weight.

Using an established porcine wound-healing model, we investigated three chitosan types of the same origin (*Pandalus borealis*) which only differ in their molecular weights (Chitopharm® L MW $0.5–5 \times 10^6$ g/mol, Chitopharm® S MW $0.05–1 \times 10^6$ g/mol, and Oligochitosan MW $<10\,000$ g/mol). The objective was to determine the influence on wound healing in general, as well as on proliferation, apoptosis, and direct cell–cell communication.

We observed that all investigated chitosan types inhibited wound healing at higher concentrations (1, 0.1%) while the high molecular weight chitosan was able to promote wound healing significantly at lower concentrations. The acceleration of wound healing was accompanied by an increase of proliferative cells at the wound margins and in the regenerating epidermis. The inhibition of wound healing at higher concentrations was accompanied by a decrease of proliferative cells and by a repression of down-regulation of Connexin 43 at the wound margins and therefore a changed direct cell–cell communication during initial wound healing.

The results show clearly that the type as well as the concentration of chitosan determines its influence on wound healing. The signal transduction pathways involved in alteration of the number of proliferative cells shall be elucidated in future experiments.

P205

Characterization of human Merkel cells at various locations

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Merkel cells are neuroendocrine cells in the skin. They are identified at electron microscopic level by the existence of dense core granules and at the light microscopic level by the presence of cytokeratin 20. They are found at various locations: disseminated in the basal cell layer of interfollicular epidermis, accumulated in the so-called 'touch domes' near hair follicles and in the basal cell layer of palmae and plantae and in the outer root sheath of hair follicles of scalp and vellus hairs. Merkel cells are ascribed to function as slowly adapting mechano receptor type 1 and are thought to be involved in growth, differentiation and homeostasis of cutaneous structures. Merkel cells are supposed to be the origin of Merkel cell carcinoma (MCC).

We wanted to characterize Merkel cells at different locations and to address the questions whether there exist different types of Merkel cells and to what extent these cells show similarities to MCC. Therefore, we investigated the presence of various proteins associated with neural or epithelial function (N-CAM, nestin, neurofilament, nerve growth factor (NGF) receptor, synaptophysin and villin), as well as the proto-oncogene CD117 (c-kit). Our results demonstrate that there exist heterogenous populations of Merkel cells in adult human skin even at the same location. They differ especially concerning NGF-receptor and CD117, but we also found significant differences for N-CAM and villin. All Merkel cells are negative for nestin. Some Merkel cells share various characteristics with MCC, while others are quite different. We discuss the possible functions of the different types of Merkel cells and their relationship to MCC.

P206

***Staphylococcus aureus* but not *Staphylococcus epidermidis* induce down-regulation of tight junction and adherens junction proteins during keratinocyte infection**

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Tight junctions (TJ) are occluding cell–cell junctions located in the uppermost living layers of the epidermis. They have been shown to play a role in barrier function of the skin.

As TJ proteins are known to be targets for bacteria and their toxins in simple epithelia and endothelia, we wanted to investigate their involvement in skin infection. Therefore, we infected human keratinocytes of the cell line HaCaT as well as a three-dimensional porcine *ex vivo* skin model with various staphylococcal strains and investigated localization and expression of TJ and adherens junction (AJ) proteins as well as of desmosomal proteins and actin. We observed a loss of TJ and, to a lesser extent, AJ proteins at the cell–cell borders of HaCaT cells after the infection with exfoliative toxin-negative *Staphylococcus aureus* dependent on the amount of bacteria and period of incubation. This down-regulation was confirmed in the keratinocytes of the 3D system. There was no change in desmosomal proteins and in actin. Infection with the commensial strain *Staphylococcus epidermidis* did neither in the aCaT cells nor in the 3D system result in any alterations. The intermediate strain *Staphylococcus lugdunensis* showed an intermediate staining pattern. Infection did not impair cell viability at the investigated points in time as was shown by the investigation of proliferation and apoptosis in the 3D system.

Summarizing the results, one might conclude that exfoliative toxin-negative *S. aureusis* able to destroy TJ in the course of skin infection while *S. epidermidis* has not this ability and that this distinction might be involved in the different pathogenicity of these strains.

P207

The focal contact protein kindlin-1 in skin and cutaneous cells: physiological expression and genetic deficiency

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A novel family of focal contact proteins, the kindlins, is involved in attachment of the actin cytoskeleton to the plasma membrane and in integrin-mediated cellular processes. The family members, kindlin-1, kindlin-2 (Mig-2) and kindlin-3, exhibit high amino acid sequence homology to each other and to the focal contact protein UNC-112 of *Caenorhabditis elegans*. Deficiency of kindlin-1, as a result of loss-of-function mutations in the *KIND1* gene, causes Kindler syndrome, an autosomal recessive genodermatosis characterized by skin blistering, poikiloderma, photosensitivity and, occasionally, carcinogenesis. Very little is known about the biochemistry and physiological functions of the kindlins. Here, we analysed expression of human kindlin-1 using real-tine polymerase chain reaction, recombinant protein expression, immunofluorescence, Western blot analysis and immunoelectron microscopy. Moderate levels of kindlin-1 mRNA were expressed in keratinocytes and HaCaT cells but not in fibroblasts or COS-7 cells. KIND-1 was overexpressed in squamous cell carcinoma cells. For protein studies, antibodies were generated against recombinant GST-kindlin-1 fusion protein and affinity purified. They recognized a 75-kDa protein in normal human keratinocytes and HaCaT cells, corresponding to the predicted molecular mass of authentic kindlin-1. No signal was detected in fibroblast and COS-7 cell extracts. Expression of recombinant, full-length kindlin-1 in HEK293 cells and subsequent immunoblot analysis confirmed the identity of the 75-kDa signal. In the skin, the antibodies produced a linear signal at the dermal–epidermal junction, and immunoelectron microscopy revealed kindlin-1 to be localized between the hemidesmosomes in basal keratinocytes. A genetic approach showed that KIND-1 mutations led to complete absence of kindlin-1 in the skin and keratinocytes *in vitro* and to abnormal morphology of the actin cytoskeleton in keratinocytes.

P208

Expression and function of 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 chloride channel is composed of different a(1–3) and a β-subunit and mediates fast synaptic transmission in the central nervous system. Recently, members of this superfamily, the nicotinic acetylcholine receptors, have been found to be involved in the regulation of various biologic functions of human skin. Because glycine, the natural ligand of GlyR, has been found to enhance epidermal barrier recovery, we wanted to know whether GlyR is 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 polymerase chain reaction. In addition, we performed immunofluorescence analysis of GlyR expression in normal human skin, eczema and psoriasis samples. 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 or the nAChR/GlyR antagonist strychnine. The β-subunit which is present in all GlyR was detected in mRNA of keratinocytes, fibroblasts and human skin. In addition, we found the a1-subunit expressed in cultured keratinocytes and fibroblasts while the a2-subunit was restricted to cultured fibroblasts. In keratinocytes, 1 mM glycine specifically induced a 50% increase in cell number after 3 days in culture, while fibroblasts proliferation was not significantly influenced. Organotypically cultured epidermis equivalents were significantly thicker than control if cultured in the presence of 1 mM glycine. In human skin, GlyR immunoreactivity (IR) was detected in the upper epidermal layers and in the inner root sheath of anagen hair follicles, while all other adnexal structures remained negative. Interestingly, in specimens of eczema and psoriasis, GlyR IR was drastically 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 of the hitherto neglected ion chloride in cutaneous physiology and pathophysiology and its regulation through GlyR.

P209

Histone deacetylase inhibitors may interfere with angiogenesis by decreasing endothelial vascular endothelial growth factor receptor-2 protein half-life

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Recent evidence suggests that histone deacetylase (HDAC) inhibitors may mediate part of their antitumor effects by interfering with tumor angiogenesis. As signaling via the vascular endothelial growth factor receptor (VEGFR)-2 pathway is critical for angiogenic responses during tumor progression, we explored whether established antitumor effects of HDAC inhibitors are mediated in part through diminished VEGFR-2 expression. We therefore examined the potential impact of three different HDAC inhibitors, trichostatin A (TSA), sodium butyrate (NaB), and valproic acid (VPA), on both mRNA and protein VEGFR2 expression. In contrast to VPA, TSA, and NaB are shown to significantly inhibit VEGFR-2 protein expression in a time- and concentration-dependent manner, whereas no such effect is demonstrated at the level of mRNA expression. Pertinent to these data, VEGFR-2 protein half-life is shown to be decreased in response

to TSA and NaB. First experimental evidence indicates that reduction of protein half-life may be mediated in part via HDAC inhibitor-induced expression of ubiquitin ligases. To further distinguish as to which of the eight different HDACs are responsible for the regulation of VEGFR-2 protein half-life, we silenced specific HDAC genes by transfecting respective siRNAs. These studies revealed that HDACs 4 and 6 are preferentially involved in VEGFR-2 expression, providing first evidence for the regulation of a tyrosine kinase receptor protein half-life by distinct HDACs. Together, VEGFR-2 protein expression may represent an important target of HDAC inhibitors in mediating antitumor effects, an assumption that is supported by data also showing significant inhibition of capillary network formation *in vitro*.

P210

Uptake of CpG oligonucleotides in *in vitro* cultured HaCaT cells and in a full-thickness skin model

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CpG oligodeoxynucleotides (ODN) are bioactive molecules that are intensively studied in immune cells where they were taken up via endocytosis. Consecutively, they accumulate in the endosomal compartment where they bind to and activate the Toll-like receptor (TLR)-9 evoking an immune stimulatory effect. Recent studies showed an expression of TLR-9 in epidermal keratinocytes suggesting a relevance of CpG-ODNs in epithelial cells.

In the present study, the trafficking of fluorescent-labelled CpG-ODN of different lengths was tested in HaCaT cells and additionally after topical application onto a full-thickness skin model (Phenion® FT skin model) using confocal laser scanning microscopy and immunohistochemistry. The Phenion® FT skin model comprises stratified keratinocyte layers grown on a fibroblast containing matrix.

We could demonstrate that both fluorescent-labelled CpG-ODN, the 20-mer and the 6-mer ODN, enter HaCaT cells instantly after a few minutes forming a corona-like structure close to the cell membrane. After 30 min, the 20-mer CpG-ODN was detected in perinuclear aggregates, whereas the 6-mer CpG-ODN accumulates in the nucleus. After topical application of CpG-ODN on top of the stratified Phenion® FT skin model, the majority of fluorescence was detected within the stratum corneum. Nevertheless, a considerable amount of the 6-mer approximates to the stratum basale after 4 h. In this experimental setup, the 20-mer accumulates in more superficial skin layers.

In conclusion, our findings show that CpG-ODNs of different lengths are able to enter epithelial cells. The subcellular distribution of both CpG-ODN tested differed, suggesting a different biological activity. Experiments using the Phenion® FT skin model showed that delivery of CpG-ODN after topical application is possible, and particularly, short molecules are able to reach the proliferative cell layer. These findings suggest CpG-ODN as pharmacological active molecules also in skin keratinocytes.

P211

Research of drug effects on epidermal stem cells *in vitro*

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Epidermal stem cells currently form an important research area in medicine. Because of the extraordinary self-regenerating properties of these cells, the hypothesis exists that epidermal stem cell errors are a likely cause of many types of human skin diseases. The effect of drugs on this specific epidermal cell type therefore needs to be researched. This could be a way to achieve long-term or curative effects in the therapy of epidermis-based illnesses such as psoriasis.

Important drugs in dermatology are glucocorticoids. In this study, we check effects of hydrocortisone on epidermal stem cells in comparison to other proliferative keratinocytes. After separation of these cells by collagen IV-mediated adherence, we cultured them preconfluent and exposed them to the glucocorticoid. While stem cells retained a proliferative state, the non-stem cell proportion was probably not able to continue cell division after withdrawal of hydrocortisone. Our conclusion is that hydrocortisone seems to be essential for the proliferation of non-stem cells *in vitro*.

P212

Fas signaling is not involved in imipramine-induced apoptosis in human melanoma cells

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The tricyclic antidepressant imipramine is capable of both reducing and inducing apoptosis in human cells. The apoptosis-reducing effect is often attributed to the inhibition of the acidic sphingomyelinase and hence a lack of ceramide generation sufficient for death signaling. On the other side, it is known that cytochrome c release and caspase activation are involved in the course of imipramine-induced apoptosis. In addition, in human lymphocytes, imipramine led to an increase in Fas expression committing the cells to die of apoptosis upon Fas clustering or Fas–Fas ligand binding.

In this study, the induction of apoptosis in human melanoma cells IGR1 and IGR39 was investigated by flow cytometry (Annexin-V-FITC/propidium iodide test) and a micro titre plate absorbance assay (crystal violet). As compared with IGR39, IGR1 cells proved to be substantially more sensitive to imipramine. However, even 50 μM imipramine for 12 h did not increase the CD95 level. The blocking of CD95/Fas by pre-incubation with an antibody (clone ZB4) did not reduce the effect of imipramine treatment for 24 h as measured by the number of viable IGR1 cells.

The results suggest that Fas signaling plays no role in imipramine-mediated apoptosis in the melanoma cells under study.

P213

Overexpression of manganese superoxide dismutase enhances contraction of a human dermal fibroblast cell line in the free-floating collagen lattice

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Manganese superoxide dismutase (MnSOD), a mitochondrially localized antioxidative enzyme, exerts its antioxidant defense by dismutation of superoxide anion to hydrogen peroxide. Reactive oxygen species (ROS)-like superoxide anion and hydrogen peroxide play an important role in the physiology of wound healing. However, prolonged generation of toxic concentrations of ROS by inflammatory cells may contribute to the pathophysiology of chronic wounds. Here, we addressed the question whether overexpression of MnSOD plays a role in wound contraction. For this purpose, we used stably transfected MnSOD-overexpressing

human dermal fibroblasts and murine MnSOD-deficient fibroblasts in the fibroblast-populated collagen lattice as an *in vitro* wound contraction assay. Using the scopoletin assay, we showed that the concentration of hydrogen peroxide is increased in MnSOD-overexpressing fibroblasts because of imbalanced overexpression of MnSOD, while hydrogen peroxide detoxifying enzymes (catalase, glutathione peroxidase) have unchanged activities. MnSOD-overexpressing fibroblasts led to a substantially increased contraction, whereas MnSOD-deficient murine fibroblasts showed a significant delay in the contraction compared with controls. Among other effectors, transforming growth factor (TGF)-β is known to stimulate collagen lattice contraction. Collagen lattices seeded with MnSOD-overexpressing cells showed high secretion levels of TGF-β1 compared with controls. Inhibition of the TGF-β1 receptor signal transduction pathway, using an inhibitor of TGF-β1 receptor kinases, resulted in a reduced contraction of the MnSOD-overexpressing fibroblasts. Incubation of vector-transfected control fibroblasts with recombinant TGF-β1 led to an enhanced contraction at late time points. Exogenously added hydrogen peroxide stimulated the contraction of vector control cells at early time points. Incubation of MnSOD-overexpressing cells with the antioxidant *N*-acetylcysteine strongly reduced the enhanced contraction at early as well as late time points.

Collectively, these data show that imbalance of MnSOD expression with an increase in hydrogen peroxide and subsequent TGF-β1 release promote collagen lattice contraction dramatically.

P214

Comparison of the *N*-methyl-D-aspartate receptor antagonists MK-801 and AP5 in keratinocytes

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Ionotropic glutamate receptors of the *N*-methyl-D-aspartate (NMDA) receptor type are expressed in keratinocytes and increase their intracellular calcium concentration. This effect seems to be involved in the differentiation of keratinocytes. Dizocilpine (MK-801) is normally used as 'standard' antagonist in neuronal cells and keratinocytes to proof the function of the receptor. Nevertheless, MK-801 is thought to be a specific antagonist with low potency especially in primary cultures of keratinocytes. This could be explained by the NMDA receptor subtype NMDA R2D in keratinocytes. The present study was performed to find a more appropriate NMDA receptor antagonist for studies in keratinocytes. Therefore, the effect of MK-801 was compared with another established NMDA receptor antagonist DL-2-amino-5-phosphonovaleric acid (AP5).

The intracellular calcium concentration of normal human epidermal keratinocytes (NHEK) was studied under the influence of NMDA and the selective NMDA antagonists MK-801 and AP5. Measurement of the intracellular calcium concentrations was performed by laser scanning microscopy.

As expected, the application of NMDA (50 μM) resulted in an elevated intracellular calcium concentration of 64.5% of all investigated cells. AP5 inhibited the NMDA effect completely. MK-801 (10–200 μM) also decreased the number of NMDA-reactive cells, but up to 56% NHEK still showed elevated intracellular calcium concentrations. Interestingly, MK-801 alone also increased the intracellular calcium concentrations in NHEK in a dose-dependent manner, while AP5 alone had no effect.

For measurements of the NMDA receptor-dependent calcium influx in NHEK, AP5 is a more potent antagonist than MK-801. Moreover, MK-801 seems to have a NMDA receptor-independent influence on the intracellular calcium level. Compared with earlier results, this is a discrepancy between NHEK and the immortalized cell-line HaCaT. This difference has to be clarified in future studies.

P215

Retinoid homeostasis in skin is dependent on expression of CYP26AI that is restricted to basal keratinocytes in normal human skin

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Retinoids are potent regulators of cell proliferation, cell differentiation, and morphogenesis. Cellular levels of all *trans*-retinoic acid (ATRA) are meticulously regulated through a balance of uptake, biosynthesis, catabolism, and efflux transport. Metabolic transformation of ATRA to 4-hydroxylated RA, a less active and more readily excretable polar species appears to be primarily catalyzed by the highly specific cytochrome P450 (CYP) enzyme 26AI. In this study, we have examined the expression and inducibility of CYP26AI by ATRA in normal human epidermal keratinocytes (NHEK) and dermal fibroblasts on RNA and on protein level. Analysis of monolayer cultures of NHEK and fibroblasts by quantitative real-time polymerase chain reaction, immunofluorescence, and Western blot analysis revealed very low basal levels of CYP 26AI mRNA expression which was enhanced by addition of 10–6 Mall-*trans* RA. Using a newly developed peptide antibody, we further examined the localization of CYP 26AI expression in normal skin, inflammatory skin diseases, and 3D skin models cultured for 21 days. In contrast to the cell culture monolayers, we showed strong constitutive expression of CYP 26AI restricted to the keratinocytes in the epidermal basal layer. These studies indicate that human skin has the capacity to metabolize RA but that substantial differences exist in CYP expression between normal skin and 3D skin models compared with monolayer cultures. Complex metabolic processes such as retinoid metabolism may therefore be better studied in model systems more closely resembling the *in vivo* situation such as 3D skin systems. In light of our prior studies showing substantial retinoid metabolism in human skin fibroblasts, our data further confirm the concept that epithelial-mesenchymal crosstalk is likely to be an important determinant of retinoid homeostasis in skin.

P216

1,25-Dihydroxyvitamin D3 modulates dose- and time-dependent cytotoxic effects induced by ultraviolet and ionizing radiation

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Both ultraviolet (UV) and ionizing radiation have been characterized as carcinogens that may induce malignant epithelial skin tumors, with UV light being the most important environmental risk factor. Increase in phosphorylated histone H2AX (γH2AX) foci has been shown to signal the presence of DNA damage following ionizing radiation, in particular, double-strand breaks. UV light does not directly produce DNA double-strand breaks but rather produces pyrimidine dimers and other photoproducts that must be

removed or bypassed to prevent arrest of the replication fork. We have now analyzed effects of the biologically active vitamin D metabolite 1,25-dihydroxyvitamin D3 in presence of pyrimidine dimers and phosphorylated γH2AX in non-malignant and malignant human keratinocytes (HaCaT and SCL-1) following UV (100–1000 J/m^2) and ionizing irradiation (IR, 1Gy−5Gy), respectively. Additionally, we analyzed cell proliferation, cell viability, and the expression of key components of the vitamin D system (vitamin D receptor, 1OHase, 25OHase, and 24OHase).

Our findings indicate that pretreatment with 1,25(OH)2D3 time- and dose-dependent (i) results in increased cell viability following UV-B or IR, (ii) modulates expression of key components of the vitamin D system, and (iii) reduces the presence of pyrimidine dimers and γH2AX foci following UV-B and ionizing irradiation, respectively. Our data support the hypothesis that the local cutaneous production of 1,25-dihydroxyvitamin D3 may represent an evolutionary highly conserved endocrine system to prevent mutagenic damage induced by UV or ionizing irradiation.

P217

Antiproliferative effects of side-chain modulated vitamin D analogs on normal and malignant melanocytes

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1,25-Dihydroxyvitamin D3 [1,25(OH)2D3] and analogs have been shown to inhibit proliferation and to induce differentiation in various cell types, including human melanocytes.

We studied cell growth inhibitory activity of new 1,25(OH)2D3-based conjugates on the growth of non-transformed and UV-C-transformed (thereby acquiring tumorigenic potential) human congenital naevus cell lines (UISO-CMN-1). Conjugation of 1,25(OH)2D3 analogs was made between polyunsaturated fatty acids (PUFAs), such as linolenic acid or γ-linolenic acid, and 1,25(OH)2D3. In previous experiments, we have shown using high-performance liquid chromatography analysis that after penetration into the skin, a major portion of calcitriol PUFA conjugate was first converted mainly into another isomer form, presumably by *trans*-esterification, and only then, it was hydrolyzed to form apparently high local concentrations of both calcitriol and PUFA. The unique biotransformation that occurred after penetration into the skin indicates that these conjugates are mutual prodrugs that are able to be bioprocessed in the skin and fully converted to the parent therapeutic agents.

Here, we demonstrate using colony-forming and cell viability assays that 1,25(OH)2D3-PUFA analogs exert strong antiproliferative effects on cell growth in non-transformed and UV-transformed human congenital naevus cell lines, indicating that these new compounds may be effective in the palliative treatment of malignant melanoma.

P218

Expression of class 3-secreted semaphorins and their receptor neuropilin-1 in the epidermis

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The first known function of neuropilin-1 (NRP1) was the role as a cell surface receptor for class 3 semaphorins on neuronal cells, mediating chemorepulsive effects and therefore regulating axonal outgrowth and migration of neurons. Recently, NRP1 has been described to be expressed by endothelial cells, where it serves as an isoform-specific coreceptor for vascular endothelial growth factor (VEGF), enhancing angiogenesis. Furthermore, there is increasing evidence that semaphorins can inhibit the migration of endothelial cells, inhibiting for example cancer angiogenesis. We found Sema3A and Sema3F to be expressed in human epidermis *in vivo* as well as by primary keratinocytes and HaCaT cells *in vitro*. Furthermore, we could show by *in situ* hybridization and by immunohistochemical staining that NRP1 is also expressed in primary human keratinocytes *in vivo* and *in vitro*. NRP1 is located mainly in suprabasal layers, and expression is strongly induced upon treatment with members of the epidermal growth factor (EGF) family, such as EGF or HB-EGF. Using HaCaT cells, we investigated NRP1 function in keratinocytes. Because semaphorin effects require plexins as coreceptors, whereas VEGF signaling is dependent on VEGF receptor tyrosin kinases such as VEGFR-1 or VEGFR-2, we analyzed the expression of these molecules. HaCaT cells expressed several plexins such as plexin A1, A2, A3, or B1 but no VEGFRs. Therefore, a function of NRP1 as a semaphorin receptor seemed likely. When cells were stimulated with Sema3A or VEGF-165, both ligands had no effect on cell proliferation. But migration in *trans*-well chambers was significantly inhibited by Sema3A, whereas VEGF-165 had no influence. The inhibitory effect of Sema3A was even stronger when HaCaT cells which stably overexpress NRP1 were exposed to this ligand. Therefore, we conclude that besides its function in neuronal guidance and angiogenesis, NRP1 is also implicated in the regulation of keratinocyte behavior. It serves as a receptor for class 3 semaphorins, and cell migration can be regulated by the receptor/ligand pair of NRP1 and Sema3A.

P219

PPAR-α and PPAR-δ agonists exert profound inhibitory effects on transforming growth factor α-induced matrix metalloproteinase 9 expression by keratinocytes

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Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors, mainly implicated in the regulation of lipid and glucose homeostasis. In addition, PPAR agonists have been shown to control inflammatory processes by inhibition of distinct proinflammatory genes. Several studies demonstrate that aberrant activation of the epidermal growth factor and/or overexpression of its ligand transforming growth factor (TGF)-α are key features of both neoplastic and inflammatory hyperproliferative epithelia. Matrix metalloproteinase 9 (MMP9) belongs to the set of genes that are effectively induced by TGF-α in keratinocytes. Induced MMP9 expression has been strongly linked to regenerative skin-repair mechanisms and inflammatory skin diseases. We therefore explored whether the known anti-inflammatory effects of different PPAR ligands are mediated in part through inhibition of TGF-α-mediated MMP9 up-regulation. PPAR-α and PPARoad agonists (e.g. fenofibrate, L165 041) are found to potently inhibit TGF-α-induced MMP9 expression by HaCaT keratinocytes, whereas predominant PPAR-γ ligands (e.g. rosiglitazone) remained without discernible effects. This inhibition is demonstrated both at the level of protein and mRNA MMP9 expression. Additional zymographic assays of culture supernatants show that PPAR-γ and PPARoad ligands significantly inhibit the catalytic activity of MMP9. As PPAR ligands do not interfere with expression and phosphorylation of the EGF receptor, we hypothesized that the inhibitory effects of PPAR-α and PPARoad agonists are mediated by suppressing the transcriptional activity of the MMP9 promoter. First transcriptional activation studies with deletional reporter gene constructs reveal that PPAR-α agonists mediate their inhibitory effects via different regulatory elements between

bp-531 and -144. In conclusion, our data provide first evidence that TGF-α-induced keratinocyte MMP9 expression is a valid target of PPAR-α and PPARoad ligands, involving distinct mechanisms of transcriptional repression.

P220

Human epidermal keratinocytes display a functional endocannabinoid/endovanilloid system and show novel signaling interactions between vanilloid receptor-1 and cannabinoid receptor-1

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Several cell populations of human skin (e.g. epidermal and hair follicle keratinocytes and dendritic cells) express the vanilloid receptor-1 (TRPV1) and cannabinoid receptor-1 (CB1). Here, we have investigated the *in vitro* effects of the endocannabinoid anandamide (AEA, which acts as an agonist for both CB1 and TRPV1) on the human epidermal keratinocyte cell line HaCaT and have defined the role of CB1 and TRPV1 in this. By Western blot, immunocytochemistry, and Q-PCR, both receptors were identified on HaCaT cells. By MTT-based colorimetric proliferation and annexin-V-based apoptosis assays, AEA inhibited proliferation and induced apoptosis of HaCaT cells (dose dependent: 0.1–30 μM). Intriguingly, these effects of AEA were equally inhibited by the specific CB1 antagonist AM-251 (1 μM) and the specific TRPV1 antagonists, iodo-resiniferatoxin (I-RTX, 50 nM) and capsazepine (1 μM). However, when the CB1 and TRPV1 antagonists were co-administered, there were no additive effects of the two antagonists. This suggested that AEA may not simultaneously act on both receptors but may sequentially activate one after the other CB1- and TRPV1-mediated signaling pathways. To test this hypothesis, we used functional calcium imaging for measuring exclusively TRPV1 activity. The results suggest that AEA primarily acts on CB1 which, in turn, activates TRPV1. Thus, human epidermal keratinocytes not only display a fully functional endocannabinoid/endovanilloid system but also exhibit novel signaling interactions between CB1 (a metabotropic, G-protein-coupled receptor) and TRPV1 (a ligand-gated, calcium permeable channel) and their intracellular signal transduction pathways.

P221

Abundance and characteristics of Schwann cells in the subepidermal nerve plexus in hairy human skin

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The subepidermal nerve plexus consists of nerve fibers composed of one or several axons ensheathed by individual Schwann cells. Apart from their function in supplying axons, dermal Schwann cells largely go unnoticed and are assumed to correspond to Schwann cells of peripheral nerves. Here, we have analyzed the extensions of the subepidermal nerve plexus in hairy skin of various body regions from 10 individuals and compared these Schwann cells to Schwann cells of larger peripheral nerves. We used immunostainings of dermal sheet preparations for NCAM, L1CAM, NGFr, CD146, S100, Vimentin, myelin basic protein, and peripheral myelin protein 22. Staining of thick skin sections and peripheral nerve sections were done in parallel. The highest number of Schwann cells per square millimeter as well as the highest total length of the subepidermal nerve plexus in millimeter per square millimeter superficial dermis was found in skin from the chest (458 ± 109 and 100 ± 18 mm) (mean ± standard deviation) and abdominal skin (415 ± 103 and 78 ± 22 mm). Intermediate Schwann cell numbers and plexus length were present in skin from inner lower arm (272 ± 70 and 66 ± 19 mm), thigh (245 ± 70 and 59 ± 16 mm), upper arm (241 ± 92 and 66 ± 20 mm), and shoulder (239 ± 65 and 73 ± 13 mm). The lowest values were seen in skin from the outer lower arm (186 ± 58 and 53 ± 10 mm) and the lower leg (178 ± 54 and 42 ± 6 mm). In the subepidermal nerve plexus, both non-myelinating as well as myelinating Schwann cells stained positive for NCAM, L1CAM, NGFr, and CD146. In contrast, myelinating Schwann cells in peripheral nerves were negative for these markers, suggesting functional differences. In summary, we show that myelinating Schwann cells of the subepidermal nerve plexus exhibit a phenotype different from their equivalents in peripheral nerves, probably reflecting different axon signals or the influence of the respective microenvironment. The high number and regular distribution of Schwann cells as well as the vast dimensions of the subepidermal nerve plexus within the papillary dermis suggest that they may not only play a role in the maintenance of the peripheral nerve fibers but also may participate in tissue homeostasis of this region.

P222

Regulation of matriptase expression in skin and skin diseases

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Matriptase is a member of the type II serine protease family, which is expressed by most cells of epithelial origin. Recently, it was shown that matriptase knockout mice die 48 h after birth because of skin defects. More importantly, skin from knockout mice transplanted on SCID mice shows severe hyperproliferation and hyper- and parakeratosis. We therefore investigated the expression and regulation of matriptase in normal skin and in skin diseases with impaired terminal differentiation. Staining of normal skin with a matriptase antibody revealed that matriptase expression was restricted to the granular layers of the epidermis. In contrast, matriptase expression in psoriasis and eczema was completely abolished. Because TNF-α is known to play a crucial role in the pathogenesis of these diseases, we investigated the regulation of matriptase by this cytokine. Stimulation of primary keratinocytes with TNF-α strongly down-regulated matriptase mRNA and protein production. Skin equivalents treated with TNF-α as well as skin equivalents generated from matriptase-deficient keratinocytes showed an impaired differentiation, displaying hyper- and parakeratosis, and a complete loss of matriptase expression. In addition, we could show that infection of keratinocytes with adenovirus overexpressing a dominant negative form of IKK-2 completely blocked TNF-α-induced down-regulation of matriptase, suggesting an involvement of the NFkB signalling pathway. Our data on the reduced expression of matriptase in skin diseases with impaired stratum corneum formation strongly support the assumption that matriptase is involved in the process of terminal keratinocyte differentiation and that its down-regulation might be part in the manifestation of these diseases.

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Evaluation of the atrophic potential and the anti-inflammatory activity of glucocorticoids using a new full-skin model

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As we have previously shown, three-dimensional full-skin models offer a huge variety of different applications. Because of their morphological correspondence, human skin by forming a stratified epidermis and therefore also permitting crosstalk between dermal fibroblasts and epidermal keratinocytes, three-dimensional skin models are the preferred system to investigate complex questions *in vitro*. Primary fibroblasts and keratinocytes were isolated from foreskin of boys less than 4 years of age. They were seeded on a collagen matrix (Phenion, Frankfurt, Germany) and were cultivated according to common protocols. The matrix was inhabited by the fibroblasts forming a dermal equivalent, whereas the keratinocytes formed a stratified epidermis containing stratum basale, -spinosum, -granulosum and -corneum at the air–liquid interface.

We tested the usability of a new full-skin model for the evaluation of the anti-inflammatory activities of gluco-corticoids. Furthermore, we analysed the potential of gluco-corticoids to induce atrophy during long-term treatment.

Pro-inflammatory cytokine release was induced by irradiating the skin models with UVB. The interleukin-6 (IL-6) and IL-8 release was measured using a commercial enzyme-linked immunosorbent assay (R&D Systems, Wiesbaden, Germany). We could show that 20 µM β-methason-17-valerate dissolved in culture medium as well as topically applied 0.1% β-methason-17-valerate (Betnesol V ointment) suppressed both the UVB-induced release of IL-6 and IL-8 and their basal levels.

The effects of systemic long-term treatment with Clobetasol-17-propionate, β-methason-17-valerate and Prednicarbate were evaluated by morphological characterization of the skin model and defining the epidermal thickness at different points during the treatment and at its end. We could show a corticoid-related decrease of the epidermal thickness.

These results suggest that full-skin models offer the opportunity to investigate glucocorticoid-mediated anti-inflammatory effects *in vitro* including evaluating the risk of atrophy.

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Effect of glycyrrhizin on growth-related signal pathways in human keratinocytes

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Glycyrrhizin is an active component of the root of Glycyrrhiza glabra (licorice) which is the most commonly used herb in Traditional Chinese Medicine. Glycyrrhizin offers a variety of pharmacological effects including a positive impact in wound healing. Focusing on repair processes, we investigated the effect of glycyrrhizin on cell proliferation in human keratinocytes (HaCaT) measuring the incorporation rate of BrdU. Furthermore, the underlying mechanisms such as growth-related signal pathways were investigated by Western blotting using phospho-specific antibodies.

In the presence of 0.05–4 mM glycyrrhizin, we found a biphasic effect on the BrdU incorporation rate in HaCaT cells. At low concentrations, we detected a concentration-dependent increase in BrdU incorporation peaking at 1 mM followed by a decrease at higher concentrations down to 70% compared with untreated controls. Confirmatory, we found a concentration-dependent phosphorylation of EGFR, ERK 1/2, Akt/PKB and PKC which was not detectable at higher concentrations. In the case of ERK 1/2 and Akt/PKB, high concentrations of glycyrrhizin reduced the phosphorylation rate beneath the basic levels of controls. Our results suppose a positive effect of glycyrrhizin on tissue-repair mechanisms in a concentration range between 0.05 and 1.00 mM and an opposite effect at higher concentrations.

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The influence of β- and γ-radiation on the binding capacity of collagen for PDGF-BB

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Introduction: Non-healing wounds lack essential growth factors, e.g. platelet-derived growth factor (PDGF). This is because of an increased proteolytic degradation by proteases such as neutrophile elastase and matrix metalloproteinases (MMPs). To support the normal wound-healing process, the protection of growth factors is essential. As a previous study has shown, a wound dressing composed of bovine collagen type I is able to bind significant amounts of PDGF-BB to release it successively afterwards. Since the binding, PDGF-BB is partly protected from proteolytic degradation. In this study, we investigated whether β- and γ- radiation modifies the binding capacity of the collagen wound dressing for PDGF-BB. Radioactive radiation is a common way to sterilize wound dressings during the production process.

Materials and methods: Wound-dressing samples were irradiated with β- or γ-radiation (20 kGy). Afterwards, the collagen sponge was cut to pieces with punch biopsies of 8 mm in diameter (corresponding to 0.5 cm^2). Each sample was incubated for 30 min at 37°C in 1 ml PDGF-BB (1 ng/ml) solution. Non-irradiated Suprasorb® C obtained from the same lot served as control. Subsequently, the supernatants were collected and the wound-dressing samples washed with phosphate-buffered saline (+0.5% bovine serum albumin) for 1 h to recover bound PDGF-BB. The concentration of PDGF-BB in both the supernatants and the washing solution was examined by enzyme-linked immunosorbent assay (R&D Systems, Wiesbaden, Germany).

Results: Our data show that the collagen wound dressing binds already after 10 min significant amounts of PDBF-BB. About 15% of the bound growth factor could be recovered. Irradiated Suprasorb® C samples exhibit nearly the same binding capacity for PDGF-BB as the non-irradiated controls. However, the elution rate after 1 h from the irradiated samples was significantly higher than from the control samples (β-irradiation: 40%; γ-irradiation: 55%).

Discussion: The physical properties of Suprasorb® C such as its porous structure and its capillary activity enable him to absorb large quantities of fluid. Furthermore, the collagen sponge absorbs substantial quantities of the PDGF-BB. Although β- or γ-irradiation partly destroys the three-dimensional structure of the collagen dressing as scanning electron micrographs reveal, radiation doses up to 20 kGy have no significant effect on the binding capacity of collagen for PDGF-BB.

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The binding capacity of β- and γ-irradiated collagen for interleukin-8

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Introduction: Non-healing wounds, e.g. venous and diabetic ulcers, contain elevated levels of pro-inflammatory immune modulators

such as interleukin (IL)-1β, IL-6 and IL-8. This condition hinders a normal wound healing and the wound retains in the inflammatory phase of the healing process. As previous studies have shown, the collagen wound dressing Suprasorb® C composed is able to bind significant amounts of these ILs. Within the presented study, we investigated whether β- and γ-irradiation modify the binding capacity of the collagen wound dressing for IL-8. Radioactive radiation is a common way to sterilize wound dressings during the production process.
Materials and methods: Wound dressing samples were irradiated with β- or γ-radiation (20 kGy). Afterwards, the collagen sponge was cut to pieces with punch biopsies of 8 mm in diameter (corresponding to 0.5 cm^2). Each sample was included in 1 ml IL-8 solution (100 pg/ml) and incubated up to 24 h at 37°C. Non-irradiated Suprasorb® C obtained from the same lot served as control. Subsequently, the supernatants were collected, and the concentration of unbound IL-8 could be determined using ELISA (Milenia, Bad Nauheim, Germany). The influence of β- and γ-irradiation on the three-dimensional structure of the collagen sponge was studied by scanning electron microscopy.
Results: Our data verify previous findings that the collagen dressing binds significant amounts of IL-8. The concentration of unbound IL-8 decreases with increasing incubation time and depends on the applied amount of the dressing. Irradiated samples show a slightly reduced binding capacity for the cytokine. However, no difference between the effects of β- and γ-irradiation was observed.
Discussion: Its physical properties such as porous structure and capillary activity allow Suprasorb® C to absorb large quantities of fluid. Furthermore, the collagen sponge absorbs considerable quantities of IL-8. β- or γ-irradiation partly destroy the three-dimensional structure of the collagen dressing resulting in a decreased binding capacity for IL-8. However, radiation doses up to 20 kGy have no significant effect on the binding capacity of the tested wound dressing for IL-8.

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Fluorescence lifetime to image epidermal ionic distributions

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Mammalian skin displays an acidic surface pH, and in human and murine skin, sequential tape-stripping experiments followed by flat pH electrode measurements have revealed a pH gradient across the stratum corneum (SC), beginning with acidic (approximately pH 5) values at the surface of the SC, decreasing towards physiologic, neutral values (approximately pH 7) at the SC/stratum granulosum interface. Unfortunately, this technique provides reliable information only about surface pH changes, without further vertical or subcellular spatial resolution; i.e. specific microdomains such as the corneocyte interstices are not resolved, and the deeper SC is inaccessible without resorting to inherently disruptive stripping methods. Furthermore, the notion of a gradient of pH through the SC stems from such stripping experiments, but other confirmation for this concept is lacking. Fluorescence lifetime imaging microscopy combines a number of advantages into a method ideally suited to visualize pH in intact, unperturbed epidermis and SC. Fluorescence lifetime is dye-concentration independent. The method requires only low light intensities and is therefore not prone to photobleaching or phototoxic artifacts, and because multiphoton lasers of IR wavelength are used, light penetrates deep into intact tissue. Our investigations into the SC pH so far have revealed the crucial role of the sodium/hydrogen antiporter NHE1 in generation of SC acidity, the colocalization of enzymatic lipid-processing activity in SC with acidic domains, and the timing and localization of emerging acidity in the SC of newborns. Together, these results have led to an improved understanding of the SC pH distribution, its origin, and regulation. Recent progress in observing tissue pH and advances in instrumentation and their implications will be presented.

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Indications that normal human hair follicles have established a hypothalamic-pituitary-thyroid axis

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Hair follicles are among the most thyroid hormone-sensitive tissues of mammals. Thyroid hormone synthesis and release are under the control of pituitary thyroid-stimulating hormone (TSH), for which thyroid epithelial cells express acognate receptor (TSH-R). Pituitary TSH release, in turn, is under control of the hypothalamic hormone, thyroid-releasing hormone (TRH), which stimulates pituitary TRH receptors (TRH-R). Recent research has indicated that the expression of TRH and TSH is not confined to hypothalamus and pituitary gland, and transcripts for several elements of the hypothalamic-pituitary-thyroid (HPT) axis have recently been identified, e.g. in cultured human skin cells (Slominski *et al*. JID 2002). Therefore, we wished to clarify whether normal human scalp hair follicles express elements of the HPT axis. Total RNA extracted from microdissected normal human anagen VI hair bulbs was analyzed by reverse transcription polymerase chain reaction (PCR). PCR primers were designed to span two exons to avoid amplification of chromosomal DNA. Specific PCR products with a size of 348 bp (TSH), 264 bp (TSH-R), 501 bp (TRH) and 582 bp (TRH-R) were identified as analyzed by agarose gel electrophoresis. Total RNA from thyroid gland, hypothalamus and pituitary gland served as template for the positive control and yielded the expected results. While these data are currently complemented by protein expression and functional analyses, they already provide the first evidence that normal human scalp hair follicles transcribe all key hypothalamic and pituitary elements of the HPT and demonstrate for the first time that normal human skin expresses the TSH and TRH-R genes *in situ*.

P229 (V30)

Human skin and hair follicles are targets and sources of erythropoietin

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The glycosylated hormone erythropoietin (EPO), primarily synthesized in kidney and liver and regulated by hypoxia, activates its specific receptor (EPO-R). However, the expression and function of the EPO/EPO-R signaling system are not limited to the hematopoietic system. This system has recently been found to be expressed in multiple non-hematopoietic tissues, e.g. for inhibiting apoptosis. The expression of EPO/EPO-R has not yet been reported in normal mammalian skin. To investigate the expression of EPO/EPO-R in human skin, we have studied

whether normal human scalp skin expresses EPO and/or EPO-R transcripts. By real time polymerase chain reaction (PCR), we detected specific PCR products for both EPO (132 bp) and EPO-R (315 bp) in human scalp skin. Even stronger EPO and EPO-R transcript signals have been found in microdissected human anagen VI hair bulbs. By immunohistology (EnVision-AP method), specific EPO immunoreactivity (IR) was confined to the skin epithelium. EPO IR was most prominent in the central outer root sheath of anagen VI hair follicles, while epidermal and hair matrix keratinocytes showed only a faint signal. We are currently investigating the EPO/EPO-R response of organ-cultured human hair follicles to hypoxia and the functional consequences of EPO-R stimulation for hair growth. These data provide the first evidence that normal human skin expresses EPO and EPO-R *in situ* and suggest that hair follicles are the predominant sources and targets of EPO/EPO-R signaling in human skin.

P230 (V06)

Dissection of nickel-regulated intracellular signal transduction pathways and identification of novel target genes in primary human endothelial cells by transcriptional profiling

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Nickel (Ni) compounds are the most common inducers of contact allergy in industrialized countries. Recent evidence indicated that Ni not only acts as a sensitizing agent but also comprises less-characterized irritant properties which are essential for elicitation of contact hypersensitivity reactions. We here studied the influence of Ni on gene expression in primary human endothelial cells and determined relevant intracellular signalling pathways. Using Affymetrix 133 A chips, the expression of 140 genes was identified to be up-regulated upon exposure to Ni, whereas 118 were down-regulated. Blocking NF-κB signalling in endothelial cells by stable expression of a dominant-negative mutant of IKK2 revealed that only a limited fraction of genes is regulated by this signalling route. Another pathway targeted by Ni resulted in activation of hypoxia-inducible factor-1α (HIF-1α). Analysis of gene regulation in cells stably expressing dominant-negative HIF-1α as well chromatin immunoprecipitation (ChIP) assays demonstrated the functional relevance of Ni-induced HIF-1α-dependent signalling. HIF-1α activation by Ni did not require functional NF-κB signalling and, vice versa, Ni-elicited NF-κB-dependent gene expression still occurred after blocking the HIF-1α pathway. Furthermore, we identified novel target genes which are up-regulated upon contact to Ni. In conclusion, our data define the irritant properties of Ni at a molecular level. We demonstrate that Ni induces parallel expression of distinct gene pools by independent activation of different intracellular signal transduction pathways which may be of relevance for the course of contact dermatitis.

P231 (V14)

CCL20 directs CCR6-positive human sperm to the oocyte

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The interaction of sperm with the oocyte is imperative during the process of mammalian fertilization. The limited number of sperm within the fallopian tube as well as the anatomic proportions suggests that human sperm-oocyte encounter is not a matter of chance but a directed process. Chemotaxis is the proposed mechanism for reorientating sperm towards the oocyte.

Chemokines are small (8–11 kDa), cytokine-like, chemoattractant proteins that mediate the directional migration of leukocytes *in vitro* and critically regulate their trafficking *in vivo*.

To asses the possible role of chemokines in gamete interaction, we analyzed the expression of all known chemokine receptors ($n = 18$) in human spermatozoa of five different healthy donors. Quantitative real-time polymerase chain reaction (PCR) analyses demonstrated that human spermatozoa express a non-random pattern of chemokine receptors and identified CCR6 as the most abundantly expressed member. Flow cytometric analyses confirmed these findings in at least 26 different healthy donors. Immunocytochemical and immunofluorescence analyses showed marked expression of CCR6 protein in the flagellum of human spermatozoa.

Quantitative real-time PCR analysis demonstrated that granulosa cells of the cumulus complex produced significant amounts of CCL20 mRNA. Subsequently, immunocytochemical analyses showed abundant expression of CCL20 protein within the cytoplasm of human cumulus granulosa cells. Next to granulosa cells, we demonstrated that human oocytes also represent a source of CCL20 production.

To determine the functional implications of our findings, we next investigated the effects of CCL20 on human sperm migration. First, we analysed the accumulation of human sperm cells in capillaries placed with gradients of CCL20 or medium-control. Results demonstrated a significantly higher percentage of migrated sperm accumulating in capillaries that presented CCL20 as opposed to capillaries containing medium alone. Results were confirmed by *trans*-well chemotaxis assays. Finally, computer-assisted videomicroscopic motion analysis showed concentration-dependent sperm chemotaxis towards gradients of CCL20.

This study for the first time identifies a chemokine ligand-receptor pair that induces chemotaxis of CCR6-positive human sperm towards the CCL20-producing oocyte and its nutrient cells, suggesting new targets for the development of non-hormonal contraceptives, fertility diagnostics or assisted reproduction.

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