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PROGRAMMHEFT

P001

Crosstalk of keratinocytes and T cells during eczematous skin reactions

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Background: Allergic contact dermatitis (ACD) and atopic eczema (AE) are eczematous skin diseases in which T cells are directly involved in the induction of keratinocytes' death. However, almost nothing is known how keratinocytes influence T cell activity in the course of allergic diseases.

Objective: The aim of this study was to investigate the outcome of keratinocyte-T cell interactions with the focus on T cell functions such as proliferation and cytokine release in an antigen-specific in vitro model of ACD to Nickel and acute AE to Phleum pratense.

Methods: T cell clones were generated of Phleum pratense sensitised ($n = 2$) or non-atopic Nickel-sensitised ($n = 5$) patients. Autologous monocyte-derived dendritic cells (DC) or EBV-transfected B cells (EBV-B) served as antigen-presenting cells (APC). Autologous primary human keratinocytes were generated by the method of suction blister. Crosstalk of keratinocytes and T cells was analysed by coincubation of keratinocytes, T cells and APC with the endpoints T cell proliferation and cytokine production.

Results: Coincubation of keratinocytes, T cells and APC revealed that keratinocytes variably influence the proliferation of T cells with 77% being blocked and 23% being induced in antigen-specific proliferation capacity. Notably, production of the cytokines IL-4 and IL-10 was regulated independently from the proliferation showing always a reduction in IL-4 and IL-10 release. IFNγ was not constantly regulated. Coincubation experiments with Phleum pratense-specific T cell clones show similar results with T cells being regulated in proliferation and cytokine release by keratinocytes. Differentiation and preincubation of keratinocytes with IFNγ did not alter the effect on T cells. However, fixation with paraformaldehyde (PFA) abrogates this immune-modulating effect.

Conclusion: Keratinocytes influence actively the effector phase of eczematous reactions. The immune-modulating effect on T cell cytokine production seems to be modified by soluble factors. Suppression of IL-4 and IL-10 in T cells could lead to a Th1 micromilieu in the skin favouring the maintenance and chronification of eczematous skin reactions. Thus, keratinocytes seem to be not only the defenceless victim of T cells in eczematous reactions but a modulator of T cell functions.

P002

A skin-like cytochrome P450 cocktail activates prohaptens to contact allergenic metabolites

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Allergic contact dermatitis is a complex syndrome representing immunological responses to cutaneous exposure to protein-reactive chemicals. While many contact sensitizers directly can elicit this disorder, others (prohaptens) require activation. Knowledge regarding the activating mechanisms remains limited, but one possibility is metabolic activation by cytochrome P450 (CYP) in the skin. We have, after microarray and real-time PCR studies of the CYP content in 18 human skin samples, developed an enriched skin-like recombinant human (rh) CYP cocktail using CYP1A1, 1B1, 2B6, 2E1, and 3A5. To validate the rhCYP cocktail, the prohaptenic conjugated diene (5R)-5-isopropenyl-2-methyl-1-methylene-2-cyclohexene was investigated using: the skin-like rhCYP cocktail, a liver-like rhCYP cocktail, single rhCYP enzymes, liver microsomes, keratinocytes, and a dendritic cell (DC) assay. The diene was activated to sensitizing epoxides in all non-cell based incubations including the skin-like rhCYP cocktail. An exocyclic epoxide metabolite was found to be mainly responsible for the allergenic activity of the diene. It also induced pronounced DC activation indicated by upregulation of IL-8.Incubation of other prohaptens like cinnamic alcohol with skin-like CYP cocktail resulted in the formation of cinnamic aldehyde, which is a more potent skin sensitizer. Cinnamic acid, which can be formed by further oxidation of cinnamic aldehyde, was not detected.The skin-like rhCYP cocktail provides a simpler alternative to using skin tissue preparations in mechanistic studies of CYP-mediated skin metabolism of prohaptens and offers the future possibility of designing in vitro predictive assays for assessment of allergenic activity of prohaptens.

P003

Impact of pollen associated lipid mediators (PALMs) from grass pollen on human mast cells

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We recently demonstrated that pollen do not only function as allergen carriers but are also a rich source of bioactive lipids. These pollen-associated lipid mediators (PALMs) act as immunostimulators and -modulators on cells of the innate and adaptive immune system.Herein we aimed to investigate the impact of water-soluble factors from grass pollen (Phleum pratense L.), their Hexane isopropanol total lipid extracts (HIP), RP-HPLC fractions (RP) from HIP and associated phytoprostanes (PPE1) on human mast cells# effector functions such as degranulation.IgE- and Calcium-Ionophore-mediated mast cell degranulation was documented by β-hexosaminidase release. The human mast cell line LAD2 as well as primary mast cells (PMCs) isolated from human foreskin were sensitized with or without human myeloma IgE for 16 h. After centrifugation, they were stimulated with anti-human IgE or Calcium-Ionophore following pretreatment with Phleum pratense L. aqueous pollen extracts (Phl.-APE), HIP, RP and PPE1. The β-hexosaminidase content in supernatants and cell pellets was measured by p-nitrophenyl-acetyl-glucosaminide formation.Water-soluble factors from pollen (Phl.-APE) dose-dependently enhanced the Calcium-Ionophore and IgE/α-IgE-mediated degranulation. Additionally, pollen derived lipids such as HIP-extracts and RP-HPLC-fractions also synergistically increased specific and unspecific mast cell degranulation. A similar potentiation of IgE/α-IgE-mediated degranulation was observed by PPE1. In the absence of IgE-receptor crosslinking only Phl.-APE was able to induce mast cell degranulation. All these outcomes were observed in LAD2 as well as in PMCs.Our results suggest that pollen-associated lipid mediators (PALMs) such as PPE1 may profoundly influence mast cell degranulation. The mechanisms leading to this effect are currently under

investigation. According to our results we suggest that under natural exposure conditions, pollen affect human mast cells not only via the known allergen-specific mechanisms but also through 'unspecific' mediators such as PALMs. These mediators could ignite a pronounced mast cell degranulation. Understanding the underlying mechanism could pave the way for new therapeutical options.

P004

Allergoid-pulsed human dendritic cells are able to induce a high production of IL-10 by autologous CD4+ T cells

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Allergen-specific immunotherapy (SIT) is a clinically efficient therapy for treatment of allergic diseases. To reduce the risk of IgE-mediated side effects allergoids with reduced IgE-binding capacity have been introduced.The aim of the study was to analyse the difference between allergens and allergoids in their ability to stimulate T cell-proliferation and cytokine production. Therefore we stimulated human monocyte-derived mature dendritic cells with timothy pollen extract (Phleum pratense) or with the corresponding allergoid and cocultured the pulsed dendritic cells with autologous CD4+ T cells. The proliferation, IL-4 and IFN-gamma production of allergoid-stimulated CD4+ T cells were reduced compared to allergen-stimulated CD4+ T cells. Interestingly, the IL-10 production was increased when CD4+ T cells were stimulated with allergoid-pulsed DC compared to allergen-pulsed DC. In order to analyse if induction of IL-10-producing regulatory T cells are responsible for these effects, we performed coculture experiments with freshly isolated CD4+ T cells together with CD4+ T cells previously stimulated with either allergen- or allergoid-pulsed DC for 1 week. We found that proliferation of freshly isolated responder CD4+ T cells was decreased by allergoid-DC- more strongly than by allergen-DC-induced CD4+ T cells, while IL-10 production was increased. After addition of anti IL-10 antibodies to the coculture the inhibitory effect was antagonized.These data indicate that regulatory T cells may be induced by allergoid-stimulated DC.

P005

Analysis of pro- and anti-inflammatory effects of weak contact allergens from phytomedicines of Arnica montana using quantitative real time PCR

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Extracts from Arnica flowerheads are traditional phytomedicines used to treat inflammatory conditions such as bruises and rheumatic inflammation. The sesquiterpene lactones (SLs), secondary plant metabolites from flowerheads, exert their anti-inflammatory effect mainly by preventing NF-KB activation due to alkylation of the p65 subunit. Despite its known immunosuppressive action, Arnica has been classified as a plant with strong potency to induce allergic contact dermatitis. We have recently shown that contact hypersensitivity could not be induced in an established mouse model, even not when Arnica tinctures or sesquiterpene lactones were applied undiluted to inflamed skin. In contrast, Arnica tinctures suppressed contact hypersensitivity (CHS) to the strong contact sensitizer trinitrochlorobenzene (TNCB) and activation of dendritic cells by LPS.Using quantitative real time PCR analysis, we are currently analysing the pro- or anti-inflammatory potential of SLs in comparison to strong contact allergens like TNCB. We could show that treatment with preparations from Arnica montana cause only a weak enhancement in RNA levels of various pro-inflammatory cytokines such as IL-6.Our future experiments aim at the definition of characteristic profiles for pro-inflammatory and effector cytokines like IFN-γ ?and their correlation with the kinetics of induction and immune regulation of CHS to weak (e.g. SLs) and strong contact sensitizers (e.g. TNCB).

P006

The role of Np1+/ Foxp3+ regulatory T-cells in the pathogenesis of insect venom allergy

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It is unknown so far, why some people develop severe anaphylactic reactions after bee or wasp stings, while others show only mild local reactions or do not react at all. We examined, whether CD4+/CD25+ regulatory T-cells (Treg) are relevant for the developement of this immunotolerance. Therefore we compared CD4+/CD25+ Treg from patients with anaphylactic reactions after insect sting with Treg from patients who developed only local swelling and with healthy controls. In proliferation experiments these CD4+/CD25+ Treg showed potent regulatory function, i.e. reacted anergic to stimulation and were able to inhibit the proliferation of CD4+/CD25-T-cells dose dependently. For further characterisation of these Treg we analysed the expression of new possible markers for Treg (Neuropilin/Np1 and Foxp3) per quantitative RT-PCR. We could show that Np1 is a highly specific marker for CD4+/CD25+ regulatory T-cells in human. Furthermore these Treg show a characteristic expression of Foxp3 compared to non-regulatory CD4+/CD25- T-cells. Interestingly CD4+/CD25+ Treg from allergic and non-allergic individuals differ in the expression of Foxp3 and Np1. Moreover we detected a specific increase of CD4+/CD25+ T-cells in non-allergic individuals after in vitro stimulation with bee or wasp venom. These results provide first evidence that non-allergic individuals are protected against type-I allergies caused by hymenoptera stings by a subset of regulatory T cells.

P007

The endogenous cannabinoid system alters the chemokine expression profile in the skin during allergic contact dermatitis

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Background and aim: Evidence has accumulated that the endogenous cannabinoid (CB) system is involved in the regulation of inflammatory responses. Using CB-receptor deficient mice, we have shown that endogenous cannabinoids attenuate allergic contact dermatitis. To investigate

how endogenous cannabinoids are able to control inflammatory responses in the skin we performed microarray experiments.
Methods: Contact hypersensitivity (CHS) was induced in C57Bl/6 mice lacking both known CB-receptors (CB1/2-/-) mice as well as in wildtype control mice by application of 0.2% DNFB on the shaved abdomen. CHS was elicited by painting one ear with 0.3% DNFB. Microarrays were performed on inflamed and normal tissue. Gene expression changes were confirmed using RT-PCR as well as in situ hybridization.
Results: In microarray experiments immune related probe sets represented the largest group of strongly upregulated genes in contact allergic skin in both mouse strains. Importantly, CCL8/MCP-2 a member of the monocyte chemotactic protein (MCP) family was differentially expressed in CB-receptor deficient animals in comparison to wildtype mice. In situ hybridization for CCL8/MCP-2 revealed keratinocytes as the major source of this chemokine.
Conclusion: Our results indicate that modulation of chemokine expression may be involved in the down regulation of cutaneous hypersensitivity responses by endogenous cannabinoids.

P008

Uptake of grass pollen allergens by epithelial cells

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Background: Epithelial cells of the respiratory tract and the dermis form a tight barrier against environmental harm. Consequently, they constitute the first exposure point of airborne allergen-carriers. In order to activate resident immune cells which then initiate a type I allergic reaction allergens first have to cross the epithelial barrier.
Objective: The aim of this study was to analyse the uptake of isolated major allergens from timothy grass pollen by epithelial cells: the group 1 allergen Phl p 1 as a protein with glycosylations and disulfide bridges and the group 6 allergen Phl p 6 lacking posttranslational modifications.
Methods: As models of respiratory tract and dermal epithelia the A549 cell line and primary human keratinocytes were used, respectively. The isolated allergens were labelled with fluorescent dyes and the uptake was analysed by flow cytometry. The intracellular localisation was determined by confocal microscopy.
Results: The uptake of allergens by A549 cells occurred rapidly and remained constant over 2–24 h. Both allergens were localised intracellulary in vesicles. Since only a small fraction of these vesicles displays an acidic pH, this might indicate that most of the allergens were intact and not processed in the lysosomal compartment. Additionally, the allergens were exocytosed by the A549 cells indicating a transcytosis mechanism for allergens to pass the respiratory epithelial barrier.Like the A549 cells, keratinocytes internalised the allergens, but the uptake was increased constantly with a maximum after 24 h. To study allergen uptake under inflammatory conditions, keratinocytes were treated with IFNγ prior to allergen exposure. IFNγ-stimulated keratinocytes showed a significantly enhanced internalisation of both allergens.
Conclusion: In this study we show clear differences between respiratory and dermal epithelial cells in the uptake of pollen allergens indicating distinct mechanisms of allergen uptake and processing in the epithelia examined. Furthermore, the increased allergen uptake by keratinocytes under inflammatory conditions may play an important role in the pathomechanism of transepidermal sensitisation during inflammatory skin reaction such as atopic dermatitis.

P009

Use of recombinant allergens to analyse cross-reactive T cell responses of birch pollen allergic patients with associated oral allergy syndrome (OAS) to hazelnuts

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In adult individuals from Northern Europe the most frequent type of food allergy appears to be pollen related OAS. A fairly high percentage of atopic patients with birch pollen allergy (>70%) experience allergic symptoms after ingestion of food, containing birch pollen allergen related proteins. The biochemical basis is a structural similarity of the involved allergens. Bet v 1 is the major allergen of birch pollen and belongs to the pathogen-related protein family 10 (PR-10). Homologous molecules were isolated from various plant families (e.g. Cor a 1.0401/hazelnuts, Dau c 1.0104/carrots, Mal d 1/apple, Api g 1/celery) and prior studies showed cross-reactive IgE in patients suffering from OAS, and allergen-specific responses of T cell clones and T cell lines generated in vitro. To date little is known about primary T cell reactivity in patients with OAS. This study analyses the T cell responses to recombinant Bet v 1a and Cor a 1.0401 in a panel of patients with birch pollen and concomitant hazelnut allergy (clinical history, skin-prick test, specific IgE). The use of recombinant allergens allows for defined preparations of a single protein species without the adjuvant effects of native pollen preparations. T cells were stimulated with autologous monocyte derived mature dendritic cells (DC) loaded with recombinant allergens in vitro. The recombinant allergens Bet v 1 a and Cor a 1.0401 were used at optimal, non-toxic concentrations (5–20 μg/ml) for loading of DC and subsequent stimulation of CD4+-T cells. In primary stimulations of CD4+-T cells with Bet v 1 a- or Cor a 1.0401-loaded DC, an allergen-specific cytokine pattern with high levels of IL-5 and IL-13 and slightly enhanced amounts of IFN-gamma was induced demonstrating a strong TH2 cytokine skewing. After primary activation, T cells of atopics with birch pollen associated OAS to hazelnut showed significant proliferation if restimulation was performed with either, the identical recombinant allergen as in primary stimulation or with the respective cross-reactive allergen. We could thus identify T cell cross-reactivity between rBet v 1a and rCor a 1.0401 in atopic patients suffering from OAS. This supports the role of T cell responses in the pathogenesis of pollen-associated OAS and emphasises the importance to target T cells with novel therapeutic approaches like the induction of tolerance using in vitro generated tolerogenic DC loaded with recombinant allergen preparations.

P010

Study of the immunogenicity and allergenicity of the cockroach allergen Per a 3 with special regard to its quaternary hexameric structure

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Cockroach allergens play a very important role in allergic diseases, especially asthma. The major allergen of the American cockroach,

Per a 3, naturally occurs as hexamer. The aim of this study was to investigate whether the hexameric structure of Per a 3 influences its allergenicity and immunogenicity. Therefore, we compared the different effects of native hexamers and dissociated monomers of cockroach hemolymph extracts, containing almost only Per a 3 proteins, on proliferation and Th1/Th2 cytokine production of CD4+ T cells in coculture with allergen-pulsed monocyte-derived dendritic cells as well as the leukotriene release of basophiles. The monomer induced a higher proliferation, a more substantial increase of IL-10 production as well as a more Th1-like immune response than the native hemolymph extracts. The leukotriene release assays revealed that the monomer is less effective than the hexamer employing the same amounts of molecules. Our data indicate that the quaternary structure can influence both allergenicity and immunogenicity. In addition, the monomeric variant of Per a 3 could be a possible candidate for a specific immunotherapy because the IgE-mediated allergic reaction is diminished and the immune response might be slightly shifted from Th2 to Th1 with high production of IL-10. (supported by NMFZ and DFG)

P011

Hindered gastric digestion represents a risk for severe reactions in food allergic patients

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Severe reactions in highly sensitized food allergic individuals represent major problems for patients and a great challenge for regulatory authorities. Accurate labelling of the main elicitors of food allergic reactions and determination of patients' allergen threshold levels is indispensable for developing avoidance strategies.We could reveal recently the fundamental role of gastric digestion in the effector phase of food allergy as it reduced the allergenic capacity of the model antigen codfish up to 10 000-fold. Moreover, elevation of pH levels in simulated gastric fluid to 2.75 and above resulted in complete abrogation of codfish digestion.Thus, in the present study we aimed to assess the absorption kinetics of fish proteins and investigated the clinical reactivity of fish allergic patients to codfish digested at physiological or elevated gastric pH.Open challenges with codfish performed in non-allergic subjects revealed absorption of biologically active fish allergens only 10 min after ingestion with maximal serum levels after 1–2 h indicating a partial pregastric absorption. Additionally, codfish allergic patients were tested with fish extracts digested with gastric enzymes at pH 2.0 and 3.0 in skin prick tests and titrated double-blind placebo-controlled food challenges. Incubation of fish proteins with digestive enzymes at pH 2.0 resulted in fragmentation of proteins leading to a reduced biological activity evidenced by a significantly smaller wheal reaction. Fish digested at pH 3.0 revealed comparable reactivity patterns as undigested extracts. Moreover, these test materials triggered reactions at 10- to 30-fold lower cumulated challenge doses in allergic patients.Our data indicate the paramount importance of gastric digestion for fish allergens as the quantitatively significant absorption and elicitation of symptoms seemed to take place in the intestine. Therefore, hindered digestion puts fish allergic patients at risk to develop severe allergic reactions at minute amounts of allergens.Supported by H220-B13 and F1808-B04 of the FWF and OeNB Jubiläumsfond grant Nr. 11375.

P012 (V18)

Critical role of TNF-α for tolerance to allergens: TNF-receptor 2 induced apoptosis of CD8+ effector T cells of contact hypersensitivity

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Specific immune suppression and induction of tolerance are essential processes in the regulation and circumvention of allergies. In this study, we use the model of low zone tolerance (LZT) to contact allergens, induced by epicutaneous application of subimmunogenic doses of haptens, resulting in the generation of regulatory CD8+ T cells (Tc2) that inhibit the development of Tc1-mediated contact hypersensitivity (CHS). Analysis of TNF-α and TNF-receptor deficient mice revealed that LZT is TNFα -dependent as TNFα -/- and receptor-double-KO-mice failed to develop LZT. Interestingly, TNFR1-/- (p55-/-) mice showed normal LZT responses, whereas no induction of LZT was observed in TNFR2-/- (p75-/-) mice. Additionally, prevention of hapten-specific clonal expansion of lymph node cells and upregulation of Tc2 cytokines after tolerization did not occur in TNFa-/-, receptor-double-KO- and TNFR2-/- mice. Adoptive transfer of T cells from tolerized TNFα -/-, TNFR2-/- or WT mice that were injected vice versa into WT, TNFα-/- or TNFR2-/- animals demonstrated that TNF-α? and TNFR2 signaling is not required for the induction and function of CD8+ suppressor T cells of LZT. However, TNF-α induced signaling via TNFR2 is essential for the effector phase of LZT. Reconstitution of TNFR2-/- mice with T cells of WT mice and adoptive transfer experiments showed that the expression of TNFR2 on CD8+ T cells of CHS is mandatory for LZT. Importantly, TNF-α induced apoptosis via TNFR2 in hapten-specific CD8+ effector T cells of CHS is essential for LZT and the inhibition of the T cell-dependent allergic inflammation.Thus, our data demonstrate a critical role of TNF-a via TNFR2 (p75) signaling in the effector phase of LZT to contact allergens, in which it inhibits the generation of CHS-promoting Tc1 cells and consequently the development of CHS and depicts the novel finding that apoptosis mediated by p75 -mediated signaling is involved in tolerance reactions to allergens.

P013 (V21)

Nickel compounds sensitize primary endothelial cells to TRAIL-induced apoptosis via down-regulation of cFLIP

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Primary endothelial cells are, in contrast to many other cell types, fully resistant to TRAIL-mediated apoptosis. However, when exposing them to the potent contact hapten nickel we noted that endothelial cells were dramatically sensitized to TRAIL-induced apoptosis as detected by hypodiploidy analysis and internucleosomal DNA fragmentation in a caspase-dependent manner. To investigate at what level nickel might sensitize endothelial cells for TRAIL-induced apoptosis, we first studied expression of TRAIL receptors 1–4 by flow cytometry. While

nickel did not affect expression of TRAIL-R1 and 3, it up-regulated TRAIL-R2 and strongly induced TRAIL-R4. Since the latter lacks a complete death domain and is therefore unable to transduce death signals, it appeared rather unlikely that nickel-mediated sensitization to TRAIL-induced apoptosis is attributable to surface regulation of TRAIL receptor expression. We thus biochemically characterized TRAIL-mediated signalling pathways in endothelial cells treated with nickel. Western blot analysis revealed rapid activation of the initiator caspase-8 in nickel-, but not diluent-treated endothelial cells. We next studied whether nickel interfered with the composition of the death-inducing signalling complex (DISC). In line with the increased surface expression of TRAIL death receptors and the initial biochemical data, the recruitment of the adapter molecule FADD to the TRAIL DISC was increased, while the amount of the apoptosis inhibitor cFLIP was decreased. These data indicated that the overall activation of caspase 8 at the DISC is enhanced which finally resulted in sensitization of nickel-treated endothelial cells to TRAIL-mediated apoptosis. Analyses at both the RNA and protein level of cFLIP expression following nickel treatment indicated that nickel leads to down-regulation of cFLIP within 8–16 h. To elucidate the functional relevance of these findings we used retrovirally expressed small hairpin RNA for efficient knock-down of cFLIP. Depletion of cFLIP led to sensitization of endothelial cells to TRAIL-induced apoptosis in the absence of nickel. Taken together, our data clearly demonstrate that exposure of primary endothelial cells to nickel sensitizes them towards TRAIL-induced apoptosis via down-regulation of the antiapoptotic caspase-8 inhibitor cFLIP. Such events may ultimately influence vascular permeability and facilitate the evasion of inflammatory cells from the circulation.

P014

The coordinated up- and down- regulation of cAMP during maturation licence 6-sulfoLacNAc-expressing dendritic cells (slanDC) to produce high levels of IL-12p70

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We previously identified a population of CD16+ myeloid cells in human blood selectively expressing 6-sulfoLacNAc on PSGL-1 and having functional features of dendritic cells, now called slanDC. SlanDC are a major source of IL-12 and TNF-α and can be found in inflamed tissue as in psoriasis vulgaris and rheumatoid arthritis. To produce IL-12, slanDC need to undergo 6 h of maturation before stimulation with LPS. This maturation is inhibited by erythrocytes and is initiated by cell–cell contacts. To get insights into the molecular mechanisms of this maturation we studied the relevance of the second messenger cAMP. Upon culture of slanDC cAMP levels peaked after 30 min and than dropped within 4 h. To study the relevance of this biphasic regulation of cAMP levels we first blocked the initial cAMP peak by inhibiting the adenylcyclase by specific inhibitors (DDA or SQ22536). Under these conditions, the upregulation of CD83, CD86, CD80, HLA-DR and CD54 as well as the production of IL-12 was blocked. The addition of exogenous 8-Br-cAMP restored this maturation. Signaling of cAMP critically involves proteinkinase A (PKA) and in line with this, we observed a reduced CD83 upregulation and IL-12 production in slanDC treated with the PKA-inhibitor H89. We next asked for the relevance of low cAMP levels after 4 h. To enhance cellular cAMP levels we stimulated the generation of cAMP by PGE2 or blocked its degradation by inhibiting the phosphodiesterase 4 (PDE4) using roflumilast. Cells treated in this way showed normal phenotypic maturation and were good stimulators of an allogeneic mixed leukocyte reaction with naïve T cells. However, slanDC treated with PDE4-inhibitors failed to produce IL-12 and displayed a reduced programming of Th1 cells. Taken together, we show that the high IL-12 production of slanDC requires the coordinated expression of high and low cAMP levels before stimulation with LPS. The phenotypic maturation of slanDC requires only the initial upregulation of cAMP levels. These data shed new light on the relevance of cAMP for the maturation of dendritic cells and helps to understand conflicting results of previous studies, where cAMP was either regarded as an inhibitor or as a stimulator of IL-12 production.

P015

Does systemic metal (nickel-) exposure in implant bearing patients induce enhanced metal (nickel) sensitivity?

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Nickel sensitivity is assessed by patch testing and may be also evaluated by lymphocyte transformation test (LTT). We wondered, if patients with arthroplasty-associated complications may show enhanced nickel sensitization rates.A total of 150 patients (111 with arthroplasty and complications; 39 patients without implants) were patch tested and their PBMC were used for metal specific LTT. Analysis of results was in addition separated according to present/absent history of cutaneous metal intolerance reactions (CMIR).Results in patients without implants and no history of CMIR (resp. positive history of CMIR) were: 100% no patch test reactivity (resp. 50% reactivity) to nickel; 100% no LTT reactivity (resp. 53% LTT reactivity) to nickel.Results in patients with implants and no history of CMIR (resp. positive history of CMIR) were: 93% no patch test reactivity (resp. 35% reactivity) to nickel; 64% no LTT reactivity (resp. 49% LTT reactivity) to nickel.We thus conclude, that in patients with complicated arthroplasty enhanced LTT reactivity to nickel as compared to patch test results can be found. This may reflect systemic nickel exposure and potentially contribute to implant-associated intolerance reactions.

P016

Carboplatin hypersensitivity in advanced melanoma patients treated with a multi-agent chemotherapeutic regimen

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Carboplatin is a cytotoxic compound frequently enclosed to multi-agent chemotherapeutic regimens used in the treatment of many different cancers including melanoma. In Ovarian cancer, a 2.5–10% incidence of documented cases of immediate-type hypersensitivity has been reported. Carboplatin is suggested to act as a hapten, binding to serum proteins, hence becoming allergenic. Carboplatin hypersensitivity was shown to develop mainly between 6th and 8th cycle of drug administration.Here, we present two cases of melanoma patients who underwent a multi-agent chemotherapeutic regimen with carboplatin, vindesine and temozolomide and subsequently developed immediate-type hypersensitivity reactions (facial flushing, itching, dyspnea and anaphylaxis). Both patients were in clinical stage IV and had previously received a minimum of 6 cycles of multi-agent chemotherapy. Skin tests composed of prick tests as well as intra-cutaneous (i.c) injection were performed with

diluted carboplatin (0.01–0.1 mg/ml), cisplatin (DDP, 0.001–0.1 mg/ml), vindesine (0.001–0.1 mg/ml) or undiluted temozolomide.Positive reactions (i.c) could only be observed to carboplatin. Total IgE levels of these patients were in normal range and patients had no previous history of allergy. As a consequence carboplatin administration was replaced by cisplatin.This report should: (i) alert clinicians treating melanoma patients with carboplatin-containing regimens to be aware of immediate-type hypersensitivity reactions to carboplatin and (ii) show that these hypersensitivity reactions are not necessarily cross-reactive to DDP.We conclude that in our cases immediate-type hypersensitivity reactions to carboplatin may be directed against the closed cyclobutane dicarboxylate moiety, which is not shared by its parental compound DDP. Therefore DDP may substitute for carboplatin in platinum compounds-containing treatment regimens which may allow to avoid other procedures associated with higher risk such as carboplatin desensitization strategies or concomitant use of steroids/anti-histamines.So far this is the first description of immediate-type hypersensitivity reactions to carboplatin in melanoma patients under multi-agent chemotherapeutic regimens containing carboplatin.

P017

Alpha-Melanocyte-stimulating hormone modulates the functional activity of human peripheral blood eosinophils

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Alpha-melanocyte stimulating hormone (alpha-MSH) is a peptide hormone derived from pro-opiomelanocortin (POMC). Albeit originally characterized as a neuropeptide its expression by many peripheral tissues including the immune system suggests biologic functions beyond melanogenesis and steroidogenesis. This is supported by the detection of melanocortin receptors (MC-Rs) in many different cell types and peripheral tissues. However, until now nothing is known on the role of the POMC system in eosinophils which are key effector cells in several allergic diseases as well as in a number of poorly understood eosinophilic disorders.We therefore purified eosinophil granulocytes from non-atopic healthy subjects and patients with atopy by CD16-negative selection (>98% purity). Expression of MC-1R was assessed by conventional RT-PCR, real-time PCR and flow cytometry using an antibody against MC-1R (FACScalibur). Expression of POMC was determined by conventional RT-PCR and real-time PCR. Release of superoxide anions in response to complement factor C5a was examined by lucigenin-dependent chemiluminescence (Hamamatsu).Peripheral blood eosinophils from both healthy individuals ($n = 8$) and atopic patients ($n = 7$) expressed MC-1R as well as POMC at similar RNA levels. However, MC-1R expression at the protein levels in eosinophils was significantly reduced in patients with atopy ($n = 5$) compared with those from normal individuals ($n = 5$). Functional studies revealed that stimulation with complement factor C5a led to a dramatic release of superoxide anions ($P < 0.001$) which was significantly inhibited by alpha-MSH ($P < 0.001$). The specificity of this effect of alpha-MSH was completely reversed by preincubation of the cells with agouti signalling peptide (ASP), a natural MC-1R antagonist ($P < 0.01$).These findings provide evidence for a modulating effect of alpha-MSH on eosinophil function. Our results may not only be important for the present understanding of the anti-inflammatory activity of alpha-MSH and related peptides in animal models of inflammation and allergy but also for the future exploitation of such peptides in the treatment of human inflammatory and allergic disorders.

P018 (V33)

Reduced in vivo allergenic reactivity of Lyc e 1 silenced transgenic tomato fruits

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Background: Profilin is a small actin binding protein which contributes to the allergenic potency of many fruits and vegetables including tomato. Two highly similar genes encoding tomato profilin have been isolated and designated as allergen Lyc e 1.01 and Lyc e 1.02.
Objective: The aim of the study was to generate profilin-reduced hypoallergenic tomato fruits by silencing of both genes in transgenic tomato plants using RNA interference.
Methods: Efficiency of gene silencing was documented by northern blotting, immunoblotting and skin prick testing.
Results: Quantification of the remaining protein revealed that profilin accumulation in transgenic fruits was decreased 10-fold as compared to untransformed controls. This decrease was sufficient to cause a reduced allergenic reactivity in tomato allergic patients as determined by skin prick tests. Since most tomato allergic patients are not monosensitized to profilin, the IgE-reactivity to the profilin-silenced tomato fruits in vivo varied widely between individuals tested.
Conclusion: We could demonstrate the efficient silencing of both profilin genes in transgenic tomato plants using RNA interference. This resulted in Lyc e 1 diminished tomato fruits, providing proof-of-concept and demonstrating that RNA interference can be used to design allergen reduced food. However, simultaneous silencing of multiple allergens will be required to design hypoallergenic tomatoes. Our findings demonstrate the feasibility of creating low allergenic food by RNA interference.

P019

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

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Patients with extrinsic atopic dermatitis (AD) are characterized, among other phenomena, by elevated levels of serum IgE and by a defect of CD8+ T cells, namely by perforin (PF)-granule reduction and Pf-hyperreleasability. Whereas CD8+ T cells are known to regulate IgE in mice and rats, their role in the human IgE-system remains unclear. IgE-levels of 45 patients with exacerbated AD were determined using the Pharmacia Cap-system. The CD8+ T cell compartment was characterized in parallel by flow cytometry. In addition, total and specific IgE-levels were measured over a time period of 10 days in culture supernatant of (i) ficoll-isolated peripheral mononuclear cells (PBMC), (ii) PBMC depleted of CD8+ T cells by Milteny beads and, as an additional control, (iii) CD8-depleted PBMC reconstituted with CD8+ T cells. Cells were obtained from six AD patients (serum IgE levels 200–5000 U/ml) and four healthy control individuals. Using SPSS for statistical analysis, a significant negative correlation was found between total serum IgE levels and the absolute number of peripheral CD8+ T cells (Pearson coeff. = −0.28, p = 0.02). Depleting >95% of CD8+ T cells in vitro resulted in higher specific and total IgE levels as compared to controls which was most significant at day 8–10. In vitro, IgE

levels correlated with the portion of Pf+ CD8+ T cells (e.g. day 9, total IgE, Pearson coeff. = 0.89, p = 0.04). Taken together we found correlative evidence that CD8+ T cells may be involved in IgE regulation in vivo. The in vitro data suggest that depleting the CD8+ T cell compartment removes an IgE-regulating population which results in elevated specific and total IgE levels. Since the level of IgE in vitro correlated with the amount of Pf+ CD8+ T cells removed, and Pf is known to downregulate immunoglobulin production in general, one may speculate that these cells are involved decisively in IgE-regulation.

P020

Perforin (Pf) is involved in the control of the IgE response to ovalbumin (OVA) in vivo

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In atopic patients, a severe defect of the perforin (Pf)-granule system was described, namely Pf-reduction and Pf-hyperreleasability. In addition, these patients are characterised by elevated serum levels of total IgE and by an exaggerated IgE-response to allergens. To elucidate, if Pf indeed regulates the IgE response to foreign proteins in vivo, we backcrossed Pf-knockout (-/-) mice from a C57BL/6 background (known low IgE responder, purchased from The Jackson Laboratory, Bar Harbor, Maine, USA; C57BL/6-Prf1tm1Sdz/J) onto a Balb/cJ background (good IgE responder, The Jackson Laboratory, BALB/cJ). 10 Pf -/- and 10 wt female F8-mice were immunized i.p. with OVA at week 7, 9 and 15 , b 1 week after birth using alumn as adjuvant. Blood was obtained at week 7, 9, 12, 15 and 17. Total serum IgE-levels (serum dilution 1:250) were measured with a commercially available ELISA in doublets (BD, Heidelberg), OVA-specific IgE levels (serum dilution 1:50) with an OVA-specific sandwich ELISA. In each group, three animals did not respond with an increase of IgE after two immunizations and were excluded. Results were analysed using the SPSS-software. After the second and third immunization, the OVA-specific IgE response as well as total IgE levels were higher in Pf -/- mice as compared to wt animals. This was significant for OVA-specific IgE at week 12, and for total IgE at week 17. This suggests that Pf is involved in downregulation of the IgE response to repeated antigen exposure in vivo.

P021

Immunohistochemical characterization of inflammatory infiltrate of intraepidermal and invasive squamous cell carcinoma of the skin in immunocompetent patients and organ transplant recipients

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Incidence of epithelial skin cancer is increasing. Organ transplant recipients (OTRs) are at a much higher risk than immunocompetent patients to develop squamous cell carcinoma of the skin. Histologically, peritumoral inflammatory infiltrate is a frequent feature and is believed to be a correlate of antitumoral immune response. Our aim was to characterize peritumoral inflammatory infiltrate in intraepidermal (actinic keratosis or Bowen's disease) and subsequent invasive squamous cell carcinoma in the same patient in organ transplant recipients ($n = 42$) and immunocompetent patients ($n = 43$). Thus, in 170 formalin fixed, paraffin-embedded tissue samples peritumoral inflammatory infiltrate was characterized by its extent, density, localisation in the dermis and by its composition focusing on immunohistochemical expression of the T-cell markers CD3, CD4, CD8 and FOXP3. Maximum diameter of infiltrate was larger in invasive lesions than in their intraepidermal precursor lesions ($p < 0.005$) but did not differ between immunocompetent patients and OTRs ($p = 0.22$). Inflammatory infiltrate in immunocompetent patients was more dense than in OTRs ($p = 0.041$) and more dense in invasive than in intraepidermal lesions ($p = 0.005$). Immunocompetent subjects showed higher rates of CD3 ($p = 0.015$) and CD8 ($p = 0.038$) immunoexpression across all lesions but comparable CD4 immunoreactivity ($p = 0.169$) to OTRs. In invasive lesions, immunocompetent patients showed higher levels of FOXP3 immunoexpression than OTRs ($p = 0.049$). In conclusion peritumoral inflammatory infiltrate was more pronounced as measured by density, CD3, CD8 and FOXP3 expression in immunocompetent patients than in OTRs and grew larger and more dense on the course from intraepidermal to invasive squamous cell carcinoma.

P022

Keratinocytes are type I IFN producers in cutaneous lupus erythematosus skin lesions

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Background: Cutaneous lupus erythematosus (CLE) is one of the most common dermatological autoimmune disorders worldwide. The pathogenesis is unclear, but several studies provided evidence for a strong pathogenic role of type I IFNs in this disease. We could recently show that a type I IFN associated recruitment of cytotoxic lymphocytes via CXCR3 <-> CXCL10 interaction is characteristic for CLE skin lesions. Plasmacytoid DCs (pDC) are supposed to be the major type I IFN producers in CLE skin lesions. However, pDCs are predominantly found within the dermal inflammatory infiltrate, while most CLE subtypes histologically are characterized by a junctional "interface" dermatitis. This prompted us to hypothesize that keratinocytes on their own might be type I IFN producers in CLE.

Patients and methods: Lesional CLE skin biopsies were characterized by in situ hybridisiation (ISH) and immunohistochemistry (IHC) for the expression of IFN alpha, IFN beta and a type I IFN associated inflammation. Keratinocyte cultures were used for functional investigations.

Results: ISH revealed a strong message for IFN alpha in the whole epidermis as well as in dermal areas with pDC invasion, while IFN beta mRNA was predominantly detectable in the basal epidermal layers. The CXCR3 ligands CXCL9 and 10 were also expressed in the epidermis of lesional CLE, but not in healthy controls. All results were confirmed by IHC.

Conclusion: Keratinocytes are type I IFN producers in CLE skin lesions. We believe that lesional type I IFN production induces the expression of several chemokines including CXCL 9 and 10, sustains the cytotoxic capacity of infiltrating lymphocytes and supports that way the recruitment of CXCR3+ "effector" CTLs.

P023 (V01)

Gene expression profiling of lichen planus reflects CXCL9+ mediated inflammation and distinguishes this disease from atopic dermatitis and psoriasis

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Gene expression profiling potentially can complement established diagnostic methods such as histopathology and may provide insight into pathogenesis and therapeutic responses in skin disease. Here we present data of a novel approach to apply the diagnostic value and pathological significance of this method in different skin disorders, using whole skin biopsies.Initially, Serial Analysis of Gene Expression [SAGE(TM)] was performed to identify frequent tags differentially expressed in various skin diseases versus healthy skin. Based on these results and literature data a skin-pathology oriented PIQOR(TM) (Parallel Identification and Quantification of RNA#s) microarray system was designed. To evaluate this system, lichen planus (LP, $n = 20$) was chosen as a model disease, since this disorder is well defined by specific clinical and histological findings. Controls included healthy skin ($n = 20$), atopic dermatitis ($n = 20$), and psoriasis ($n = 20$).SAGE(TM) analyses revealed 60 genes whose expression was specifically upregulated in LP. Gene expression analyses using the new topic-defined microarray chip followed by unclassified clustering was able to discriminate LP from AD and Pso. Genes significantly expressed in LP included type I IFN inducible genes and a specific chemokine expression pattern. The CXCR3 ligand CXCL9 was the most significant marker for LP. In situ hybridisation and immunohistochemistry confirmed the results of gene expression profiling. Additionally they revealed the new finding, that keratinocytes are primary type I IFN producers in LP skin lesions.Our results show that gene expression profiling using a skin specific chip with 1542 genes followed by unclassified clustering is a reliable method to identify patients with LP in the chosen context. Cytokine and chemokine expression patterns reflect recent models concerning the pathogenesis of this disease. These findings support our hypothesis, that gene expression profiling usefully completes the diagnostic spectrum in dermatology and may provide new starting points for therapeutic targets.

P024

In vivo evidence for oxidative damage and persistent activation of macrophages with enhanced nitric oxide and metalloelastase release contributing to the hostile microenvironment and accelerated aging of ulcer-adjacent fibroblasts of chronic venous leg ulcers

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Reactive oxygen/nitrogen species produced by macrophages in presence of iron released by erythrocytes have been suggested to contribute to disturbed healing in chronic venous leg ulcers. So far, little in vivo evidence for ROS-induced impaired healing is available. Here we analysed the implications of oxidative damage in inflammatory cells infiltrating chronic venous leg ulcers.Skin biopsies from patients with chronic venous leg ulcers revealed a persistent inflammatory infiltrate mainly consisting of macrophages. In contrast, in acute wounds, inflammatory cells mainly represented by CD66+ PMN at day 1 and CD68+ macrophages at day 2 decreased and eventually disappeared at day 5 after wounding. In chronic, but not in acute wounds, macrophages stained strongly positive for 8-hydroxy-2-deoxyguanosine (8OHdG) and nitrotyrosine (NT), indicating oxidative stress-induced DNA and protein damage. Exposure of human macrophages to H2O2 and iron simulating the Fenton reaction in vitro also led to generation of 8OHdG and NT. Macrophages subjected to H2O2/iron showed an increased, iron-dependent release of NO and MMP-12. Higher expression of MMP-12 and iNOS was detected by immunostaining in chronic venous leg ulcers when compared with normal skin or acute wounds.In the dermis adjacent to chronic venous leg ulcers, but not in acute wounds or normal skin of age-matched controls, a high number of fibroblasts stained positively for p16, an inhibitor of cyclin-dependent kinases 4/6 which was recently accepted as a robust in vivo marker for skin aging. This suggests a causal relationship between increased ROS generation and cell senescence.We here identify a novel mechanism for NO release in human macrophages with a major impact for disturbed wound healing and for NO physiology in general. In chronic venous leg ulcers, the enhanced release of NO in conjunction with ROS results in oxidative/nitrosative damage and activation of macrophages with subsequent enhanced release of MMP-12 leading to degradation of extracellular matrix and oxidative stress-induced senescence of resident fibroblasts, key events which further amplify dysfunctional wound healing.

P025

The impact of aging on the expression of selected genes (heat shock proteins, elastin, fibrillin, and steroid hormone receptors) in human skin

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Changes in steroid hormones and their receptors, extracellular matrix proteins, and heat shock proteins (HSP) have been discussed as causes and/or consequences of intrinsic skin aging. Thus, in the present study mRNA expression of estrogen receptors 1 and 2 (ESR1, ESR2), progesterone receptor (PGR), elastin (ELN), fibrillin 1 (FBN1), and heat shock proteins 27, 72, and 90 (HSP27, HSP72, HSP90) were quantified in the skin of healthy female volunteers. 44 volunteers (phototypes II and III) were included in the study; ages were between 19 and 28 (group I, $n = 22$) and between 55 and 65 (group II, $n = 22$). Punch biopsies were taken from the upper inner arms and the expression of the genes of interest was quantified by real-time RT-PCR. Immunohistochemistry was used to investigate HSP27, HSP72 and fibrillin 1 before and after heat stress. Elastic fibers were visualized by Weigert's stain. We found that expression of ESR1, ESR2, PGR, HSP27, HSP72, and HSP90 did not show significant differences between age groups. Expression of ELN and FBN1 was significantly higher in group II (ages 55–65years)

($p = 0.037$ and 0.001, respectively; *U*-test). Ex vivo heat shock induced an identically strong increase in the intensity of hsp72 immunostaining in both groups. For hsp27 a less pronounced but statistically significant induction was only observed in group I, but not in group II. Immunohistology and Weigert's stain demonstrated age dependent degeneration and reduced stress resistance of fibrillin rich microfibrills in the upper dermis. These results demonstrate that in sun protected healthy female skin the expression of steroid receptors and HSP remains remarkably stable between 19 and 65 years indicating that these factors are essential for skin physiology. The reduced ability to respond to stress with induction of hsp27 and the increased expression of the major components of elastic fibres might correspond to an impaired adaptive capacity and to a dysregulation of extracellular matrix formation that contribute to the phenotype of aged skin.

P026

Hornerin is expressed in healthy and inflamed skin

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Epidermal differentiation results in the cornified envelope, building up the frontline physical defense shield against diverse environmental hazards. A number of genes specifying structural proteins expressed late during epidermal differentiation have been identified and found to be clustered on chromosome 1q21, among them S100 proteins and so-called "fused" members of CE precursor proteins such as profilaggrin, trichohyalin, repetin, cornulin and hornerin. We have previously shown that the S100-protein psoriasin has antimicrobial properties. In another study we could show, that defined peptide fragments of Hornerin, which we had isolated from healthy persons stratum corneum, have antimicrobial properties. Because a recent study has shown immunoreactive hornerin by immunohistochemistry in human psoriatic skin and wounds, but not in healthy skin, we analysed the immunohistological distribution of hornerin in healthy and inflamed skin.Antisera against recombinant rather N-terminal and C-terminal hornerin peptides (rHRNR2, (residues 1075–1172) and rHRNR3 (residues 2591–2662) were raised in two goats and antibodies were subsequently affinity-purified. Specificity was tested against recombinant hornerin peptides as well as hornerin peptides obtained from stratum corneum extracts by immunoblotting techniques. As a result immunohistochemical analyses of healthy skin revealed hornerin expression – in contrast to a previous study – in the epidermis, preferentially at the apical part, and prominently in the stratum corneum at different localizations like face, trunk, arms, hands, legs and feet. Both antibodies gave the same profile. Analyzing 12–15 samples in each group, varying individual expression levels of hornerin were observed. In lesional skin of atopic dermatitis, psoriasis and verrucae vulgares hornerin immunoreactivity showed similar extent and patterns as seen in non-lesional areas of the same patient.Taking together hornerin is expressed constitutively in the epidermis at various localizations of healthy skin and in lesional skin of inflammatory diseases. Expression levels vary individually by a yet unknown mechanism.

P027

Tumor environmental dose radiotherapy induces lymphangiogenesis in the skin

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We studied the time course of microvascular changes in the environment of radiated tumors in a standardized human protocol. 80 skin biopsies from 40 patients with previously-treated primary breast cancer were taken from radiated skin and corresponding contra-lateral non-radiated control areas either 2–8 weeks, one, or more than 2 years after radiotherapy (skin equivalent dose 30–40 Gy).There was an increase of total podoplanin+ lymphatic microvessel density resulting mainly from a duplication of the density of smallest lymphatic vessels (diameter $< 10\ \mu$m) in the samples taken 1 year after radiation. Our findings implicate radiogenic lymphangiogenesis during the first posttherapeutic year. This effect was less in older patients and in chronic lymphedema. The numbers of CD68+ VEGF-C+ cells were highly elevated in radiated skin in the samples taken 2–8 weeks after radiotherapy. Thus, VEGF-C expression by invading macrophages can be supposed to be the major pathogenetic route of induction of radiogenic lymphangiogenesis. In contrast to the lymphatics, blood microvessels showed no significant changes in calibers or densities after radiotherapy with an exception of a transient increase of large venules shortly after radiotherapy that might reflect increased postexpositional blood-flow.Summarizing, we first report lymphoproliferation induced by radiotherapy. We introduce the term "radiogenic lymphangiogenesis" – a process that might be important in the development of local recurrences or metastases in case of surviving tumor cells in the environment of the radiated tumor.

P028

The subepidermal nerve plexus analysed by electron microscopy

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As the most distal part of the nervous system, the subepidermal nerve plexus is located in the papillary dermis. Its network of fine nerve fibers embraces our whole body surface and enables us to perceive stimuli from our environment. The fibres are composed of axons ensheathed by terminal Schwann cells and in their delicateness they are predominantely found in the subepidermal nerve plexus as they form larger nerve bundles to cross the reticular dermis and subcutaneous tissue on their way to join the spinal cord.We were interested in the ultrastructure of the subepidermal nerve plexus and performed electron microscopy on immunohistochemically stained

horizontal sections of papillary dermis. Native dermal sheet preparations were incubated with Nerve Growth Factor receptor antibody and fixation was done after incubation in primary antibody and completed with postfixation at the end of the staining procedure. A total of nine sections containing 61 nerve fibers were analysed in detail. The thickness of the single nerve fibers ranged from 1244 nm to 35303 nm with a median diameter of 4737 nm. The median axon number ensheathed per Schwann cell was two with a range from 1 to 27. Axon diameter ranged from 197 nm to 1925 nm with a median caliber of 610 nm. Approximately 15% of axons were found to be only incompletely invaginated by terminal Schwann cells being closely associated to the extracellular space. All of the nerve fibers found were unmyelinated. But five nerve fibers contained axons which were wrapped by two or three layers of the mesaxon and strikingly all of these fibers additionally contained further axons wrapped by only one layer of mesaxon. Potentially, this rudimental multi-layered sheath depicts the beginning of myelination in the periphery. However, myelinated and unmyelinated axons have never been shown to occur in the same Schwann cell. So far, ultrastructural descriptions mostly concentrate on larger nerve fibers, being sparse in response to the details of the fine fibers in the subepidermal nerve plexus. We were able to specify morphological data being useful in the further evaluation of physiological and pathological changes of the superficial dermal nerve plexus. As skin biopsies are increasingly discovered an exciting tool to evaluate peripheral neuropathies, this is of emerging importance.

P029

Akkuranz der klinischen Beurteilung melanozytärer Läsionen: Korrelation mit histologischer Diagnose

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Melanozytäre Läsionen verursachen viele diagnostische Hautbiopsien. Wenige Studien sind über die Akkuranz klinischer Beurteilung erschienen. Wir untersuchten daher 3822 klinisch als melanozytär beurteilte Hautbiopsien. 10% waren nicht melanozytäre Läsionen wie seborrhoische Keratose, Histiozytom, Spinaliom, während >1% entzündlich waren. Die häufigste Fehleinschätzung war die seborrhoische Keratose in 5%. In 8% hielten Kliniker melanozytäre Läsionen für Histiozytom, seborrhoische Keratose oder Basaliom. Die Akkuranz der klinischen Beurteilung war am besten für gutartige Naevi (64–78%), am schlechtesten für atypische Naevi (6–12%), mittel für Melanom (58–43%). Zumindest bei gutartigen Naevi urteilten Dermatologen signifikant besser als Nichtdermatologen. Mit geringerer Akkuranz wurden benigne Naevi (72–86%), atypische Naevi (88–93%) und Melanom (66–78%) als melanozytäre Läsionen ungeachtet der Dignität erkannt. Schließlich wurden 97–99% benigner Naevi, 99% atypischer Naevi und 91–99% der Melanome korrekt als Tumor beurteilt. Klinisch komplett exzidierte melanozytäre Läsionen waren histologisch nicht in toto in 10–18%, aber mit klinisch zunehmendem Verdacht auf Malignität stieg die Totalitätsrate der Exzision. Zusammengefasst urteilten in unserem Kollektiv Dermatologen klinisch geringgradig besser als Nichtdermatologen in der korrekten Identifikation melanozytärer Läsionen, aber alle zeigten substantielle Fehleinschätzungen in Typ und Dignität der Läsionen sowie in der Totalität der Exzision.

P030 (V19)

CCL18 is expressed in bullous pemphigoid and correlates with disease course

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The autoimmune skin disease bullous pemphigoid (BP) is characterized by subepidermal blister formation and a strong dermal infiltrate of mononuclear cells and eosinophils. The T cells in BP secrete mainly Th2 cytokines and proliferate in response to peptides of the BP autoantigen BP180. Thereby, they contribute to the concurrent infiltration of eosinophils and disease manifestation. To characterize the mechanisms of T cell homing to BP skin, we investigated the role of the chemokine CCL18 in this disease. The secretion of CCL18 by antigen presenting cells is induced by the TH2-cytokine IL-4. CCL18 has the potential to recruit naïve T cells as well as memory Th2 cells. Analyzing the CCL18 expression in BP patients we detected high concentrations of CCL18 in blister fluid which were 5-fold increased to normal CCL18 levels in blood of healthy individuals. In blood of BP patients the CCL18 levels were increased by 84% of the concentration in healthy individuals. We demonstrate the chemoattractive function of CCL18 by showing that T cells isolated from fresh blood mononuclear cells from untreated BP patients migrated in response to CCL18 as well as to blister fluid. By immunofluorescence staining we identified Langerhans cells and antigen presenting cells of the dermis as main CCL18 producers in BP skin. To investigate the relevance of CCL18 as a biomarker in chronically relapsing BP we analysed CCL18 serum levels in eight patients following their disease course over a maximum period of 5 years. Extent of skin lesions was assessed on a 4 point clinical scale. Importantly, CCL18 levels correlated with the disease course in 75% of the patients whereas titers of indirect immunofluorescence staining to basement membrane deposition of autoantibodies, the main diagnostic tool in BP, did not correlate in most of the patients. Thus, we suggest CCL18 as a new diagnostic tool for bullous pemphigoid that can be easily detected by commercially available ELISA systems. Furthermore, our results implicate that CCL18 is a highly concentrated chemokine in BP serum and blister fluid which has the potential to recruit blood cells from BP patients.

P031

Massive influx of CD8dim+ perforin+ T lymphocytes into alopecia areata scalp skin lesions

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We recently reported a significant reduction of CD8dim+ Pf+ peripheral lymphocytes in AA. Since this may be caused by extravasation into the scalp skin, the inflammatory infiltrate of AA-lesions ($n = 7$) as compared to normal scalp skin from healthy controls ($n = 5$) was analysed by flow cytometry using a FACScan after mechanical disintegration of punch biopsies. Peripheral blood was analysed in parallel. In the AA-infiltrate, CD8dim+ lymphocytes outnumbered CD8high+ cells. This was significantly different from controls as well as from peripheral blood. In addition, significantly more perforin (Pf)-containing

lymphocytes were detected in AA-lesions as compared to controls. In comparison to peripheral blood CD8dim+ lymphocytes, lesional CD8dim+ AA-lymphocytes expressed less Pf per cell as judged semi-quantitatively by mean fluorescence intensity. Our results indicate that the significant reduction of CD8dim+ Pf+ peripheral lymphocytes in AA is caused at least in part by a massive influx of these cells into lesional scalp skin where they release Pf and contribute decisively to the pathogenesis of AA.

P032

Filaggrin mutations p.R501X, c.2282del4, p.R2447X and c.6867delAG in ichthyosis vulgaris

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Ichthyosis vulgaris (IV) is the most common hereditary disorder of cornification in humans, characterized by generalized fine scaling of the skin, palmar hyperlinearity with or without keratosis pilaris and atopy. Recently, the molecular basis of IV was ascribed to loss-of-function mutations in the gene encoding filaggrin (FLG), namely p.R501X and c.2282del4 in the first of the 10–12 filaggrin repeats of exon 3. We here confirm the presence of these two mutations in FLG in 15 out of 21 IV-patients including eight affected individuals of a four-generation family, showing homozygosity, compound heterozygosity or heterozygosity for p.R501X and c.2282del4. Notably, two of the 16 pedigree members with no discernible phenotype are heterozygous for p.R501X. An extended search for additional mutations in the distal portion of exon 3 of the FLG gene, uncovered compound heterozygosity due to p.R2447X in filaggrin repeat 7 in three out of eight affected family members and five asymptomatic individuals. Moreover, in an isolated case of severe IV a novel 2 bp deletion in filaggrin repeat 6, namely c.6867delAG, is found in addition to p.R501X. Surprisingly, this compound heterozygous patient clearly shows an apparent granular layer and FLG expression on immunohistochemistry. Taken together, these novel FLG mutations confirm that FLG is a major IV gene and point to the existence of modifiers of the disease.

P033

Cystic sebaceous gland tumor caused by an underlying germline mutation in MSH6

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Clinical criteria for diagnosis of Muir-Torre syndrome include synchronous or metachronous occurrence of at least one sebaceous gland neoplasia (adenoma, epithelioma or carcinoma) and at least one internal neoplasm in a patient. To date, Muir-Torre patients with mutations in MSH2 and MLH1 have been reported in the literature, with the majority of mutations located in MSH2. Here, we present the first MSH6 mutation in a Muir-Torre patient with a cystic sebaceous gland tumor and provide an update on mutations identified in our large Muir-Torre patient sample.

P034

Fc receptor-like 3 gene (FCRL3) in alopecia areata: Investigation of the functional variant c.−169C>T

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A common genetic background for several autoimmune diseases has been demonstrated by an overlap of susceptibility alleles at different loci, including HLA and non-HLA genes. One of these non-HLA-regions is located on chromosome 1q21-q23 which contains a novel cluster of FcR-like receptors, also called FCRLs. A functional variant in the FCRL3 gene (c.−169C>T) has been implicated in susceptibility to autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus and autoimmune thyroid disease. Investigation of the functional variant in the FCRL3 gene in a large case-control sample of alopecia areata patients fails to support the hypothesis that this variant is also involved in susceptibility to alopecia areata.

P035 (V23)

TNF, LTA and TNF receptor polymorphisms in psoriasis vulgaris and psoriatic arthritis: Evidence for an association of psoriatic arthritis with the promoter polymorphism TNF-857 independent of the PSORS1 risk allele

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Objective: Single nucleotide polymorphisms (SNPs) of TNF gene at positions −238 and −308 have earlier been associated with psoriasis vulgaris and psoriatic arthritis (PsA). The strong linkage disequilibrium

(LD) at chromosomal region 6p21 – a region known to harbour also other risk factors for psoriasis (PSORS1) than SNPs of TNF gene – renders the interpretation of these findings difficult. Therefore in this study several SNPs of TNF gene and of its neighbouring LTA gene were analysed independently and dependently on carrying the PSORS1 risk allele.
Methods: SNPs in the promoter of TNF gene (−238G/A, −308G/A, −857C/T, −1031T/C), one of LTA gene (+252A/G), of TNLFRSF1A gene (+36A/G) and of TNLFRSF1B gene (+676T/G), respectively, were genotyped in 375 Psoriasis-patients, 375 PsA-patients, and 376 controls. The tryptophan-tryptophan-cysteine-cysteine haplotype of the CCHCR1 gene (CCHCR1*WWCC) was used to estimate the PSORS1 risk allele.
Results: Whereas the earlier described strong association of allele TNF*−238A with psoriasis could be confirmed, our study revealed that this association was completely dependent on carrying the PSORS1 risk allele. For PsA, but not psoriasis vulgaris without joint involvement strong association with the allele TNF*−857T was detected (OR = 1.956, 95% CI 1.33–2.88; pcorr = 0.0019) also in patients negative for the PSORS1 risk allele.
Conclusions: Our results indicate genetic differences between psoriasis vulgaris patients with and without joint manifestation. While the previously reported association between TNF*−238A and psoriasis seems to primarily reflect linkage disequilibrium with PSORS1, TNF*−857T may represent a risk factor for PsA independent of PSORS1.

P036

Spectrum of FGFR3 mutations in multiple intraindividual seborrheic keratoses

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Activating mutations of the fibroblast growth factor receptor 3 (FGFR3) cause skeletal dysplasia syndromes in germline. Somatic FGFR3 mutations have been identified in various cancers and in benign skin tumors such as seborrheic keratoses (SK). These lesions represent one of the most common tumors in man. Affected individuals often show a large number of disseminated SK. The spectrum of FGFR3 mutations in patients with multiple SK has not been determined yet. We therefore screened 78 SK of four patients (19, 19, 21, and 19 SK/patient, respectively) for the presence of FGFR3 mutations. A previously described high throughput SNaPshot multiplex assay was used covering all FGFR3 mutations reported in human skin tumors so far. FGFR3 mutations were detected in 46 of 78 SK (59%). However, the mutation frequency varied considerably in the patients, ranging from 26% to 89%. Four SK revealed a double and one a triple FGFR3 mutation. The different FGFR3 mutations were rather stochastically distributed among the patients, who showed at least four distinct mutated loci. Interestingly, all mutations detected in this series of human SK were linked to a severe skeletal dysplasia phenotype in germline (thanatophoric dysplasia and SADDAN syndrome). Our results show that FGFR3 mutations are common genetic alterations in multiple SK with a varying mutation frequency between individuals but without specific intraindividual mutational hot spots. The mechanism leading to the high frequency of FGFR3 mutations in human SK as well as details of FGFR3 signaling in mutant cells remains elusive.

P037

Pili annulati- genetic analysis of 4 additional families with pili annulati and expression analysis of genes in the critical region using hair follicle RNA

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Pili annulati is an autosomal dominant transmitted hereditary hair disorder characterized by alternating light and dark bands in the hair fibre of affected individuals. Recently, a locus for pili annulati was mapped to chromosome 12q24.32–24.33 by linkage analysis in five families segregating this trait. Recombination events defined a critical region of 8 Mb. The aim of the current study was to reduce the size of the candidate region by analysis of further families and to investigate the expression of possible candidate genes in hair follicles and scalp tissue. Genomic DNA was extracted from 96 individuals of four families, after examination to establish their phenotype. Fine mapping was performed in all 96 individuals using 26 microsatellite markers spanning a 20 cM region at the telomeric end of chromosome 12. Candidate genes were analysed for their expression in hair follicles, derived from plucked hair follicles, scalp and other tissues by RT-PCR.In family I, seven individuals were affected, five unaffected. The largest family so far described for pili annulati in the literature was family II, with 26 affected and 39 unaffected family members over three generations. Family III and IV were smaller families with three and six affected and eight and two unaffected individuals, respectively. In family I, a recombination between D12S1714 and D12S367 was identified which reduced the region by half from 8 to 4 Mb, containing 45 known and putative gene loci. We have analysed a majority of the genes in this region by RT-PCR and have found that 18 were expressed in plucked hair follicles.In summary we confirmed the locus for pili annulati in four further families, reduced the critical interval to 4 Mb between the marker D12S367 and the telomeric end of chromosome 12, and identified possible candidate genes expressed in the human hair follicle.

P038

Common mutations in the filaggrin gene in ichthyosis vulgaris and concomitant atopic diseases in German patients

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Ichthyosis vulgaris (IV) is an autosomal-dominant cornification disorder with incomplete penetrance having a prevalence of 1:250–1 000 and a worldwide occurrence. The ~400 kDa polypeptide profilaggrin is the major protein of keratohyalin granules and its proteolitically processed end-product filaggrin is essential for the formation of the skin barrier function of the stratum corneum

(SC). Recent genetic studies revealed 14 causative FLG mutations in IV patients with R501X and 2282del4 being the most frequent (~80%). We studied the frequency of these two FLG nonsense mutations in a large German group of IV patients (47 individuals out of 37 families). Moreover, histological, immunochemical and/or ultrastructural analyses of IV skin has been performed ($n = 24$). 23 patients had concomitant atopic diseases. For R501X 7.1% ($n = 3$), 29.3 ($n = 11$) and 62.2% ($n = 23$) of all index patients ($n = 37$) showed a homozygous, heterozygous or negative genotype, respectively. For the mutation 2282del4 2.7% ($n = 1$) were homozygous, 46.0% ($n = 17$) were heterozygous, and 51.3% ($n = 19$) were negative. 14% ($n = 5$) of the 37 IV patients were R501X/2282del4 compound heterozygous. 48.6% ($n = 18$) were heterozygous for one of the alleles – either R501X or 2282del4. At least 72.9% ($n = 27$) of all index patients showed one or two mutation alleles. Ten index patients (27%) did not display any of the mutations. The frequency and distribution of R501X and 2282del4 in the subgroup of patients with IV and atopic diseases ($n = 23$) did not show significant differences when compared with patients suffering from IV only ($n = 14$). The study of 100 alleles of healthy controls did not show the mutation R501X, the mutation 2282del4 was present on two alleles. This observation is in accordance with the recently described combined allele frequency of ~4% in the European population. We thus can confirm that filaggrin mutations underlie IV and at the same time predispose to atopic manifestations. The analyses clearly confirm the fulminate pathogenetic impact of FLG mutations. Future work focussing on the SC barrier defect in IV may provide valuable clues for the understanding of atopic diseases.

P039

Analysis of a functional serotonin transporter polymorphism in psoriasis vulgaris

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Serotonin is a monoamine acting as a neuromediator in the central and peripheral nervous system. Recently, serotonin has also been shown to influence T- and B-cell function. The serotonin transporter is central in the regulation of the serotonergic system and is widely expressed on cells of the immune system. A functional promoter length polymorphism of the serotonin transporter termed 5-HTTLPR has been implicated in the genetic background of depression.Psoriasis is a complex disease with a polygenetic inheritance. Because of the role of T-cell mediated inflammatory processes in psoriasis and the increased prevalence of depression in psoriatic patients we speculated that genetic variations of the serotonin transporter may be associated with psoriasis. Analysis of 294 patients with psoriasis vulgaris and 315 healthy control individuals revealed no significant differences in genotype and allele distribution of the investigated serotonin transporter polymorphism. In a subgroup of 137 psoriatic patients the Hamilton Rating Scale for Depression was performed. No significant difference in this score between carriers of different genotypes could be shown. This argues against a major role of this genetic variation of the serotonin transporter in the susceptibility to psoriasis vulgaris and occurrence of depressive symptoms among psoriatic patients.

P040

TGM1 mutations in bathing suit ichthyosis: a functional understanding

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Bathing suit ichthyosis (BSI) is a striking and unique clinical form of autosomal recessive congenital ichthyosis characterized by pronounced scaling affecting the trunk and scalp but sparing the extremities and the central face. Here we report on a series of ten BSI patients. Our genetic, ultrastructural and biochemical investigations show that BSI is caused by transglutaminase-1 (TGase-1) deficiency. We identified 13 mutations in TGM1, among them seven novel missense mutations and one novel nonsense mutation. Digital thermography validated a striking correlation between warmer body areas and presence of scaling in patients suggesting a decisive influence of the skin temperature. In situ testing of TGase activity in skin of BSI patients demonstrated a marked decrease of enzyme activity when temperature was increased from 25 to 37°C. We conclude that BSI is caused by TGase-1 deficiency and there is evidence to suggest that it is a temperature sensitive phenotype. To verify this we expressed nine of the new BSI mutations in HEK 293 cells. The recombinant proteins were currently tested upon their activity by fluorescence spectroscopy at different temperatures ranging from 20 to 40°C.

P041

Molecular cytogenetic investigations on venous tissue from patients with recurrent varicosity

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Objective: Varicose veins are a common disorder, exhibiting a high grade of recurrence even after correct surgical treatment. Almost half of the patients reveal a familial history of varicosity, however little is known on the pathophysiological correlation so far. Analysis of cytogenetic changes is a well-established method in the investigation of hereditary disorders. However, for familial varicosity, despite its high frequency of occurrence, only very few such investigations exist. Therefore, we screened for chromosomal aberrations in venous tissue from patients affected by recurrent varicosity with the molecular cytogenetic technique Multicolour-Fluorescence-in-situ-Hybridisation (M-FISH).
Method: We established short-term primary cell cultures directly from biopsies of varicose veins. After chromosome preparation hybridisation was carried out with M-FISH DNA probes. Chromosome aberrations were analysed with a fluorescence microscope using M-FISH software. M-FISH allows the clear identification of all 24 different human chromosomes and therefore is a sensitive approach for the detection of both numerical and structural chromosome aberrations. Clonal numerical aberrations were confirmed with conventional FISH on interphase nuclei.
Results: We found structural chromosome aberrations in 3/6 so far investigated cases. In one patient M-FISH analysis revealed different

structural aberrations consisting of deletions (chr. 12), translocations affecting several chromosomes [e.g. t(4;6), t(4;15;5)], and even an insertion of part of chromosome 5–13. In a second case a clonal reciprocal translocation t(2;9) was observed in 32% (8/25) of cells. Another case revealed clonal trisomy of chromosome 7.
Conclusion: We succeeded in the detection of numerical and structural chromosome aberrations in cells derived from biopsies of varicose veins with M-FISH technique for the first time. There are hints that structural chromosome aberrations are found mainly in varicose vein tissue from patients with familial history of varicosity, however are missing in biopsies from sporadic cases. Our findings could therefore be helpful in the future elucidation of genetics and heredity of varicosity. Furthermore, our results suggest that an examination of patients and, if so, relatives could be reasonable in order to estimate the risk of recurrence of varicosity.

P042

Comparison of manual versus computer-aided, automated evaluation of cell-interactions under dynamic flow conditions

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Recruitment of leukocytes from blood to tissue is a perquisite of inflammation. These interactions are ideally investigated under flow conditions, as those are encountered in vivo. Despite the unquestionable quality of results obtained from flow-chamber experiments, evaluation is time-consuming and from our experience we hypothesized that inter-individual comparisons of results may yield a high degree of variation. We hence evaluated the use of a program for computer-aided evaluation of flow-chamber experiments. Clips investigating interaction of peripheral-blood-mononuclear cells with endothelial cells, showing different rolling and sticking interactions, were chosen without knowledge of specific experimental conditions; and sent to investigators, who were asked to evaluate rolling velocity, rolling- and sticking fractions. Simultaneously, clips were analysed using Imagoquant Software. Data on rolling velocities was almost identical between manual and computer-aided evaluation (e.g. film A: 220.3 ± 127 mm/s (manual) versus 249.3 ± 7.0 mm/s). Hence, the program is suited for analysis of velocity. As standard deviation in computer-aided evaluation is lower, the software also allows to detect subtle changes. Due to the great discrepancy with regard to rolling fraction (both within the manual evaluation group and manual versus computer-aided evaluation; e.g. film B: 27.38 ± 8.71 cells/cm^2/s (manual) vs 788.56 ± 111.60 cells/cm^2/s), the value of this program needs to be tested by more groups before final conclusions can be drawn. Relative changes in rolling fractions between manual (1.64 ± 0.39-fold increase from A to B) computer-aided (2.12 ± 0.75-fold) evaluation pointed in a similar direction. Hence (hopefully), Imagoquant Software can be used in future studies to analyse the results of flow-chamber experiments, making results comparable and also shortening time required for evaluation.

P043

Tumor-induced immunotolerance in HGF x CDK4R24C mice bearing autochthonous melanoma in the skin – a matter of DC dysfunction?

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Mice over expressing hepatocyte growth factor (HGF) and carrying a mutated cyclin dependent kinase 4 (CDK4R24C) are prone to autochthonous melanoma in the skin which grow rapidly and metastasize to the lymph nodes and the lungs. During evaluation of melanoma vaccine strategies we obtained evidence for tumor-induced immunotolerance in HGF x CDK4R24C mice. Here we investigate whether the function of dendritic cells (DC) is impaired in mice bearing autochthonous melanoma. Isolation of DC from primary melanomas, tumor draining metastatic lymph nodes and spleens of HGF x CDK4R24C mice following digestion with collagenase and magnetic cell sorting was established. These DC preparations are being immunophenotyped using flow cytometry. Furthermore, allogeneic MLR are performed to determine their immunostimulatory function. Mice bearing autochthonous melanomas are compared with wild type mice bearing transplanted B16 and with untreated controls. An improved understanding of the mechanisms of tumor-induced immunotolerance is of crucial importance for further development of immunotherapies.

P044 (V12)

In vivo optical imaging of macrophage recruitment in cutaneous granuloma formation using fluorescence labelled macrophages

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Extravasation of monocytes/macrophages (MØ) is one of the earliest events in inflammation. Analysis of this process in vivo is of high clinical and scientific interest. We established in vivo tomographic imaging of MØ labelled with membrane selective fluorochromes for quantitative spatial and temporal analysis of MØ migration using cutaneous granuloma formation as model system. Bone marrow derived MØ were labelled with the membrane selective carbocyanine fluorochrome DiR. This did not result in apoptosis and functionality of labelled cells was retained as shown by measuring adhesion, secretion of nitric oxide and phagocytosis of labelled MØ. For in vivo studies, cutaneous granuloma formation was induced in the flank of mice by s.c. injection of polyacrylamide gel pellets with or without LPS. DiR labelled MØ (1x10Exp7) were injected intravenously and mice were imaged up to 7 days post injection by fluorescence reflectance imaging (FRI) and fluorescence mediated tomography (FMT). FRI and FMT studies revealed maximum fluorescence intensities 72 h after injection with significantly higher signal – to – noise ratios at the LPS containing pellets compared to controls. FMT showed distribution of labelled cells in the periphery of the pellets. To confirm transendothelial migration of labelled cells, gel pellets were harvested and the cellular infiltrate was analysed by flow cytometry which revealed presence of approximately 20% DiR positive MØ expressing typical markers (F4/80, CD115).In

conclusion, tagging of MØ with DiR allowed non-invasive tracking of MØ migration for several days in vivo. We observed significantly higher MØ migration in granulomas containing LPS. This is in agreement with the known induction of TNFα by LPS which has been shown to be of crucial importance for the induction of MØ chemotaxis during granuloma formation (1). Thus optical imaging techniques such as FRI and FMT allow reliable quantification and localization of inflammatory cell response in vivo and present a fascinating new tool both for basic inflammation as well as for clinical orientated research.

1 von Stebut E *et al.* Blood. 2003: 101: 210–5.

P045

S100A8 and A9 proteins control autoreactive CD8+ T cell development during CD40L-induced systemic autoimmunity

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CD40-CD40L signaling is involved in the development of autoimmunity. Within the skin transgenic (tg) overexpression of CD40L in basal keratinocytes spontaneously leads to systemic autoimmunity as evidenced by autoantibodies, nephritis, proteinuria, and autoimmune dermatitis, which can be adoptively transferred by injecting CD8+ T cells into naive recipient mice. To identify genes involved in the development of MHC class I-restricted autoreactivity gene expression profiling of sorted CD8+ T cells from CD40L tg mice before and after onset of disease was performed. Surprisingly, an increased expression of S100A8 and A9 genes both members of the S100 family of Ca-binding proteins was detected in CD8+ T cells after onset of autoimmune disease. Elevated mRNA levels of S100A8/A9 in CD8+ T cells from CD40L tg mice compared to controls were confirmed by quantitative real time PCR. To determine the functional relevance of S100A8/A9 expression for the development of autoimmunity in vivo, CD40L tg mice were crossed to S100A8/A9-/- mutants. Interestingly, CD40LXS100A8/A9-/- mice showed significantly reduced autoimmune dermatitis and markedly decreased numbers of skin lesion infiltrating lymphocytes. Since CD40L tg mice show renal IgG/IgM depositions, glomerulonephritis, and proteinuria, renal function was analysed in CD40LXS100A8/A9-/- mice. Importantly, CD40LXS100A8/A9-/- mice showed a complete loss of nephritis, renal immunoglobulin depositions, and proteinuria indicating normal kidney function. Additionally, the activation status of asplenic and lymph node CD8+ T cells was determined in CD40LXS100A8/A9-/- mice demonstrating a decreased expression of cytotoxic/autoreactive markers like CD43 and granzyme B. CD8+ T cells isolated from CD40LXS100A8/A9-/- mice produced significantly reduced amounts of IL-17 a cytokine which has been suggested to mediate the inflammation associated with several autoimmune diseases. These data indicate that the expression of S100A8/A9 proteins may be critically involved in the pathogenesis of autoreactive MHC class I-restricted T cells in CD40L-induced systemic autoimmunity.

P046 (V07)

Galectin-2 suppresses contact allergy by inducing apoptosis in activated CD8+ T cells

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Galectins are a family of animal lectins known to affect cell survival, to modulate cell adhesion, and to induce cell migration. Since galectin-2 was shown to regulate cell-mediated inflammatory bowel disease in mice, we were interested to investigate the role of galectin-2 in cutaneous immunity. Therefore, groups of mice were systemically treated with galectin-2 before sensitization to contact allergens to analyse the effects of galectin-2 on contact hypersensitivity (CHS) responses. Interestingly, upon epicutaneous immunization followed by a local challenge at the ear, galectin-2 treated mice demonstrated a significantly decreased CHS response compared to controls. Even after re-challenge with the same hapten after a 2-week interval galectin-2 injected mice mounted a reduced CHS response suggesting the induction of tolerance. Since CHS responses in mice can be controlled by CD4+CD25+ regulatory T cells we analysed the numbers and suppressive activity of regulatory T cells in galectin-2 treated mice. Surprisingly, numbers and function of CD4+CD25+ T cells were normal but we detected dramatically decreased levels of total CD8+ T cells in the challenged ears and in skin draining lymph nodes of galectin-2 treated mice compared to non-treated controls. This reduction in cell numbers was even more pronounced in activated compared to naive or memory CD8+ T cell subsets. To further characterize the effects of galectin-2 on T cells we cultured purified total CD4+ and CD8+ T cells in the presence of galectin-2. Galectin-2 treated and activated CD8+ but not CD4+ T cells showed a significantly reduced proliferation upon T cell receptor stimulation with mitogenic antibodies. Additionally, galectin-2 exposed and activated CD8+ T cells demonstrated an increased expression of annexin V indicating that galectin-2 treatment induced apoptosis in CD8+ T cells. Importantly, the pro-apoptotic effects of galectin-2 on activated CD8+ T cells could be reversed by addition of the pan-caspase-inhibitor zVAD. Together, these data indicate that galectin-2 is an inducer of apoptosis in activated CD8+ T cells. These results furthermore suggest a potential therapeutic use of galectin-2 in CD8 mediated skin disorders such as contact allergy.

P047

Dimethylfumarate treatment reduces leukocyte rolling in vivo via decreased expression of adhesion molecules

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Fumaric acid esters (FAE) has been used for treatment of psoriasis for many years. It becomes evident, that compared to the mixture of FAEs used in clinic so far, dimethylfumarate (DMF) as monosubstance provides the therapeutic potency. The modes of action by which DMF act in improving symptoms of this mainly Th1-driven inflammation are not fully understood.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 showed that DMF treatment affected expression of different activation

markers and adhesion molecules, including CLA, CD25 and CD69. Reduced CLA-expression resulted in decreased binding to P- and E-selectin. DMF had no effect on expression of HLA-DR, CD54, PSGL-1 and VLA-4 and didn't change the binding to VCAM-1. Intravital microscopy experiments showed that DMF reduced rolling of human PBMC in mouse ear vasculature in vivo, and using P-selectin KO mice and anti-E-selectin Ab we could show that this reduction was mainly E- and P-selectin-dependent. We further investigated the effects of DMF on the regulation of important fucosyltransferases using RT-PCR as well as on NF-κB binding activity via EMSA technique. Preliminary data suggest that DMF seem to reduce expression of FucT7 on mRNA level. Taken together, 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.

P048

Regulatory T cells suppress the psoriasiform skin disease through TGF-β signals in the CD18 hypomorphic murine model of psoriasis

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Dysfunctional CD4+CD25+ regulatory T (Treg) and CD4+CD25- effector T cells have been identified in human psoriasis, however, their role in its pathogenesis remains unclear. We previously showed that CD18 hypomorphic (CD18hypo) PL/J mice with reduced levels of the common chain of β2 integrins (CD11/CD18) to only 2–16% of wild-type levels develop a psoriasiform skin disease strongly resembling human psoriasis. Here, we report that the reduced expression of CD18 is responsible for an impaired cell–cell contact between CD4+ and dendritic cells (DCs) as shown by 4PI microscopy, leading to a defect in expansion and activation of antigen-experienced Treg cells, eventually resulting in a dysfunctional Treg cell population. In contrast, low expression of CD18 does not disturb the cell–cell contacts between dendritic cells and CD4+CD25- responder T cells in that they are antigen specifically activated and converted into pathogenic memory T cells. Dysfunctional CD18hypo Treg cells do not produce TGF-β 1, whereas CD18 wild-type (CD18wt) Treg cells produce a large amount of TGF-β 1 after adoptive transfer into affected CD18hypo mice. CD18wt Treg cells, but not CD18hypo Treg cells, suppress pathogenic T cells, thus resulting in a significant improvement of the psoriasiform skin disease as assessed by adapted PASI score. The suppressive function of CD18 wild-type Treg cells is mediated through TGF-β 1. These findings may imply a potential immunotherapeutic strategy to boost immunity by TGF-β-producing Treg cells in autoimmune diseases, such as psoriasis.

P049

DC isolated from Friend retrovirus infected mice express impaired maturation markers, show elongated contact duration to naïve T cells and prefer the expansion of regulatory T cells

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Retroviruses, particularly HIV, have developed immunomodulatory mechanisms to avoid being attacked by the immune system. The mechanisms of this retrovirus-associated immunosuppression are far from clarified. Since dendritic cells (DC) may play a decisive role in these pathogenic processes, we have used the Friend virus (FV) mouse model to analyse the role of DC in retrovirus-induced immunosuppression. DC were generated from bone marrow of uninfected or FV-infected mice. The infection was productive, and infected DC transmitted the virus both in cell culture as well as in vivo. FV-infected DC expressed lower levels of costimulatory molecules (CD40, CD80, CD86) and maturation markers (CD83), whereas expression of MHC molecules was not altered in FV-infected DC. Live imaging analysis of the cell contact between DC and T cells in a 3-D collagen gel revealed longer contact duration of T cells with infected DC compared to uninfected DC. Surprisingly, flow cytometric analysis showed that naïve T cells were still activated by FV-infected DC and expressed high levels of activation markers (e.g. CD69, CD25) but this activation did not result in antigen-specific T cell proliferation. Interestingly, infected DC expanded the population of Foxp3-positive regulatory T cells in vitro twice as much as uninfected DC. From these results we suggest that the sustained contact between FV-infected DC and naïve T cells results in tolerance rather than in immunity. Thus, retroviral infection of DC results in preferential expansion of antigen-specific regulatory T cells, which might serve as an escape mechanism of the virus from immune recognition.

P050

Skin-specific reduction of Foxp3+ regulatory T cells in patients with lupus erythematosus

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Naturally occurring regulatory CD4+CD25+ T cells (Treg) have been shown to contribute to immunologic self-tolerance by suppressing potentially auto-reactive T cells. Recent data have suggested that a lack or suppressive defect of Treg is involved in a variety of human autoimmune diseases. In this project, we analysed the phenotype and function of CD4+CD25+ Treg in patients with various subtypes of lupus erythematosus (LE), a heterogeneous autoimmune disease with a broad clinical spectrum reaching from primarily cutaneous lesions to severe systemic organ manifestation. The number of Treg in lesional skin specimens of patients with this disease was investigated using anti-Foxp3 and anti-CD4 monoclonal antibodies for immunohistochemistry. Furthermore, characterization of peripheral blood CD4+CD25+ Treg from normal healthy donors (NHD) and from patients with various subtypes of LE was carried out by flow cytometry, analyzing the expression of Foxp3

and Treg subpopulations. We also purified CD4+CD25hi Treg and tested their sensitivity to CD95L-mediated apoptosis. The quantitative analysis of CD4+ T cells in skin lesions of patients with LE revealed a similarly high number when compared to other chronic inflammatory skin diseases, such as psoriasis, atopic dermatitis, lichen planus, and chronic eczema. However, the frequency of Treg in skin lesions expressed as percentages of Foxp3+ cells from CD4+ cells was significantly lower in the inflammatory infiltrate of LE than in control diseases. There was no correlation between disease activity and the frequency of Foxp3+ Treg in the skin of patients with LE. Interestingly, no significant differences were found regarding the phenotype and number of CD4+CD25+Foxp3+ Treg or the sensitivity to CD95L-mediated apoptosis of CD4+CD25hi Treg in NHD and patients with a primarily cutaneous subtype of LE. In conclusion, our data suggest an organ-specific reduction of Treg in the skin of patients with cutaneous LE rather than a global peripheral dysfunction as reported for patients with active systemic organ manifestation of the disease. These results might give insight into the different pathogenetic mechanisms of autoimmune diseases.

P051

Physiological role of toll-like receptor (TLR) 2, 4 and IL-12Rb2 in hapten-induced contact hypersensitivity

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Contact hypersensitivity (CHS) is a T cell mediated disease triggered by exposure of the skin to chemicals or metal ions. The induction of inflammation by these contact allergens is crucial for sensitization and elicitation of CHS. However, the mechanism by which this innate immune response is induced are poorly understood. Using mice with defects in the innate immune receptors TLR2, 4 and IL-12Rb2, we have now identified a physiological role for these receptors in CHS. Mice lacking both TLR4 and IL-12Rb2 as well as mice lacking both TLR2 and TLR4 were resistant to CHS induced by contact allergens and irritant. The defect crucially affected the potential of dendritic cells (DC) to induce sensitization and resulted in the prevention of CHS by regulatory T cells. In the process of identifying the potential mechanism and ligands that trigger the relevant TLR, our studies revealed that CHS was normal in germ-free mice. These results imply that endogenous ligands for TLR2 and 4 are important in CHS.We are currently elucidating the identity of these self-ligands for TLR2 and 4 and study their generation following contact allergen application to the skin.

P052

CCL21 as an inflammatory chemokine in the skin; impact on dendritic cell maturation and migration

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Recently, CCL21 has been suggested to act as an inflammatory chemokine in the skin. We have dissected the contribution of CCL21 to skin inflammation, particularly its impact on antigen uptake, maturation and migration of skin-residing dendritic cells (DCs). CCL21 induces antigen-uptake by and CD40 expression on DCs, phenomena typically ascribed to inflammatory cytokines, but not maturation as defined by morphology (granularity and size), phenotype (CD80/86-, CCR7-expression) and function (allostimulation). The latter data were confirmed by ex vivo analysis of DCs derived from plt/plt mice, which lack CCL21 expression in lymphatic tissues. plt/plt DCs exhibited surface expression of MHC class II-, CD86- and CCR7-molecules, and an allostimulatory capacity at levels comparable to wild-type mice. Thus in skin inflammation, CCL21 acts as a distinct regulator of CD40, a master regulator of DC function. In addition, skin-expressed transgenic CCL21 induced preferential recruitment of CCR7-expressing CD11+DCs and CD4+/CD62L+ naïve/central memory T cells into the draining lymph nodes. By regulating antigen-uptake by and CD40 expression on DCs and by induction of encounter of spatially and functionally segregated cell populations in a defined immunological compartment, expression of transgenic CCL21 in the skin could have important implications on the design of immunotherapeutic strategies including DC-based vaccination.

P053

Defining tissue-specific parameters for the development of in vitro assays to identify potential contact allergens

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Substitution of animal testing for the risk assessment of drugs and chemicals is one of the vital topics in life and health sciences. In course of the adoption of the new EU regulatory framework for Registration, Evaluation and Authorisation of Chemicals (REACH) the development of ethically acceptable and cost reducing in vitro cell based systems for the (re-) evaluation of the toxicity and sensitising potential of a wide range of chemically different substances has gained an even more substantial importance. Due to the complex interactions of different cell types involved in sensitisation processes and the subsequent development of allergic diseases, in vitro models capable of differentiation between irritants and contact allergens comprising stromal and immune system cells have to be designed. We established a co-culture model of skin primary cells in comparison to cell lines together with different sorted dendritic- and T-cell subsets for the evaluation of the sensitising potential of a defined testing set of chemicals. For these chemicals covering known extreme, mild and non-sensitisers we have accomplished solubility and toxicity studies to achieve a concentration range covering toxic and non-toxic concentrations for the induction of sensitising responses. Our readout parameters cover measurements of cytokine levels and surface markers to deliver a robust assay for the differentiation between sensitising and irritant compounds. First results show a stimulus-independent upregulation of IL-8 release from skin epithelial cells with an increased release at toxic concentrations. This suggests that IL-8 release is a common response to general skin danger signals that precludes the differentiation between irritants and contact allergens.

P054

Antigen delivery into dendritic cells (DCs): The choice of melanoma cell preparation for DC loading influences the efficacy of antitumor T cell responses

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Vaccination protocols that utilize dendritic cells (DCs) in order to elicit immunity against tumors are the subject of intense research. The heterogeneous functions of DCs in immune regulation depend on the diversity of DC subsets, on their functional plasticity, and on the quality/quantity of antigen uptake, processing, and presentation. Various strategies for loading of DCs with antigen including peptides, DNA, or whole tumor cell preparations have been suggested. In order to determine parameters that guarantee optimal efficacy for T cell stimulation we systematically analysed the uptake of apoptotic or necrotic melanoma cell preparations by DCs at various maturational stages and the implications for the immunostimulatory function. After an optimal loading interval of 4–10 h an average of 25 ± 9% of immature DCs showed internalized necrotic melanoma cell material. Apoptotic melanoma cell material was phagocytosed by a lesser percentage of DCs (41 ± 1.5%). Maturation inducing cytokines could be added simultaneously with the tumor cell preparations to immature DCs without affecting the uptake, while DCs maturated for 24 or 48 h before loading showed a markedly reduced phagocytotic activity. Loading with melanoma cell material clearly decreased the maturational status of DCs in a dose dependent manner when compared to unloaded controls. Cryopreservation of loaded and maturated DCs was feasible (>75% cell yield) without alterations of the surface expression of maturation and costimulation markers. In an attempt to assess the immunostimulatory capacity of the loaded DCs at various maturational stages, DCs were used for two cycles of autologous T cells stimulation before measuring antigen driven IFN-gamma secretion in an ELISPOT assay. Interestingly, DCs loaded with apoptotic cell material induced the highest IFN-gamma secretion of autologous T cells when being matured, whereas DCs loaded with necrotic cell material performed best when being used in an immature status. As recently reported by others, this may be due to the fact that only necrotic cells liberate heat shock proteins, which lead to DC maturation by CD40 interaction and bind tumor-derived peptides and thereby promote processing and MHC class I and II presentation by DCs.

P055

In vitro expanded CD4+CD25+ regulatory T cells show up regulation of Foxp3 and augmentation of the suppressive activity

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In an experimental model of contact hypersensitivity (CHS) we have previously shown that freshly isolated CD4+CD25+ regulatory T cells suppress the elicitation phase of a CHS reaction by blocking the interaction of the CD8+ effector T cells and the endothelium. However the low numbers of endogenous Tregs have hampered our studies. Therefore we have established a method to expand naïve CD4+CD25+ regulatory T cells in vitro, using anti-CD3, anti-CD28 and rmIL-2. Using this novel culture method we were able to expand Tregs up to 180-fold (as compared to the originally applied Tregs) within 21 days. Compared to freshly isolated Tregs, the expression of the characteristic cell surface markers for regulatory T cells, i.e. CD25, CD62L and Foxp3 remained unchanged, whereas the mean fluorescence intensity (MFI) of Foxp3 increased significantly during expansion. This increase correlated with the time spent in culture (d0: MFI = 1651, d5: MFI = 5774, d15: MFI = 7150; control-CD25-: MFI = 314). In vitro and in vivo the expanded Tregs showed an increased suppressive activity. For instance, in standard suppression assays, expanded regulatory T cells were able to block proliferation of stimulated CD4+ T cells up to 70% in a dilution of 1:50 in a cell–cell-contact dependent manner. However, freshly isolated or CD3/CD28 activated Tregs did not exert any suppressive function when applied in this ratio.In our in vivo CHS models injection of expanded Tregs reduced the ear swelling reaction as well as the interaction of CD8+ effector T cells with the endothelium more vigorously than freshly isolated regulatory T cells. Thereby, the expanded Tregs displayed an unchanged migration pattern compared to freshly isolated T cells. Thus our data indicate that in vitro expanded CD25+ regulatory T cells exert a stronger regulatory function as compared to freshly isolated Tregs and further studies will determine the underlying mechanisms and whether in vitro expanded regulatory T cells provide us with a tool to suppress allergies and autoimmune diseases more effectively.

P056

Dermal fibroblasts induce maturation of dendritic cells

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To trigger an effective T cell-mediated immune response in the skin, dendritic cells (DC) migrate into locally draining lymph nodes where they present antigen to naive T cells. During their migration to secondary lymphoid organs, DC have to travel through the stromal microenvironment comprised of the extracellular matrix and stromal cells such as fibroblasts, macrophages and endothelial cells. Little is known about the interaction of DC with these various cellular microenvironments. Here, we show that DC are located in close proximity to stromal fibroblasts in inflamed skin. In accordance, a Thy-1- and ICAM-1-dependent adhesion of DC to fibroblast could be detected in vitro. Co-culture experiments demonstrate that fibroblasts are effective in inducing both phenotypic and functional maturation of DC in a manner that is dependent on both direct cell–cell contact (ICAM-1-dependent) as well as on soluble mediators (TNF alpha). The resulting fibroblast-matured DC are able to support T cell driven immune responses reflected by CD25 expression and enhanced T cell proliferation. Together these data demonstrate that dermal fibroblast which DC can encounter during their trafficking from skin to lymph node may act as potent regulators of DC differentiation and function, and thus may actively participate in the regulation and outcome of DC-driven cutaneous immune responses.

P057 (V30)

Role of integrin alphaE(CD103)/beta7 for lymphocyte morphology and motility

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Tissue-specific recruitment of lymphocytes is pivotal for their functions. The integrin αE(CD103)/β7 has been implicated in epithelial localization of T lymphocytes under normal and pathological conditions through binding to E-cadherin.In order to investigate the role of CD103 for epithelial lymphocyte recruitment in detail, we studied CD103-deficient mice. These animals showed a significantly reduced number of dendritic epidermal T cells (DETC) as compared to wildtype. Interestingly, fluorescence microscopy of epidermal sheets revealed that the dendrites of wildtype DETC spanned an average area of 494 μm^2, which is 52% larger than that of CD103 deficient DETC (325 μm^2; $P = 0.0006$). These observations raised the hypothesis that CD103 is involved in morphology and (intercellular) locomotion of intraepithelial lymphocytes rather than merely retention of these cells within the epidermis.To approach this hypothesis, K562 cells were transfected with the murine integrin αE(CD103) and β7, and plated on rmE-cadherin. As determined by time-lapse microscopy, the number of cells showing an amoeboid movement was increased by 50.0% in cultures transfected with αE/β7 as compared to mock transfectants ($P = 0.01$). When the cells were transfected with a point-mutated CD103 chain that is, 'locked open' in an active conformation, the difference to the mock-transfectants was even increased to 84.6% ($P = 0.0003$). In contrast, transfection of an inactive 'locked closed' mutant did not result in enhanced motility of the transfectants. In addition, function-blocking anti-CD103 antibodies or cytochalasin D disrupting the actin-based cytoskeleton abrogated the enhanced motility. Next we coupled YFP to the constructs and studied the transfectants on rmE-cadherin by confocal microscopy. Cells with the wildtype constructs showed longer dendrites and spanned larger areas as the mock-transfected cells or the 'locked closed' transfectants. This difference was even more pronounced with the constitutively active 'locked open' transfectants, resembling the difference seen between wildtype and CD103-deficient DETC in vivo. Based on these results, we propose that the integrin αE/β7 not only plays a role for retention of epithelial T cells, but is also involved in their locomotion and morphogenesis, presumably through interactions with cytoskeletal components.

P058

Therapeutic efficacy of antigen-specific vaccination and toll-like receptor stimulation against established transplanted and autochthonous melanoma in mice

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Antigen-specific immunotherapeutic approaches for melanoma have been primarily investigated using the transplantable B16 melanoma cell line in mice. To more closely mimic the clinical situation in patients with melanoma, we use genetically modified mice overexpressing hepatocyte growth factor (HGF) and carrying an oncogenic mutation in the cyclin dependent kinase 4 (CDK4R24C). HGF x CDK4R24C mice rapidly develop multiple invasive melanomas in the skin following neonatal carcinogen treatment which spontaneously metastasize to lymph nodes and lung. We used a vaccine strategy consisting of recombinant adenovirus encoding human tyrosinase-related protein 2 (Ad-hTRP2) and application of CpG DNA and synthetic dsRNA against engrafted B16 melanoma or primary autochthonous melanomas in the skin and in the lung of HGF x CDK4R24C mice. Both Ad-hTRP2 vaccination and peritumoral injections of TLR ligands were required for rejection of established B16 melanoma in the skin. Vaccination with Ad-TRP2 followed by injections of TLR ligands resulted in delayed growth of autochthonous primary melanomas in the skin and a reduction in the number of spontaneous lung metastases but did not induce tumor regression. Carcinogen-treated HGF x CDK4R24C mice bearing multiple autochthonous melanomas did not reject transplanted B16 melanoma despite treatment with Ad-hTRP2 and TLR ligands. These results suggest the development of a profound tumor immune tolerance in mice carrying primary autochthonous melanomas.

P059 (V34)

Generation of T cell memory critically depends on cell volume regulation by the taurine transporter TAUT during T cell activation

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After antigen specific activation only surviving cells may become memory type B or T lymphocytes, but the mechanisms that control the decision of apoptosis versus memory are poorly understood. We hypothesized that regulators of cell volume contribute to this process. Cellular accumulation of organic osmolytes including the beta-amino acid taurine is one important mechanism of cell volume regulation and the carrier accomplishing concentrative cellular uptake of taurine is the taurine transporter (TauT). To characterize the role of cell volume regulation by TauT for the generation of immune memory, we analysed taut-/- in comparison to wildtype (wt) mice. Contact hypersensitivity responses (CHS) to TNCB were significantly reduced in taut-/- mice as determined by ear swelling and cellular skin infiltrate. Interestingly, while no difference was detected in lymph node cells of nave mice, exclusively CD4+ and CD8+ T cells were significantly reduced in skin draining lymph nodes after CHS induction in taut-/- mice. This indicates a possible role for TauT in rescuing T cells from activation induced cell death (AICD). Indeed, after in vitro activation, CD4+ and CD8+ T cells from taut-/- mice expressed significantly higher levels of the apoptosis marker phosphatidylserine (Annexin V+) and survival of taut-/- T cells was significantly decreased pointing towards enhanced AICD in T cells lacking TauT. To prove an in vivo role of TauT for T cell survival, we adoptively transferred TNCB specific CFSE loaded T cells from taut+/+ and taut-/- mice into nave wt recipients. Consecutively, these mice were challenged with TNCB and analysed. Strikingly, recovery of dividing CFSE+ CD4+ and even more pronounced of dividing CFSE+ CD8+ taut-/- T cells was significantly reduced compared to wt indicating increased AICD of taut-/- T cells. In summary, we could show for the first time that cell volume regulation by TauT is crucial during T cell activation, modulates induction of T cell apoptosis, and plays a role for T cell survival and the selective process of generation of T lymphocyte memory.

P060

Indirect Th1 polarizing capacity of IL-4, mediated through IL-12p70 secretion by human dendritic Cells

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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 by murine dendritic cells consequently inducing interferonγ(IFN-γ) producing Th1 cells. These findings are of importance especially for the understanding of the regulation of atopic immune responses, where early IL-4 dominated inflammation is followed by IFN-γ expression and the regulation of this process is still not understood. To elucidate the role of IL-4 in humans, the phenotype of activated human monocyte-derived dendritic cells (DC) and their priming effect on naïve, autologous Th cells was analysed as a function of IL-4. In all cases CD11c+ CD14- HLA-DR+, CD86+/-, CD83- immature MoDC, generated under a wide range of IL-4 concentrations (from 5 to 50ng/ml), were activated with various maturation stimuli. As expected, HLA-DR and CD86 expression was vigorously upregulated and CD83 appeared within 24 h upon stimulation, as the capacity of the dendritic cells to upregulate cell-surface molecules upon maturation was not dependent on IL-4 concentration. However, in contrast to low or intermediate doses of IL-4, high doses of IL-4 markedly altered the LPS induced cytokine production of human DC, resulting in significantly enhanced IL-12p70 production. Investigating the underlying mechanisms, we found that enhanced IL-12p70 production induced by high dose IL-4 depended on simultaneous 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 upregulated and IL-10 was downregulated resulting in an IL-12/IL-10 ratio >1. Further functional experiments were carried out by priming autologous naïve CD4+CD45RA+ T cells by a 12-day-coculture with either high dose IL-4 or low dose IL-4 treated DC. Analysis revealed that IFN-γ? but not IL-4 production was significantly increased in T cells after coculture with high dose IL-4 treated DC. 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 and illustrate an indirect Th1 polarizing capacity of IL-4. This is of great clinical relevance in regard to the role of IL-4 in atopic diseases and may explain the transition from IL-4 to IFN-γ predominance in chronic lesions.

P061

Epidermal p65/RelA is essential for the crosstalk between keratinocytes and immune cells in an IκBα-mediated model of psoriasis

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Previously we have shown that the development of a psoriasis-like skin disease in IκBα-deficient mice requires simultaneous aberrant NF-κB signalling in the immune cells and epithelial cells. Extending the analysis of this model, we found by microarray analysis, that the cytokine expression in IκBα-deficient mice is similar to that described for human psoriasis. Important target genes such as S100A8 and S100A9 are found strongly upregulated. Moreover, we show by conditional ablation of the genes encoding IκBα and RelA in epidermal keratinocytes and in different immune cell populations that initiation and maintenance of a psoriasis like skin disease depends on RelA mediated interactions between IκBα deficient epidermal keratinocytes and IκBα deficient lymphocytes. The inflammatory response is initiated in a RelA dependent manner by epidermal keratinocytes, as epidermis-specific deletion of RelA in an IκBα-deficient background prevented the development of the psoriasis-like inflammation which otherwise developed in IκBα-deficient mice. As we found a constitutive and concomitant activation of RelA in the epidermis and in the invading immunocytes in 14% of patients with active psoriasis, our model expands the view on the understanding of the pathogenesis of psoriasis and may lead to a novel therapeutic strategy.

P062

Immunomodulation by non-pathogenic gram-negative bacteria operates via TLR2 on dendritic cells and IL-10 producing T cells

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Non-pathogenic bacteria as part of the normal flora remain on skin and gut epithelium without inducing inflammation in healthy individuals. Moreover, some strains of non-pathogenic bacteria have even been used to treat or prevent allergic inflammation. One of these strains of non-pathogenic bacteria is the gram-negative bacteria *V. filiformis* (Vf), which has been used to treat atopic patient skin and proved effective compared to placebo. The underlying mechanism, however, remained unknown. We therefore investigated the effects of Vf extracts on immunity to disclose a general principle of the immune balance on epithelial surfaces. Initial studies revealed that several Vf preparations led to maturation of DC as monitored by upregulation of CD80, CD86 and MHC II. However, marked differences were seen in regard to cytokine production. As expected, Vf lipopolysaccharide (LPS) induced IL-12 in DC and consecutive co-cultures of these DC with T cells led to the induction of IFN-γ producing Th1 cells. In contrast to IL-12, IL-10 levels remained low in LPS-stimulated DC. However, when the complete bacterial extract was investigated, the effects of LPS were minor. DC activated with Vf bacterial extract secreted only moderate levels of IL-12 but large amounts of IL-10. Co-cultures of these DC with T cells resulted in the induction of remarkably high levels of IL-10 together with some IFN-γ in CD4+ T cells, a phenotype characteristic for inducible regulatory T cells. To investigate the mechanism of IL-10 production, DC generated from several knock-out and wildtype mice were analysed. Strikingly, IL-10 production was almost abolished in DC lacking TLR2 suggesting a hitherto unknown but dominant TLR2 ligand with tolerogenic activity. Our data demonstrate a possible scenario of immunomodulation by gram-negative non-pathogenic bacteria like Vf. These bacteria control proinflammatory responses by stimulating DC to orchestrate induction of IL-10 producing T cells. We identified TLR2 as the responsible receptor mediating this immuno-dominant activity of these bacteria that have the potential to maintain or even restore the immune balance on epithelial surfaces such as in atopic dermatitis patients. Thus, TLR2 activating compounds of these bacteria could be a new class of immune-modulators.

P063

Dendritic cell based antitumor vaccination: impact of indoleamine 2,3-dioxygenase expression

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Background: Recent reports have demonstrated that the enzyme indoleamine 2,3-dioxygenase (IDO) is upregulated in human dendritic cells (DCs) upon in vitro maturation. IDO is supposed to convey immunosuppressive effects by degrading the essential amino acid tryptophan, thereby downregulating T-cell functions. Moreover, IDO expressing DCs are supposed to induce regulatory T cells. Hence, we evaluated IDO expression in DC preparations used for therapeutic DC vaccination in vitro and in vivo.

Patients, methods and results: IDO expression was detected by real time-PCR in a series of human clinical grade DCs ($n = 28$) prior to subcutaneous vaccination of 11 melanoma patients with advanced disease demonstrating a high expression in all samples. Indeed, IDO in human DCs was strongly upregulated on RNA and on protein level upon in vitro maturation by Interleukin-1β(IL-1β), tumor necrosis factor-α(TNF-α), Interleukin-6 (IL-6) and Prostaglandin E2 (PGE2) over a time course of 24 h. The enzymatic activity of induced IDO was demonstrated by measuring tryptophan degradation. Moreover, in biopsies of vaccination sites obtained 24 h after application of the DC vaccine a prominent infiltrate of IDO-positive cells was observed by immunohistochemistry. Notably, the inflammatory infiltrate of these sites stained positive for Forkhead box P3 (FoxP3), suggesting an IDO-mediated induction/recruitment of regulatory T-cells. All analysed melanoma patients receiving DC based immunotherapy exhibited rapid disease progression with a short overall survival.

Conclusion: Our data suggest a potential clinical relevance of IDO expression in DC-based therapeutic vaccines via the attraction and/or induction of FoxP3+ T-cells.

P064

High expression of the antimicrobial peptide psoriasin by human mammary glands during lactation

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Psoriasin (S100A7) has been shown to be the major Escherichia coli killing antimicrobial peptide in the skin. Its expression appears focally especially in areas with high bacteria concentrations. Furthermore it was suggested that psoriasin is secreted by eccrine sweat and sebaceous glands of the skin. Since mammary glands are evolutionary skin appendices and since the presence of antimicrobial peptides in mother's milk represents a potentially interesting mechanism to protect neonates from gastrointestinal infections, we investigated the capacity of mammary gland epithelial cells to produce psoriasin.By Western blot analysis we found very high concentrations of psoriasin in human breast milk. Immunohistochemical staining of mammary glands revealed little if any positive psoriasin staining in non-lactating mammary glands. In contrast, psoriasin expression was strongly induced in lactating human mammary glands. These results were confirmed in mice, were we found an induction of psoriasin expression during pregnancy and a further increase during lactation, compared to virgin mice, which showed no psoriasin expression. In addition to the in vivo data, stimulation of primary human mammary epithelial cells with a cocktail inducing lactation (consists of prolactin, dexametasone and insulin) in vitro, led to a strong induction of both psoriasin mRNA and protein expression. Our data suggest that psoriasin production and secretion by mammary glands could be important to prevent E. coli infection of the breast during the lactation period. Furthermore, the high psoriasin concentration in human breast milk could also be a protective factor for newborn babies.

P065

Dietary prebiotics interfere with dendritic cell maturation

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The intestinal microflora of breast-fed infants is dominated by bifidobacteria and *lactobacilli*. Because this characteristic is considered to be an important physiologic factor for the development of the immune system, great efforts are undertaken to modify the flora of bottle-fed infants to resemble that of breast-fed infants. Recent evidence suggests that infant formula supplemented with dietary prebiotics helps to adjust the microbial intestinal flora to that of breast-fed infants promoting the postnatal maturation of the immune system. However, it is not clear whether the immunomodulatory capacity of prebiotic ingredients is mediated indirectly by the propagation of distinct subspecies of bacteria of the gut flora or whether it depends on direct effects on immune cells. Dendritic cells (DC) are pivotal for the activation and differentiation of effector T lymphocytes as well as for the maintenance of peripheral tolerance. We investigated whether prebiotics directly affect the activation and maturation of DC. To this end immature DC were generated from murine bone marrow precursors (BM-DC) in the presence of different prebiotic oligosaccharides and stimulated with lipopolysaccaride (LPS) to develop into mature DC. We show that neutral oligosaccharides (NOS) resembling core structures of human milk oligosaccharides interfered with the maturation of LPS-stimulated BM-DC in a dose-dependent manner as demonstrated by reduced expression of MHC class II and costimulatory molecules (CD40, CD86). LPS-matured DC cultured with NOS had a lower stimulatory capacity for allogenic T cells in a mixed lymphocyte reaction as well as for syngeneic OVA-TCR-transgenic T cells. Our results suggest that this prebiotic oligosaccharide might facilitate the generation of tolerogenic DC.

P066

Mouse models of TMA-induced contact hypersensitivity with improved chronic character aiming at improved clinical relevance

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Atopic dermatitis is a chronic, eczematous skin disease with steadily increasing incidence especially in children. There is a high medical need for new therapies, and improved animal models better reflecting human disease are required to support efficient drug discovery.Atopic

dermatitis represents a T cell-dependent inflammatory dermatosis with crucial role of Th2 cytokines such as IL-4, IL-5 and IL-13 as well as IgE production. In mice, TMA (trimellitic anhydride) is routinely used to trigger T cell-dependent contact hypersensitivity (CHS) reactions and to induce a Th2 profile of cytokine secretion. State of the art schedules mainly focus on acute inflammation but usually do not address chronic character and only allow prophylactic treatment. In this study we compared a standard acute model with new models of TMA-induced skin inflammation focusing on the influence of repeated challenges on chronic character. We measured ear thickness over the time course and several endpoint parameters such as: immune cell infiltration in the skin, weight of corresponding lymph nodes and cytokine secretion profile of lymph node cells, cytokine production in skin as well as IgE levels. We developed new subchronic and chronic TMA-induced CHS-models with excellent treatment response to the clinical gold standard prednisolone and, in chronic model, with treatment of an established chronic skin inflammation.Taken together, we outline a way to improve similarity of mouse T cell-dependent skin inflammation to atopic dermatitis. Hopefully, this will help to increase the predictive value of animal models for drug discovery projects for atopic eczema in the future.

P067

Recruitment of immature mast cells to peripheral tissue is mediated by E-selectin, VCAM-1, and PECAM-1

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Numbers of skin mast cells, constitutive tissue residents, are markedly increased at sites of chronic inflammation or immune responses. However, the underlying mechanisms of mast cell progenitor trafficking to peripheral tissues remain to be identified in detail. In this study we show that unstimulated immature murine mast cells (iMCs) adhered to skin microvascular endothelial cells (sEND). This adhesion of iMCs to sEND was inhibited by neutralizing antibodies against the endothelial cell adhesion molecules E- and P-selectin, VCAM-1, ICAM-1, ICAM-2, JAM-B, or PECAM-1. Furthermore, iMCs adhered to immobilized recombinant E-selectin, VCAM-1, or PECAM-1, demonstrating that the adhesion of iMCs is mediated by these endothelial cell adhesion molecules. To identify iMC adhesion molecules involved in this process, we blocked iMC adhesion to both sEND and immobilized recombinant adhesion molecules. We found functionally relevant interactions between PSGL-1 and E-selectin, VLA-4 (CD49d/CD29) and VCAM-1, and between CD51/CD61 and PECAM-1. Furthermore, adhesion of iMCs to sEND and to immobilized E-selectin was enhanced by the proinflammatory mediator TNF-alpha and by complement component C5a. In contrast, the adhesion of iMCs to VCAM-1 and PECAM-1, representing the subsequent steps of the extravasation cascade, was dominantly regulated by 'outside-in signalling' of VLA-4 and CD51/CD61 integrins induced by crosslinking of PSGL-1. Most interestingly, iMCs were found to transmigrate across an endothelial barrier directionally and dose-dependently in response to TNF-alpha and complement component C5a. Our results demonstrate that iMCs can adhere to sEND, which is orchestrated and tightly regulated by interactions with selectins, VCAM-1, and PECAM-1 and followed by directed transendothelial migration. Our ongoing studies are aimed at characterizing the in vivo relevance of these mechanisms for mast cell progenitor recruitment in settings of chronic cutaneous inflammation including allergic and pathogen-driven inflammatory responses. A better understanding of the mechanisms of mast cell recruitment to sites of pathologic situations may reveal possibilities for a direct modulation of mast cell numbers in peripheral tissues.

P068 (V10)

Maturation of dendritic cells is induced by cellular contact to mature mast cells

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Mast cells (MCs) are important players in inflammatory reactions and innate immune responses. In this study we show that mature murine peritoneal mast cells (PMCs) and immature murine bone marrow derived mast cells (BMMCs) can establish cell–cell contact with immature murine bone marrow derived dendritic cells (BMDCs). This cellular interaction is mediated in part by the MC adhesion molecule CD31, as MC adhesion to dendritic cells was significantly inhibited by a CD31 blocking antibody. Cellular contact of mature PMCs to immature dendritic cells induced the expression of the costimulatory signals CD80 and CD86 on the cell surface of BMDCs. Furthermore, the expression of CD40, CD38, CD31, and CD54 was strongly increased, suggesting the induction of BMDC maturation. Moreover, the contact of PMCs to BMDCs induced the expression of the chemokine receptor CCR7 by BMDCs, which is required for the initiation of DC migration to T cell areas of draining lymph nodes. The induction of BMDC expression of costimulatory signals and of CCR7 by PMC contact was not further enhanced by previous stimulation of MC with the proinflammatory mediator TNF-alpha or by previous IgE-induced MC activation. Interestingly, the initiation of BMDC maturation by contact to MCs depends on the maturation status of MCs, as the expression of CD86, CD40, CD38, CD54, and CCR7 on BMDC surface was not enhanced by cellular contact to immature BMMCs. Taken together, cellular contact of mature MCs to immature dendritic cells leads to the induction of dendritic cell maturation. This effect depends on the status of MC maturation but not on previous MC activation. Our results show, for the first time, that a cellular contact between immature DCs and mature MCs can induce the maturation of DCs and facilitate DC migration. Thus, MCs are not only involved in innate immunity but may also take part in the initiation of adaptive immune response by enhancing the stimulatory capacity of dendritic cells.

P069

Antigen-experienced double-negative CD3+CD4-CD8- T cells with a distinct regulatory function are highly increased in β2 integrin-deficient mice

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Beta2 integrin adhesion receptors (CD11/CD18) are known to importantly participate in transendothelial migration of leukocytes and lymphocyte activation providing firm intercellular adhesion and co-stimulatory signaling during antigen presentation. We recently reported that lymphocytes obtained from CD18-deficient (CD18-/-) mice showed an aberrant homing to primary and secondary lymphoid tissues. This was due to an altered phenotype and activation pattern of lymphocytes

in absence of beta2 integrins. In particular, $\beta 2$ integrin deficiency led to the generation of 10–20-fold increased numbers of TCR$\alpha\beta$ and $\gamma\beta$ CD3+CD4-CD8- double-negative (DN) T lymphocytes in peripheral lymphoid and non-lymphoid tissues as compared to wild-type (WT) mice.We now analysed phenotypic and functional properties of TCR$\alpha\beta$ DN T lymphocytes from spleens and lymph nodes of CD18-/- mice using in-vitro proliferation, suppression and cytotoxicity assays. Interestingly, these cells showed an antigen-experienced phenotype along with a vigorous in vitro proliferation upon low-dose IL-2 or to soluble CD3 alone, whereas they were completely unresponsive to potent mitogens such as PMA/ionomycin, ConA or allo-MHC. Furthermore, TCR$\alpha\beta$? ?DN T cells revealed a dose-dependent suppressive effect on in vitro proliferation of WT T cells in co-culture experiments. As shown by transwell co-culturing, this effect was neither contact dependent in vitro nor mediated by membrane TGF-β, as reported for natural Treg cells. In addition, TCR$\alpha\beta$ DN cells did not express the transcription factor Foxp3. Hence, our data indicates that TCR$\alpha\beta$ DN T cells from CD18-/- mice constitute a regulatory cell population different from natural Treg cells. Currently, experiments are undertaken to investigate immunosuppressive mechanisms of these cells in vivo using the CD18-hypomorphic (CD18hypo) psoriasis mouse model for autoimmunity. These studies may elucidate the role of the highly increased regulatory cell population with regard to the distinct susceptibility of CD18-/- mice for bacterial and fungal infections.

P070

In vivo depletion of CD4+CD25+Foxp3+regulatory T cells during development of B16 melanoma accelerates tumour growth

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Naturally occurring regulatory T cells (Treg) are essentially involved in the suppression of effector T cells (Teff) to prevent the onset of autoimmune diseases such as multiple sclerosis, rheumatoid arthritis or lupus erythematodes. Furthermore, Treg have an impact on immune responses directed against various tumors, in that depletion of CD4+CD25+Foxp3+ cells before tumour-challenge reduces tumour growth. However in melanoma-bearing patients this preventive treatment cannot be applied, as the tumour is already established. Therefore, we seek to generate a murine model in which we are able to treat existing tumours. For this purpose we want to combine depletion of Treg in mice after tumour-challenge with the stimulation of effector T cells. Mice were inoculated s.c. with B16 melanoma cells and 5 or 10 days later the mice were injected i.p. with anti-CD25 antibody (clone PC61) to deplete the Treg. Treg depletion at these time points led to an increased rate of tumour growth. To investigate the reason for this development, we analysed the efficiency of Treg depletion in the animals. We found that in lymphoid organs and blood regulatory T cells were completely removed, whereas inside the tumor Treg were still detectable. To determine whether the CD25 antibody treatment had additional effects on CD4+ effector cells, which upregulate CD25 during activation, we injected preactivated splenocytes into naïve mice or activated transgenic T cells in vivo. Afterwards anti-CD25 antibodies were injected and 3 days later the activated cells were isolated and analysed. Our data indicate that PC61 does not deplete Teff and that it does not affect any other cell subsets, e.g. CD8+ cells. Moreover, a combination of CD4+CD25+ depletion and repeated injection of tumor antigen-loaded DC#s showed no effect on the tumor growth compared to untreated mice. In summary we conclude that the increased rate of tumor growth does not arise from a co-depletion of effector T cells but rather from the inability of the antibody to enter the tumor and reach the Treg that reside at this site. Thus the tumor creates its own immunosuppressive environment and depletion of tumor residing Treg may further boost anti-tumor immunity.

P071

Influence of apoptosis on immune response in systemic lupus erythematosus

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Systemic lupus erythematosus (SLE) is a heterogeneous autoimmune disease associated with abnormal immune responses including production of autoantibodies and immune complexes. It has been proposed that the accumulation of apoptotic cells in the tissue and circulation of patients with this disease might be due to a defect in the clearance system. In addition, several reports suggested that a complete and safe disposal of apoptotic remnants is crucial for the maintenance of peripheral tolerance. The aim of this study was to investigate whether an impaired clearance of apoptotic cells or a defect in the apoptotic pathway itself is responsible for the manifestation of SLE. We utilized primary human DCs derived from peripheral blood mononuclear cell samples and analysed the phenotypic and functional changes in DCs after uptake of apoptotic material and stimulation via Toll like receptor ligands. In this in vitro co-culture system, phagocytosis of apoptotic neutrophils as well as apoptotic Jurkat T cells from normal healthy donors (NHD) led to a substantial inhibition of lipopolysaccharide-induced DC-maturation. Interestingly, inhibited DCs from NHD showed a reduced expression of co-stimulatory surface molecules (CD86, MHC class II) as well as significantly reduced secretion of pro-inflammatory cytokines (e.g. TNF). Further data suggest that DCs derived from SLE patients are highly sensitive to inflammatory stimuli as measured by cytokine secretion in contrast to NHD. In addition, we observed that the activation of patients' DCs could still be suppressed by apoptotic tumor cells. To determine if apoptotic cells of SLE patients can also reduce cytokine secretion, we co-cultivated apoptotic neutrophils with U-937, a tumor cell line displaying DC properties. We observed an increase of inflammatory parameters in some of the patients with SLE in contrast to NHD when apoptotic neutrophils were used. These data support our hypothesis that there is a molecular defect in peripheral tolerance of SLE patients which might be due to impaired apoptosis. Further analysis is necessary to understand the mechanism and impact of these findings and to allow therapeutic approaches in the future.

P072

Indoleamine 2,3-dioxygenase (IDO): the antagonist of type I IFN driven skin inflammation?

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Recent studies have provided evidence that a type I interferon (IFN) driven immune response might play an important role in the pathogenesis of lichen planus (LP), an inflammatory disorder of the skin of

unclear etiology. Plasmacytoid dendritic cells (pDCs) in affected skin from LP have been proposed to produce IFN$\alpha\beta$?locally which leads to the expression of IFN-inducible chemokines such as IP10/CXCL10 in the epidermis. This chemokine recruits chemokine receptor CXCR3-expressing T-lymphocytes into the skin via CXCR3/IP10 interactions. Indoleamine 2,3-dioxygenase (IDO), which degrades the essential amino acid tryptophan and suppresses T cell proliferation in vitro, is induced by IFNs and other inflammatory cytokines. Here, we demonstrate a strong expression of IDO in cells at the site of LP-induced inflammation. In the same area, the expression of MxA, a protein specifically induced by type I IFNs, was seen. Thus, both pro-inflammatory and counter-regulatory mechanisms are operative in cutaneous lesions of LP. We propose that the balance of these mechanisms may be involved in the pathogenesis of this disorder.

P073

Functional indoleamine 2,3-dioxygenase (IDO) activity in human monocyte-derived dendritic cells

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Professional antigen presenting cells (APC), in particular dendritic cells (DC), are key regulators of the choice between tolerance and immunity. It has been proposed that immature DC (iDC) may present antigen in a tolerogenic fashion, whereas mature DC (mDC) drive T cell immunity. Alternatively, it has been hypothesized, that a specialized subset of mDC can also induce tolerance. It was postulated that a certain subsets of DC seems competently to express Indoleamine 2,3-dioxygenase (IDO). IDO is a rate-limiting enzyme that degrades the essential amino acid tryptophan into kynurenine. The IDO activity of mouse placenta has an essential role in preventing rejection of allogeneic foetuses. These observations introduced the concept that IDO expression could suppress immune responses by blocking T-lymphocyte proliferation. Expression of IDO was observed in cells exposed to interferons and in certain types of activated macrophages and DCs, suggesting a role of IDO in the regulation of immune responses. In the literature, there is controverse discussion as to which subsets of human DC produce IDO, and if so, which are the stimuli that induce functional IDO. Therefore, we re-evaluated human DC for their ability to express and produce functional IDO.Our most recent detailed analysis of IDO-expression by human DCs indicates that human monocyte-derived immature DCs do not express IDO constitutively. However, maturation of DCs with different maturation stimuli leads to gene expression, protein expression and functional IDO-activity in these cells. Thus, maturation per se renders DCs putatively capable of tolerance induction. Furthermore we could show that not IFNγ, but LPS is the strongest inducer of functional IDO activity in DCs.

P074 (V24)

Organization and function of a dynamic homeostatic synapse between T lymphocytes and dendritic cells

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Antigen-independent interactions between T cells and dendritic cells (DC) not only promote antigen scanning but also control survival, (slow) proliferation and antigen sensitivity of the peripheral T cell pool (homeostatic interactions). In vivo and ex vivo data suggest that these homeostatic interactions are very dynamic and short-lived. In contrast to the well established immunological synapse during antigen-specific T cell activation the molecular composition of homeostatic contacts remains poorly understood. As resolution limits together with the complex situation in a lymph node so far prevent detailed in vivo analysis, we here used co-cultures of preactivated T cells and DC in 3D collagen lattices together with confocal reconstruction and flow cytometry to study protein distribution and cell signalling events in homeostatic interactions. Like in vivo, homeostatic contacts between T cells and DC were short-lived (approximately 3–5 min) and T cells continued to migrate during these interactions (median velocity: 10 μm/min). While the T cell kept its elongated morphology and a clearly segregated distribution of LFA-1 and CD43 to the front and the back of the cell, respectively, the actin cytoskeleton polarized towards the leading edge of the interaction plane. Although neither the T cell receptor (TCR) nor MHC-complexes accumulated in the interaction plane, these short lived and dynamic contacts facilitated signal transduction with increased tyrosine phosphorylation along the interaction plane peaking at the back of the T cell (uropod). MHC-/- DC, in contrast to wildtype DC, failed to induce a slow proliferative response. However, MHC-/- DC sustained reduced tyrosine phosphorylation along the junction and prolonged survival suggesting the importance of TCR dependent and -independent signalling in the homeostatic synapse. Taken together, the homeostatic synapse represents a prototypic dynamic cell–cell interaction mode that integrates high migration velocity with TCR-dependent and -independent signals and implicates graded receptor scaffolds along the mobile interaction plane.

P075

Effects of Interferon-alpha on peripheral tolerance induced by tolerogenic IL-10-modulated dendritic cells

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The induction of tolerance is crucial for the progression of malignant diseases. Tumor-derived factors like IL-10 drive cancer immunosuppression by inhibition of dendritic cell maturation and T-cell activation. The beneficial effect of Interferon-alpha (IFN-a) adjuvant therapies of patients suffering from stage II/III malignant melanoma was widely evaluated in several studies. Multiple activities of IFN-a on immune cells have been described but no information is available on the impact of IFN-a on tolerance mechanisms. Here we investigated effects of IFN-a on immature (iDC) and IL-10 modulated tolerogenic DC (IL-10 DC) generated from peripheral progenitor cells and their respective stimulatory capacity on T-cells. As IL-10 DC are known to induce a population of anergic regulatory T-cells, we further analysed the effects of IFN-a on anergy induction. In iDC, IFN-a induced an enhanced maturation and increased stimulatory capacity demonstrated by upregulation of the differentiation marker CD83 as well as of co-stimulatory molecules. In addition, we observed a strengthened response of allogeneic CD4+ T-cells cocultured with INF-a-treated iDC. In IL-10 DC the expression of differentiation and co-stimulatory molecules was decreased as compared to mature DC. Addition of IFN-a to these tolerogenic DC provoked a significant higher expression of CD80, CD83 and CD86 molecules. Furthermore, CD4+ as well as CD8+ T-cells showed an enhanced proliferation when stimulated with IFN-a-treated IL-10 DC in contrast to T-cells activated with untreated IL-10 DC, indicating the restoration of the antigen presenting function of human tolerogenic DC in the presence of IFN-a. However, addition of IFN-a did not induce full differentiation of tolerogenic DC as triggered by a maturation cocktail

(IL-1beta, TNF-alpha, IL-6, PGE2). Notably, analysis of the anergic potential of T-cells primed with IFN-a-treated IL-10 DC in anti-CD3/CD28 restimulation assays, revealed that IFN-a abolished the generation of anergic T-cells by tolerogenic IL-10 DC and restored the T-cell response up to 95%. These experiments demonstrate that IFN-a treatment of IL-10 DC in vitro abrogates their tolerogenic properties and prevents the induction of T-cell anergy. Our results suggest that adjuvant effects of IFN-a in cancer patients may act via modulation of tolerogenic DC and inhibition of anergy induction in T-cells.

P076 (V05)

HLA class II-restricted autoaggressive T cells shape the autoantibody response against desmoglein 3 (dsg3) in an HLA-transgenic mouse model of pemphigus vulgaris (PV).

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PV is a severe autoimmune blistering disease primarily characterized by IgG autoantibodies against the desmosomal adhesion molecule, dsg3. PV is strongly associated with the HLA-DRB1*0402/DQB1*0301 alleles, providing the rationale for using HLA class II-transgenic mice to study the pathogenic relevance of immunogenetic factors. HLA-DRB1*0402/DQB1*0301, human CD4+, murine MHC class II (I-Aß)-deficient mice were immunized with human dsg3 leading to the induction of dsg3-specific IgG reactive with human and mouse epidermis and human dsg3 by ELISA. Dsg3-specific IgG1 and IgG2a initially recognized the presumably 'apathogenic' COOH-terminal extracellular domain 5 (EC5) and shifted later to 'pathogenic' NH2-terminal epitopes within the EC1 and EC2 domains. Autoreactive T cells isolated from draining lymphnodes showed the same pattern of dsg3 reactivity, i.e. recognition of COOH-terminal epitopes early on followed by recognition of NH2-terminal epitopes. Moreover, dsg3-reactive CD4+ T cells were immortalized by fusion with a TCR-deficient murine thymoma cell line (BW5147) allowing the demonstration of an HLA-DRß1*0402-restricted T cell response to defined dsg3 epitopes that were identical or highly homologous to the T cell epitopes of dsg3 identified in PV patients. Clinically, the HLA-transgenic mice developed extensive patchy hair loss as previously described in a dsg3-deficient mouse model. Of note, this phenotype was also induced when the HLA-transgenic mice were immunized with T cell-derived dsg3 epitopes. In conclusion, our observations clearly demonstrate that PV is a T cell driven, HLA class II restricted autoimmune disorder against the major autoantigen, dsg3. The present HLA-transgenic mouse model of PV holds great promise as an ideal in vivo tool to develop antigen-specific, T cell targeted therapeutic strategies in PV.

P077

TLR-mediated inflammatory responses in keratinocytes: expression of TSLP and IL-31

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In atopic dermatitis (AD) bacterial colonization with *Staphylococcus aureus* and recurrent bacterial and viral infections are a common phenomenon. Keratinocytes, known to express toll-like receptor (TLR) 2 and 3, may act as first-line defence against these pathogens by initiating innate cutaneous immune responses. Thus we hypothesized that keratinocytes activated by microbial stimuli produce factors important for modulating the allergic cutaneous inflammation in AD. To address this issue we analysed the effect of the TLR3 ligand poly(I:C), a synthetic dsRNA, and staphylococcal cell wall components [peptidoglycan (PGN), lipotechoic acid (LTA), Pam3CSK4], that act as TLR2 ligands, on the activation profile of cultured primary normal human keratinocytes (NHK) by quantitative real time PCR and/or ELISA. In addition, we compared the effects mediated by TLR2/3 ligation to those induced by type 1 and type 2 T-cell effector cytokines (IFN-γ, IL-4).Stimulation of NHK with poly(I:C) resulted in strong induction of thymic stromal lymphopoietin (TSLP) and IL-6 and, to a lesser extent, of IL-8 and GM-CSF. In addition we detected IL-8 and GM-CSF, but no TSLP and IL-6 secretion upon TLR2 ligation. Comparatively low amounts of GM-CSF, IL-8 and TSLP were found in supernatants conditioned by NHK after incubation with the Th1/Th2 cytokines IFN-γ and IL-4. Investigation of the newly described cytokine IL-31, which is known to be overexpressed in AD skin, revealed an upregulation of IL-31 mRNA strictly dependent on LTA or IL-4 activation. These results clearly show that keratinocytes are able to provide pathogen-specific inflammatory signals, which may have an influence on the course of the cutaneous allergic inflammation in AD skin.

P078

RNAi mediated gene silencing in primary T cells: a new approach for validation of target genes in inflammation

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T cells play a pivotal role in chronic dermatological diseases such as psoriasis and atopic dermatitis. The identification and validation of disease-relevant and T cell-specific target genes will advance the development of new improved dermatological therapeutics. RNA interference (RNAi) represents a powerful technology to investigate and validate the role of such target genes. RNAi relies on double-stranded RNAs of 21 nucleotides, so-called small interfering RNAs (siRNAs), that are capable to induce specific gene silencing in mammalian cells.We examined siRNA transfection of human T cells in vitro with the Amaxa nucleofection technology. siRNA-transfected T cells were compared to T cells transfected with non-silencing control siRNA and gene silencing was determined by real time polymerase chain reaction (RT-PCR), Western blot analysis and functional T cell assays in vitro.Additionally, we want to characterize the impact of RNAi-mediated gene silencing on T cell function in vivo. For this purpose, we are currently optimizing siRNA transfection of murine T cells and establishing an adoptive transfer system of murine contact hypersensitivity (CHS). This adoptive transfer system offers the unique advantage to evaluate the role of target genes in their corresponding in vivo environment of skin inflammation. Our in vitro data indicate that nucleofection efficiently delivers siRNA into human and murine T cells. Furthermore, we found that T cell stimulation, the half-life of the target mRNA and the protein need to be considered in order to achieve optimal silencing.We presently evaluate the functionality of siRNA-transfected murine T cells in vivo by adoptive cell transfer in our CHS model.

P079

Cyclosporin A abolishes CD28-mediated resistance to CD95-induced apoptosis in T lymphocytes 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 calcineurin inhibitor Cyclosporin A (CsA) fully reverses resistance to CD95-mediated cell death after TCR/CD28 costimulation or superagonistic anti-CD28 mAb stimulation of primary rat lymph node 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 (XIAP), was unaffected by CsA. Apoptosis triggered by CD95 cross-linking was characterized by robust capase-3 activation. 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 signalling 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 effector mechanisms of immune suppression conferred by CsA treatment.

P080

IgG autoantibodies targeting distinct regions of the non-collagenous domain 1 (NC1) of Collagen VII (Col VII) correlate with the clinical activity of epidermolysis bullosa aquisita (EBA)

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EBA is caused by autoantibodies against the major component of anchoring fibrils, Col VII. Utilizing a baculovirus-derived ELISA with three overlapping recombinant proteins of the NC1 of Col VII (NC1(1), aa17-610; NC1(2), aa273-857; NC1(3), aa611-1253), EBA and control sera were tested for the presence of Col VII-specific IgG autoantibodies. We investigated a total of 30 patients with autoimmune bullous diseases [EBA ($n = 9$), BP ($n = 17$), PV ($n = 1$), MMP ($n = 1$), bullous lupus erythematosus ($n = 1$), pemphigus erythematosus ($n = 1$)] and healthy controls ($n = 20$). All (9/9) of the tested sera of patients with active EBA showed IgG reactivity with the Col VII-NC1 (aa17-1253 of Col VII). Two patients with severe mechano-bullous EBA showed a direct correlation of IgG reactivity with Col VII and disease activity as determined by the autoimmune bullous disease severity and intensity score (ABSIS). Moreover, Col VII-reactive T cells were detected in a patient with EBA and recognized epitopes within aa611-1253 of the Col VII while IgG reacted with several epitopes of Col VII-NC1 (aa17-1253). IgG reactivity with Col VII was specific for EBA since none of the 18 pemphigoid sera, two pemphigus sera and healthy control sera ($n = 20$) reacted with the Col VII-NC1 recombinants by ELISA. A serum from a bullous lupus erythematosus patient showed IgG reactivity with aa611-1253 of the Col VII-NC1 domain. Our findings suggest, that (i) the established baculovirus-derived Col VII-NC1 ELISA is a powerful tool for diagnosis of EBA; (ii) IgG autoantibodies recognize, in the majority of cases, epitopes within aa611-1253 of the NC1 domain; (iii) Col VII-specific IgG titers correlate with disease activity and 4) IgG reactivity against Col VII is associated with T cell recognition of similar or identical epitopes of the NC1 domain.

P081

Role of proteinase-avtivated receptor-2 (PAR2) in human neutrophil transendothelial migration and apoptosis in vitro

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Recent findings suggest a crucial role of Proteinase-activated receptor-2 (PAR2) in inflammation and innate immunity. PAR2 is the second member of a novel G protein-coupled receptor subfamily with seven putative trans-membrane domains. This subfamily is characterized by a unique mechanism of receptor activation. Accessible serine proteases cleave the receptor to expose a new, previously cryptic, N-terminal sequence ('tethered ligand') which further interacts with the same receptor and activates it. Tryptase, trypsin, and bacterial serine proteases are capable of directly activating PAR2. PAR2 is expressed by human neutrophils, however its functions on these cells remained unclear. We reported that PAR2 stimulation leads to significant changes of neutrophil cytokine production and cell adhesion molecules expression. In the present study, we demonstrate that PAR2 agonists (serine proteases as well as synthetic activating peptides) down-regulate transendothelial migration of neutrophils and prolong their life in vitro. Moreover, PAR2 agonists enhance interferon gamma (IFNγ)-induced up-regulation of cell surface FcγRI, one of the key receptors involved in neutrophil phagocytic activity. Additionally, there is a significant increase of PAR2 expression on the neutrophil cell-surface in the case of septic patients as compared to cells from healthy volunteers. Together, our results indicate that PAR2 may be involved in the pathophysiology of sepsis in humans potentially by regulating apoptosis, transendothelial migration of neutrophils and their Fcγ-receptor expression.

P082

Th1 and Th2 cells differ in their rolling ability in vivo

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T helper subsets, such as T helper type 1 (Th1) and T helper type 2 (Th2) cells, are key players in distinct inflammatory skin diseases, e.g. psoriasis versus atopic dermatitis. Infiltration of Th1 or Th2 cells into the skin is a prerequisite for disease manifestation. While much is known about their surface adhesion molecule expression and in vitro binding abilities to endothelial receptors, little is known about their actual migration behaviour in vivo. Here we demonstrate that Th1 and Th2 cells differ not only in their expression of surface antigens but also in their consecutive in vivo rolling ability, one of the first steps in the transmigration cascade. We polarized human naïve CD4+ T cells towards Th1 or Th2 phenotype and confirmed this by their cytokine profile using a cytokine bead array, as well as chemokine receptor and adhesion molecule expression by flow cytometry. After fluorescent labelling, cells were injected retrogradely into the right carotid artery of C57Bl/6 mice. The fraction of rolling T-cells was determined in the left ear by intravital microscopy of skin postcapillary venules ($n = 14/5$ vessels/mice). Th1 cells, which compared to Th2 cells expressed higher levels of CLA (28.4 ± 4.3 vs. 10.7 ± 4.4) CD15s (12.4 ± 2.0 vs. 3.8 ± 1.2) and displayed a higher binding to sE-selectin (10.7 ± 1.9 vs. 2.9 ± 1.2) also showed significantly more rolling interactions with vessel endothelium in vivo (rolling fraction Th1: 21.8 ± 4.6 vs. Th2: 9.0 ± 1.8, $P < 0.05$). Our results indicate that Th1 cells preferentially roll along skin microvasculature and therefore should be more prone to home into skin. Further factors seem required to confer skin homing potential to differentiated Th2 cells.

P083

Profiling lymphocyte subpopulations in peripheral blood under efalizumab treatment of psoriasis by MELC robot microscopy

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CD11a-blocking efalizumab has recently been approved as a systemic treatment of moderate to severe chronic plaque psoriasis. When treating six psoriasis patients with efalizumab over 12 weeks in the present study, we observed an overall good tolerability and five treatment responders characterized by a decrease of PASI from 21.3 ± 5.4 to 3.9 ± 0.6. The accompanying significant increase of peripheral blood lymphocytes from 1.9 ± 0.7 to 4.3 ± 1.0 x10exp9/L ($P < 0.05$) was analysed by multi epitope ligand cartography (MELC) robot microscopy. Thereby a high-dimension simultaneous multiplex immunophenotyping was pursued using 39 fluorophore-labelled antibodies including labelled efalizumab and 3 other affinity reagents such as lectins. Due to efalizumab treatment there was a substantial decrease of the cellular expression of CD11a (detected by mab clone 25.3.1) and efalizumab binding sites (EfaBSs). This was paralleled by an increase of the number of EfaBS- and EfaBS+ lymphocytes by a factor of 2.4x and 2.2x, respectively. The latter effect was mainly derived from a subpopulation showing a low degree of EfaBS expression. Efalizumab treatment led furthermore to an increase of the numbers of CD3+, CD4+, CD8+, CD44+, CD45+, CD45R0+, CD45RA+, CD52+, CD58+, CD247+, HLA-DR+ and Sambucus nigra lectin-reactive lymphocytes (by factors from 2.0 to 3.3x). In terms of a combinatorial molecular phenotype we identified a CD3+/CD4+/CD44+/CD52+ lymphocyte subpopulation which accumulated most predominantly from 0.824 ± 0.270 x10exp9/L up to 1.616 ± 0.152 x10exp9/L under efalizumab treatment ($P < 0.01$). Thus, the current study extends the knowledge of efalizumab-dependent perturbations of recirculating blood lymphocyte subpopulations in psoriasis patients.

P084

Electroporation of TCR encoding RNA into T cells as a new approach for anti-tumor immunotherapy

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Cancer immunotherapy has focused mainly on the generation of CD8+ cytotoxic T cells, although tumor-specific CD4+ T cell-mediated help is required to mount an efficient anti-tumor response. Therefore, the possibility to provide tumor-specific CD4+ T-cells, either for transfer together with tumor-specific CTL, or to support the in vivo generation of anti-tumor CTLs, should be beneficial. Until now effective T cell receptor (TCR) transfer required stable retroviral transduction, which poses many risks; e.g. insertional mutagenesis, stable genetic alteration (possibly causing long-lasting autoimmunity), and unwanted additional effects of virus. Here we used an optimized RNA transfection protocol for the transient introduction of T cell receptor (TCR) alpha and beta chains, with a known antigen/MHC-specificity, into CD8+ or CD4+ T cells. A high transfection efficiency (≥85%) of EGFP-RNA for both cell types was obtained. electroporation of primary CD8+ T cells with RNA coding for a gp100/HLA-A2-specific TCR resulted in functional CTLs, recognizing both peptide-loaded and melanoma cell line targets, and responding with cytokine production or cytolysis. Furthermore, the electroporation of primary CD4+ T cells with RNA coding for either a MAGE-3/HLA-DP4-specific TCR or the gp100/HLA-A2-specific TCR resulted in antigen-specific pro-inflammatory cytokine production. Mainly IL-2, TNF and IFNγ were produced, but also low amounts of IL-4 and very little IL-10 were observed, indicating that TCR-transfected T cells mostly have a Th1 phenotype. These TCR-transfected CD8+ and CD4+ T cells, even after freezing and thawing, can be used simultaneously, or separately to induce tumor lysis and to provide T cell help. Our method for transient TCR transfer using RNA electroporation into CD8+ and CD4+ T cells is a promising new strategy to induce more efficient CD8+ T cell responses for the immunotherapy of cancer.

P085

Mass spectrometric comparison of antimicrobial active psoriatic scale and healthy stratum corneum extracts: are there more skin-derived antimicrobials?

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Why is healthy skin not infected and why are psoriatic lesions remarkably free of microbial infections? A number of skin-derived antimicrobial peptides have been discovered in the past. 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 analysed and compared mass spectrometric profiles and antimicrobial activities of fractionated psoriatic scale and healthy stratum corneum extracts. HPLC fractionation of heparin-affinity purified scale and healthy stratum corneum extracts and subsequent testing for antimicrobial activity revealed antimicrobial activity against various microorganisms in many fractions. While scale extracts are a rich source of antimicrobials higher amounts of healthy stratum corneum material were necessary to isolate and analyse antimicrobial activity. Major mass signals (revealed by ESI-QTOF mass spectrometric analysis) in antimicrobial active fractions of both healthy stratum corneum and psoriatic scale extracts correspond to known heparin-binding antimicrobial peptides/proteins identified as psoriasin, hBD2, lysozym and RNase 7. The mass spectrometric signal for psoriasin was the most prominent signal in both healthy stratum corneum and psoriatic scale extracts. Mass spectrometric signal intensities for hBD2, RNase 7 and lysozyme relative to the psoriasin mass signal appeared reduced in healthy stratum corneum extracts when compared to psoriatic scale extracts. Less intense mass signals in other HPLC fractions with antimicrobial activity were found in both extracts and indicate the presence of not yet identified antimicrobials differentially expressed in healthy stratum corneum and psoriatic lesions. Further, search for the cathelicidin hCAP-18/LL-37 and its truncated forms in both, extracts of chronic plaque psoriasis and healthy person's stratum corneum, failed, suggesting that it is only present in minute amounts. Following further purification and re-evaluation of antimicrobial activity future mass spectrometric mapping and sequencing experiments with not yet identified molecules may provide first clues on their identity.

P086

Biological drug binding biochip assay using the MELC robot technology as explored for the affinity of Efalizumab to psoriatic skin tissue

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Multi epitope ligand cartography (MELC) robot technology allows sub-cellular point-precise in situ co-location of large numbers of epitopes giving insight into toponomics. Thereby, a given skin tissue section is processed through successive cycles of (i) labelling with fluorophore-tagged antibodies, (ii) fluorescence imaging and (iii) soft bleaching. Pixel-precise overlay of images provides combinatorial co-location data.We present details of our development of MELC to a biological drug binding biochip assay inaugurated for the antipsoriatic efalizumab. For this purpose efalizumab was covalently conjugated to FITC (by its 5-Ex-succinimidyl ester) to be subsequently integrated into a MELC library of more than 40 antibodies. SDS-PAGE analysis revealed successful binding of FITC to efalizumab. FITC was found to be bound to the heavy and light chains of the protein following separation by mercaptoethanol. The binding specificity of FITC-labelled efalizumab using MELC was investigated first on PBMC samples. Preincubation of the cells with unlabelled efalizumab caused a nearly complete loss in the binding of FITC-labelled efalizumab. FITC-labelled efalizumab and clone 25.3.1 also directed to CD11a, both, revealed a nearly identical staining pattern. Then, we performed a detailed analysis, by multivariate gating, of efalizumab binding site co-location with selected markers (particularly those characterizing T-cells, dendritic cells and NK cells) in defined skin microcompartments.Example, in the epidermo-dermal compartment of affected psoriatic skin we observed that co-location of the memory phenotype CD45R0 was very similar in CD4+/CD3+ T-cells and in CD8+ T-cells. There was a high degree of efalizumab binding site co-location (98.1 ± 2.5 % and 97.8 ± 1.8 %) in CD4+/CD3+ and CD8+ memory T-cells, respectively.The biological drug binding MELC biochip assay may be especially useful for this new class of medication with regard to (i) preclinical ex vivo predictive testing and (ii) clinical treatment-related monitoring of skin lesions, thereby contributing to an increase of drug safety.

P087

The bulge – a second site of epithelial immune privilege within the human anagen hair follicle?

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The hair follicle epithelium rhythmically generates, maintains and deconstructs an area of relative immune privilege (IP) in the anagen hair bulb, i.e. namely in the cyclically regressing hair matrix, which may serve to sequester anagen- and/or melanogenesis-related autoantigens from immune recognition and autoaggressive inflammation. Recent gene profiling data from putative epithelial hair follicle stem cells isolated from the bulge region of mouse and human hair follicles, however, has raised the possibility that a second immunoprivileged site may exist in the bulge region. However, in prior studies, using standard immunohistology, we had failed to obtain evidence that the bulge region in man or mice, the seat of epithelial hair follicle stem cells, is immunoprivileged.Using more sensitive immunohistological staining methods like tyramide signal amplification (TSA) immunofluoresence and Envision®, we have collected new protein expression evidence to support or refute this concept. In the current study, we provide the first evidence that, in contrast to the epidermis and the distal part of hair follicle epithelium, MHC class Ia and MHC class II expression are indeed downregulated in the bulge region of human scalp hair follicles, whereas both CD200 receptors and locally generated immunosuppressants like TGF-β1 and α-MSH are upregulated. These findings (which are complemented by additional immunohistological indicators of IP) parallel the IP of the human anagen hair bulb and the proximal human nail matrix, and strongly suggest that the human hair follicle bulge region represents an additional area of relative IP in human skin. A defective bulge IP may play an important, previously unrecognized role in the development of many types of scarring alopecia,

where an autoaggressive inflammatory cell attack on this region damages and, ultimately, destroys the hair follicle's stem cell-based regenerative capacity, thus causing permanent hair follicle destruction. Therapeutic restoration and protection of this newly discovered bulge IP should, therefore, become a new strategy in the – still very frustrating – management of scarring alopecia.

P088

Natural killer (NK) and NKT cells may be crucial in the herpes simplex virus (HSV) immune responses

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Episodes of HSV reactivation as symptomatic disease, but also asymptomatic shedding and transmission, can be suppressed but not cured by antiviral medications. Manipulation of immune responses seem to be a critical component to decrease disease burden. The objective of our experiments was to compare the T cell immunity of HSV I-seronegative subjects (HSV neg) with HSV I-seropositive individuals with silent infection (HSV pos) -no episodes in the past 5 years and with recurrent infections (HSV rec)-more than two episodes per year. Lymphocyte subpopulations were analysed performing four-colour flow cytometric analyses (Becton Dickinson Multitest) at the beginning of reactivation in HSV rec, analogue any time point (tp) 0 in HSV neg and HSV pos, and over a time period of 2 and 6 weeks (tp2, 6), respectively. 6 HSV neg had a lower median frequency of 105/μl NK cells (CD16+CD56+) and of 30/μl NKT cell (CD3+CD16+CD56+) than compared to 12 HSV pos of 170/μl NK cell and 45 NKT cell and to 12 HSV rec of 170/μl NK cell and 50 NKT cell. Of HSV rec, the median frequencies of CD8 T cell (50/μl and 45/μl, respectively; $P = 0.042$; Wilcoxon-test) and NKT cell (560/μl and 495/μl, respectively; $P = 0.028$; Wilcoxon-test) decreased significantly between tp0 and tp6. No significant differences of the other lymphocyte subpopulations of the groups were noted. This study, so far, demonstrates that the lymphocyte subsets NK and NKT cells may be crucial in the HSV immune responses. Further experiments for quantification and functional assessment of HSV-specific T cell responses measured by the enzyme-linked immunospot (Elispot) assay are ongoing.

P089

Eukaryotic expressed human osteopontin and its fragments interact with various receptors to induce dendritic cell migration

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Osteopontin (OPN) is a secreted phosphoglycoprotein with Th1 cytokine functions that is involved in the initiation of CHS by guiding DC into lymph nodes and by transiently inducing their Th1 polarizing phenotype. OPN functions are altered by posttranslational modification and thrombin cleavage, dividing the protein into a C-OPN and N-OPN fragment. The OPN molecule contains binding domains for CD44, probably in both fragments. The N-terminal fragment contains the RGD av-integrin binding sequence and the SLVVGLR a4b1/a9b1 binding site. We found that DC differentially express CD44v isoforms upon maturation. To further investigate pathways of the different OPN functions, we determined the regulation of OPN integrin receptors in DC. Upon terminal DC maturation avb3 and avb5 are upregulated while a9b1 and a4b1 are constitutively expressed. Previously we had generated murine eukaryotic OPN. To investigate the function of more physiological eukaryotic human full length OPN (fl-OPN) that may functionally differ substantially from the previously tested prokaryotic protein, we expressed 6xhis-tagged eukaryotic human fl-OPN and its N- and C-terminal fragments in HEK-293-EBNA cells. Interestingly, the C-OPN transfected cells do not adhere to plastic and are reduced in their viability. Because his-tag specific western blot did not detect C-OPN, we speculate that either C-OPN accumulates intracellularly or that small amounts of surface bound C-OPN block adhesive functions of cell receptors. Concentrated supernatants of fl-OPN and N-OPN transfected cells were purified by nickel affinity chromatography. To generate C-OPN, fl-OPN was cleaved by thrombin and C-OPN was separated by the C-terminal 6xhis-tag. The OPN variants containing the different receptor binding sites were compared in their function to induce DC migration. fl-OPN and OPN fragments potently induce DC migration. Withdrawal of Ca2+/Mg2+, inhibiting integrin function only partially reduces DC migration towards the variants, underlining the importance of CD44 in OPN induced DC migration. In conclusion eukaryotic human OPN and OPN fragments induce DC migration, indicating that multiple domains of OPN, which interact with different OPN receptors, contribute to regulated DC migration.

P090

Optimized peptide delivery to dendritic cells (DCs): Increased T cell stimulatory capacity by loading DCs with complexes of cationic, antigenic peptides and Poly I/C dsRNA

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In recent years dendritic cells (DCs) have become popular candidates in cancer vaccine development because of their crucial role in inducing T-cell responses. The aim of the present study is to develop a novel vaccination strategy utilizing DCs loaded with synthetic, cationic (positively charged), and antigenic peptides complexed to negatively charged Poly I/C dsRNA. The positively charged protein transduction domain of the HIV-1 TAT protein was shown to rapidly translocate across the plasma membrane of living cells. This property can be exploited for the delivery of proteins, drugs, and genes into cells. Although the mechanism of translocation is not yet understood we adapted this system by fusing an antigenic, major histocompatibility complex-class I-binding epitope (Melan-A/Mart-1 sequence: ELAGIGILTV) with a cationic sequence derived from the HIV tat transduction domain (tat49-57: RKKRRQRRR). The cationic fusion peptide compound is then mixed with a negatively charged Poly I/C dsRNA compound to quantitatively form peptide/nucleic acid complexes. We found that the loading of DCs with complexes of cationic, antigenic peptides and Poly I/C dsRNA or cationic, antigenic peptides alone did not negatively affect the DC viability. The peptide-Poly I/C complexes readily induced a full DC maturation as measured by surface expression of CD83, CD80, and HLA-DR. When using peptide-Poly I/C

complex loaded DCs for two cycles of autologous T cell stimulation a quantitatively superior epitope specific IFN-gamma secretion in comparison to DCs maturated by a cocktail of cytokines and loaded with peptide could be measured in an ELISPOT assay. In conclusion, complexes of cationic, antigenic peptides and Poly I/C dsRNA might be used for a TLR-3 mediated DC maturation and intracellular peptide targeting in a single step. The improved T cell stimulatory capacity of such DCs might be due to a prolongated presentation after intracellular delivery of peptides.

P091

Analysis of NOD1 and NOD2 expression in human skin

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Human skin is able to initiate a defense response upon contact with potential harmful bacteria leading to an increased expression of antimicobial proteins and proinflammatory cytokines. Recognition of bacteria and their products (so-called 'pathogen-associated molecular patterns, PAMPs') is mediated via specific receptors (so-called 'pattern recognition receptors, PRRs') which are able to recognize PAMPs. In addition to Toll-like receptors a novel class of intracellular PRRs, the NOD-like receptors, have recently been identified. NOD ('nucleotid oligomerization domain') -like receptors contain C-terminal leucine-rich repeats (LRRs) which may mediate the recognition of intracellular bacterial PAMPs (e.g. peptidoglycan). Recently we identified expression of NOD2 in human primary keratinocytes and demonstrated that activation of NOD2 mediates the expression of the antimicrobial peptide human beta-defensin-2. Since nothing is known about the protein expression of NOD1 and NOD2 in human skin, we analysed protein expression of NOD1 and NOD2 in human skin using immunohistochemistry. As a result we observed distinct immunoreactivity of NOD1 and NOD2 in the keratinocytes of the outer, more differentiated layers of human skin. In contrast to NOD1, we detected strong immunoreactivity of NOD2 in sebaceous glands. NOD1 and NOD2 expression was upregulated in psoriatic epidermis which is in concordance with data showing induction of NOD1 and NOD2 expression through proinflammatory cytokines.In summary our results demonstrate that the PRRs NOD1 and NOD2 are expressed in human skin where they may participate in the recognition of potential pathogenic bacteria.

P092

Blocking the interaction with complement and Fc receptors abolishes antibody-induced blistering in experimental epidermolysis bullosa acquisita

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Epidermolysis bullosa acquisita (EBA) is a severe autoimmune skin disease characterized by tissue-bound and circulating autoantibodies to type VII collagen, the major component of anchoring fibrils. When passively transferred into mice, rabbit IgG against type VII collagen induces Fc-dependent activation of complement, the recruitment of leukocytes into the skin and subepidermal blistering. In addition to these inflammatory mechanisms, it was speculated that antibodies against type VII collagen might induce blisters by disrupting the ligand function of type VII collagen by an Fc-independent mechanism. In the present study, to address this question, we injected mice with anti-type VII collagen chicken IgY antibodies, which cannot bind to complement and Fc receptors of mammalian origin. Like rabbit IgG, chicken IgY antibodies against type VII collagen bound to the dermal-epidermal junction of skin sections. However, in contrast to rabbit IgG, IgY antibodies did not fix complement C3 in ELISA and immunofluorescence complement-binding assays. In addition, in contrast to IgG, IgY antibodies did not recruit granulocytes and induce dermal-epidermal separation ex vivo. Following their passive transfer into Balb/c mice ($n = 4$), chicken IgY antibodies against type VII collagen bound at the dermal-epidermal junction, but did not fix complement C3 or recruit granulocytes. Importantly, these mice did not develop skin blisters. In contrast, BALB/c mice ($n = 4$) injected with rabbit IgG against type VII collagen developed a blistering phenotype, associated with deposits of IgG and complement C3 as well as granulocytic infiltration at the dermal-epidermal junction. Control mice injected with normal rabbit IgG or chicken IgY ($n = 2$ per group) did not develop skin disease. These findings demonstrate that Fc-dependent engagement of humoral and cellular inflammatory factors by pathogenic antibodies is required for subepidermal blistering in experimental EBA.

P093 (V14)

NADPH oxidase is required for neutrophil-dependent tissue damage induced by autoantibodies against type VII collagen

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Autoimmunity against type VII collagen is associated with epidermolysis bullosa acquisita (EBA), bullous systemic lupus erythematosus and inflammatory bowel diseases. We previously demonstrated ex vivo that antibodies to type VII collagen induce dermal-epidermal separation in cryosections of human skin when co-incubated with human granulocytes. In addition, antibodies against type VII collagen, when passively transferred into mice, cause a subepidermal blistering skin disease reproducing the findings in the skin of EBA patients. Our previous studies further revealed that granulocytes are required for antibody-induced blistering both ex vivo and in mice. On the other hand, NADPH oxidase-derived reactive oxygen species are thought to be critically involved in granulocyte-mediated tissue injury. In the present study, we demonstrate that NADPH oxidase is a prerequisite for the antibody-induced granulocyte-dependent blistering in both ex vivo and in the mouse model of EBA. Both specific inhibition of the NADPH oxidase from human granulocytes or the use of granulocytes from chronic granulomatous disease patients, defective in NADPH oxidase, accounted for the lack of dermal-epidermal separation in vitro. In addition, mice deficient in the p47 subunit of the NADPH oxidase (p47phox-/-; $n = 10$), in contrast to wild type mice ($n = 10$), failed to develop blistering by injection of antibodies against type VII collagen. Transfer of p47phox -sufficient granulocytes into p47phox -deficient mice ($n = 5$) restored their susceptibility to tissue damage by antibodies against type VII collagen demonstrating that granulocytes

provide the NADPH oxidase required for tissue damage. Our findings identify granulocyte-derived NADPH oxidase as key molecular effector engaged by pathogenic autoantibodies and provide relevant targets for prevention of tissue damage in granulocyte-dependent autoimmune diseases.

P094

Desmocollin IgG and IgA autoantibodies are restricted to atypical and paraneoplastic pemphigus (PNP)

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Desmogleins (Dsgs) and desmocollins (Dscs) are transmembrane cadherins that are components of desmosomes adhesions structures critical for tissue integrity of stratified epithelia and their appendages. Dsgs and Dscs have four and three isoforms respectively, Dsg1-4 and Dsc1-3, respectively. The autoimmune blistering disorders of the pemphigus group are characterized by autoantibodies targeting Dsg1 and Dsg3, and occasionally, Dsc1-3. In this study we investigated the incidence of IgG autoantibodies against Dsc1-3 in a cohort of patients with pemphigus vulgaris (PV; $n = 76$) who were clinically characterized by predominant mucosal ($n = 15$), cutaneous ($n = 7$), or mucocutaneous ($n = 54$) phenotype and sera from patients with PNP ($n = 3$), IgA pemphigus ($n = 3$) and atypical pemphigus ($n = 1$).
Methods: Recombinant proteins consisting of the entire extracellular domains of human Dsg3, Dsc1, Dsc2 and Dsc3 were produced in the baculovirus expression system and sera were analysed by enzyme linked immunosorbent assay (ELISA).
Results: All of the tested PV ($n = 76$) and PNP sera ($n = 3$) showed IgG reactivity with Dsg3 while only 1/3 IgA pemphigus sera showed IgA reactivity with Dsg3. In contrast, none of the PV sera showed IgG reactivity with Dsc1-3. However, 1/3 of the PNP sera was IgG reactive with Dsc3 and an atypical pemphigus serum showed both, IgG and IgA reactivity with Dsc1. Conclusion: IgG reactive with Dsc is not commonly found in PV and is restricted to cases of PNP and atypical pemphigus.

P095

Anti-cancer activity of TLR7/8 activated dendritic cells

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As a synthetic agonist to toll-like receptor 7/8, imiquimod has been successfully used for the treatment of certain skin neoplasms. In order to gain insight into the underlying mechanisms of tumor clearance, we analysed lytic molecules expressed by the inflammatory infiltrate induced by the imiquimod treatment of basal cell carcinomas.Results obtained showed that CD11c+HLA-DR+CD14-TNF+iNOS+ myeloid dendritic cells, but not NK-cells or CD8+ T-cells, were the main source of perforin and granzyme B in the peritumoral imiquimod-induced infiltrate. Expression of TRAIL was observed on a broader spectrum of cells including plasmacytoid dendritic cells around tumor cell islets. In the peripheral blood, freshly isolated CD11c+ myeloid dendritic cells were essentially devoid of lytic molecules. Upon toll-like receptor 7/8 stimulation, however, CD11c+ myeloid dendritic cells exhibited anti-perforin and anti-granzyme B reactivity and were able to secrete these molecules in the culture supernatant. The same activation protocol led plasmacytoid dendritic cells to express TRAIL on their surface. More important even was the finding that both activated myeloid and plasmacytoid dendritic cells could use these molecules to effectively lyse cancer cell lines.These data suggest that myeloid and plasmacytoid dendritic cells are directly involved in imiquimod-induced tumor destruction. The attractive possibility exists that these DC populations, when appropriately activated, can act as effector cells in anti-cancer immunity.

P096

Characterization of subchronic versus acute mouse models of DNFB-induced skin inflammation

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This study evaluates whether an elaborated 2,4-dinitrofluorobenzene (DNFB) dosing schedule in comparison to an acute standard schedule (i) is suitable for a subchronic mouse model of contact hypersensitivity (CHS), (ii) exhibits improved chronic characteristics of skin inflammation, and (iii) closer reflects the therapeutic response pattern of chronic T cell-dependent skin diseases like psoriasis. The type 1 cytokine-dominated DNFB-induced CHS model is structured in sensitization phase (first exposure), leading to establishment of T cell memory, and challenge or elicitation phase (second exposure) leading to T cell-dependent skin inflammation. We compared single challenge (acute model) versus repeated challenges with reduced allergen load (subchronic model) and searched for chronic features of skin inflammation.In subchronic model ear edema formation and treatment response could be followed over time courses of 3–4 days pursuing ear thickness. Strong cutaneous T cell infiltration as well as disability of athymic nude mice to respond to subchronic DNFB treatment demonstrate the pivotal role of T cells. Moreover, only in subchronic setting characteristics of psoriasis as epidermal thickening, hyper vascularization and a strongly enhanced cytokine expression are observed. Systemic or topical treatment with standard glucocorticoids over the elicitation phase is dose-dependently efficacious. Remarkably, antagonization of cytokines is anti-inflammatory active in psoriasis and in subchronic model but not in acute CHS. Taken together, repetitive hapten challenges facilitate the development of hallmarks of chronic skin inflammation in DNFB-induced CHS model in mice and increase the similarity to chronic inflammatory skin diseases like psoriasis.

P097

TLR ligands modulate skin for improved dendritic cell migration. A model for adoptive immunotherapy

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Adoptive immunotherapy using tumor-antigen charged dendritic cells is severely limited by ineffective migration of injected cells from the site of injection to the draining lymph nodes. We established a mouse

model, adapted to human vaccination protocols, that may serve as a tool to improve dendritic cell migration from skin to lymph node.- Congenic (CD45.1) dendritic cells were injected intradermally into ear skin. Numbers and phenotype of injected dendritic cells arriving in the lymph nodes were evaluated. As previously described, epicutaneous pretreatment of the injection site with inflammatory cytokines (conditioning) 24 h before injection of dendritic cells is crucial for enhanced cell migration. Based on these data, we investigated the effects of diverse TLR ligands (e.g. poly(I:C),CpG, R848) on skin conditioning prior to dendritic cell injection. Treatment of skin with TLR ligands (epicutaneous or injected) caused an increase of dendritic cells arriving in the lymph node and, as a consequence, improved antigen-specific T-cell responses. Combinations of TLR ligands and mechanical stimuli for inflammation proved particularly efficient. Additional combinations will be tested. This mouse model should allow to optimise adoptive dendritic cell therapy via the skin.

P098

Mast cell carboxypeptidase A is the essential protection factor against endothelin-1 and snake venom proteins of the sarafotoxin group in vivo

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Mast cells have been shown previously to provide in vivo protection against snake venom sarafotoxins and endothelin-1 (ET-1), a potent vasoconstrictor and mediator in systemic and cutaneous inflammatory processes. Mast cell deficient mice challenged with ET-1 or sarafotoxin succumb to the toxic effect of these 21 amino acid peptides whereas if mast cells are present in the mouse, the animal survives. There has been some evidence that proteases stored in the mast cell granules are involved in this protective effect of mast cells, but the exact way in which the proteases exert their function and the type of enzyme(s) that are required for protection of mice from the lethal action of sarafotoxin and ET-1 have not been definitively identified. We have generated mice with a targeted deletion of the mast cell-carboxypeptidase A (Mc-cpa) gene, that do not only lack the Mc-cpa protein but also a chymase, mast cell protease-5 (Mcp-5), possibly due to instability of Mcp-5 in the absence of Mc-cpa. We show now that these mutant mice, double-deficient for Mc-cpa and Mcp-5 expression are as vulnerable to sarafotoxin and to ET-1 as mice lacking mast cells. Further, we can show that a crucial biochemical detoxification step catalysed by Mc-cpa is the removal of the C-terminal tryptophan-21 essential for receptor binding of ET-1 and sarafotoxin. To address the possible contribution of Mcp-5 in this process we generated by gene knock-in a second mouse mutant in which the wild type Mc-cpa allele was replaced by a catalytically inactive Mc-cpa allele (Mc-cpaY248L + E270A). Expression of the Mc-cpaY248L + E270A protein was permissive for expression of Mcp-5. These mice also succumb to sarafotoxin. To ET-1 they show an attenuated response that finally still results in death in most cases. This suggests that Mcp-5 is only of partial importance for the detoxification of ET-1 related peptides. Thus, a single mast cell enzyme, carboxypeptidase A, is sufficient for protection of mice against snake venom sarafotoxin and ET-1. We propose that, during evolution, snake venom sarafotoxin has been unable to evade Mc-cpa's C-terminal degradation specificity because the same area of the peptide is required for its toxicity.

P099

Development of protein-contact dermatitis depends on tissue origin of activated T cells

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Epithelial surfaces of skin and intestine form barriers to the external environment and are permanently exposed to multiple potential antigens. Clinical observations suggest that oral antigen exposure may activate antigen specific T cells not only within the gut associated lymphoid tissue (GALT) but also to distant sites. As seen in patients with atopic dermatitis, food allergen-reactive T cells could be isolated from the skin. Antigen-presenting dendritic cells from skin and gut have been shown to imprint specific T cells in vitro for selective homing to their corresponding organs after transfer in vivo. Therefore we investigated the potential of GALT-derived antigen-specific T cells to migrate to the site of antigen-exposure. Ovalbumin (OVA)-specific T lymphocytes from OVA T cell-receptor transgenic mice fed with OVA-protein were transferred into naïve BALB/c mice exposed to OVA either by oral administration or epicutaneous application. After oral OVA challenge we observed specific enrichment of OVA-reactive T lymphocytes only in the GALT whereas random distribution of OVA-specific T lymphocytes was seen in control mice. Antigen-specific homing was associated with an upregulation of $\alpha 4\beta 7$ integrin and concomitant downregulation of L-selectin and P-selectin ligand (PSGL-1) expression on OVA-specific T lymphocytes. Interestingly, epicutaneous OVA exposure directed gut derived OVA-specific T lymphocytes predominantly into all skin draining lymph nodes accompanied by reduced L-selectin and PSGL-1 expression. In contrast to activation by feeding OVA, no $\alpha 4\beta 7$ expression was detected after epicutaneous OVA exposure. In parallel, skin primed OVA-specific T lymphocytes were transferred into naïve BALBc mice and migration was analysed after epicutaneous or oral challenge. We observed an antigen-specific enrichment of OVA-specific T lymphocytes in the skin draining lymph nodes after epicutaneous OVA exposure. After oral antigen administration OVA-reactive T lymphocytes migrate predominantly in the GALT. Importantly, no clinical characteristics of protein-contact dermatitis could be observed in mice after transfer of GALT-primed OVA T lymphocytes and epicutaneous antigen exposure. In contrast, skin-primed OVA-specific T lymphocytes were able to induce eczema after adoptive transfer and epicutaneous OVA challenge.

P100

Influence of effector T cells on the micro milieu of lymphatic organs

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Immigration of effector T cells during inflammation is an important process in different skin diseases and during wound healing because it is essential for the curing of the disease. To understand the pathology of these processes in more detail we started to investigate the effects of migrating T lymphocytes on the micro milieu and we took the spleen as an example. Until now it is not clear whether migrating cells are able to influence directly the micro environment of lymphatic organs. Therefore, we used an established animal model and injected naïve or memory T cells as well as in vitro activated lymphocytes into congenic rats. Immunological analyses allowed us to observe these different cell types during their migration to study their effects on endogenous

populations inside the spleen. All investigated cell types showed a similar migration pattern. Interestingly, only effector T lymphocytes were able to increase the expression of IFN-gamma, IL12 and IL-10 mRNA in different compartments of the spleen. Furthermore, in contrast to naïve and memory T cells, effector T cells induced proliferation of endogenous cells. They caused the induction of germinal centres, a clear sign for a T cell dependent B cell immune response. Therefore, our results clearly indicate an influence of effector T cells on the micro environment of the spleen.

P101

Investigation of the in and ex vivo expression of the human antimicrobial protein RNase 7

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Healthy human skin protects itself by the permanent release of endogenous antimicrobial proteins. Recently, we identified RNase 7 as a major antimicrobial protein of healthy skin. To gain more insight into the in vivo expression of RNase 7 we investigated the RNase 7 concentrations on the skin surface of healthy volunteers as well as patients suffering from psoriasis. In vivo washing fluids derived from various skin locations were analysed by ELISA using a novel generated RNase 7 antibody. As a result RNase 7 expression could be detected on various skin surfaces (e.g. arm, leg, sole of foot, hand, axilla, forehead). Surprisingly, the non-affected skin surface of patients suffering from psoriasis showed induced levels of RNase 7 as compared to the same skin locations of healthy persons. Highest RNase 7 amounts could be recovered from psoriatic lesions. These results indicate that RNase 7 may contribute to the low infection rate of psoriatic skin. Recently we found that keratinocytes express induced levels of RNase 7 upon contact with bacteria. To analyse whether bacterial contact results in increased amounts of RNase 7 on the skin surface we treated the surface of skin explants with bacterial culture supernatants. This ex vivo approach revealed that bacterial supernatants can induce the release of RNase 7 on the skin surface. Together, these results show that RNase 7 is not only constitutively expressed in high levels in healthy skin but also inducible expressed at sites of bacterial infection thus contributing to enhanced innate cutaneous defense.

P103

Expression and function of CD137 receptor and ligand in human mast cells

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Mast cells are traditionally viewed in the context of anaphylactic reactions due to their ability to release preformed and rapidly synthesised substances. However, mast cells are also involved in innate and adaptive immune responses. During these processes, mast cells and T cells have to communicate with each other. Here, we investigated whether the CD137 receptor, a well known T cell co-stimulatory molecule, and its ligand may be involved in these cell-cell interactions.In the human mast cell line HMC-1, we found a constitutive mRNA and protein expression of the CD137 ligand and its receptor. Upon cell stimulation, the expression levels of CD137 receptor increased significantly, whereas the expression of the ligand remained unaffected. Activation of mast cells via CD137 ligand induced a selective cytokine response in HMC-1 cells, reflected in increased levels of TNF-α ?and IL-6 secretion, but not of IL-8 secretion. In contrast, stimulation via CD137 receptor did not result in secretion of these cytokines. These findings suggest different roles for the CD137 receptor and its ligand in mast cell activation. Since, the CD137 ligand may act as an early mast cell stimulator, the CD137 receptor may gain importance later on in the activation process, when it may, perhaps, function as a negative regulator of cell activation.

P104 (V20)

A Novel peptide antibiotic derived from human hornerin

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Human skin is constantly exposed to microorganisms, but normally stays healthy. During the last years it became clear that our skin serves not only as a physical barrier against infection but also as a chemical barrier ± by constitutively and inducibly producing antimicrobial peptides and proteins (AMPs). With a biochemical/functional strategy, we previously identified RNase7 and S100A7/psoriasin as principal AMPs in extracts of healthy persons stratum corneum. Since we further detected several non-characterized antimicrobially active peptides, we aimed in this study to determine their molecular structure. By HPLC-analyses, we purified from extracts of healthy persons stratum corneum an antimicrobial peptide (AMP), which by Edman degradation, electrospray-ionisation-mass spectrometry (ESI-MS) analyses and mass spectrometrical sequencing showed high sequence similarity to mouse hornerin. We therefore suggested that it possibly originated from a human ortholog. To test this hypothesis, we cloned the complete hornerin cDNA sequence and revealed that hornerin possesses a unique protein structure with a S100 domain, an EF-hand calcium-binding domain, a spacer sequence and two types of tandem repeats, suggesting that it represents a novel member of the fused S100 protein family. Hornerin mRNA was detected to be constitutively expressed in foreskin-derived primary keratinocytes cultures, but surprisingly rather location-dependent in skin tissue. To follow the hypothesis, that hornerin fragments represent AMPs, we recombinantly expressed three hornerin peptides rHRNR2 (tandem repeat unit B), rHRNR3 (tandem repeat unit A) and rHRNR4 (C-terminus) and subsequently analysed their antimicrobial activity using the microdilution assay system. The rHRNR3 peptide, containing the sequence motif found in the purified natural hornerin fragment isolated from stratum corneum, exhibited antimicrobial activity at low micromolar concentrations against *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans*. The other peptides were found to be not or nearly not antimicrobially active.Our results suggest that hornerin may have a yet unknown protective function in healthy human skin as part of the chemical barrier ± with preformed AMPs, which are generated from parts of the tandem repeats of a hornerin precursor molecule by a yet unknown cleavage mechanism.

P105

Essential role of hypoxia-induced angiogenesis in delayed-type hypersensitivity reactions

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Angiogenesis plays a major role in organ-specific autoimmune diseases caused by delayed-type hypersensitivity reactions (DTHR). The exact mechanisms involved in neoangiogenesis in DTHR remain enigmatic. Hypoxia can induce angiogenesis via stabilization of the transcription factor hypoxia inducible factor (HIF)-1α in resident and infiltrating cells. After dimerization of HIF-1α with HIF-1β, HIF-1 binds to the hypoxia-responsive elements in the regulatory regions of proangiogenic mediators such as vascular endothelial growth factor A (VEGF-A). For better understanding of mechanisms involved in angiogenesis we investigated the role of hypoxia in acute and recurrent episodes of cutaneous DTHR using 18F-fluoroazomycin arabinoside (FAZA) that selectively accumulates in hypoxic tissue. In our experiments C57BL/6 mice were sensitized and challenged with trinitrochlorobenzene (TNCB) to induce and elicit cutaneous DTHR. Mice were injected 12 h after challenge with 18F-FAZA and scanned in vivo using positron emission tomography (PET) and computed tomography (CT). Moreover tracer uptake was determined by autoradiography. In addition we performed H&E-staining, HIF-1α- and HIF-2α-immunhistochemistry and real-time PCR (RT-PCR) analysis of gene expression patterns. 64Cu-PTSM and PET-CT was used to exclude a perfusion effect. In-vivo PET-CT images confirmed intense 18F-FAZA uptake already in the acute phase of cutaneous DTHR persisting at the same level even after recurrent episodes of cutaneous DTHR. H&E staining confirmed enhanced blood vessel formation in chronic DTHR. During chronic DTHR 2% of infiltrating lymphocytes and some endothelial cells showed nuclear expression of HIF-1α- and HIF-2α protein. RT-PCR analysis confirmed an enhanced expression of pro-inflammatory and pro-angiogenic genes, such as TNF, IL-1β, VEGF, HIF-1α- and HIF-α 4–24 h even after the first TNCB-challenge. Thus, non-invasive in vivo examination of hypoxia-induced angiogenesis using 18F-FAZA appears to be a new powerful tool to examine early phases of angiogenesis in autoimmune diseases such as rheumatoid arthritis.

P106

Expression profiles of chemokine receptors in melanoma metastases

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Site-specific metastases in melanoma patients are reported to be due to the selective expression of certain chemokine receptors by melanoma cells and the presence of their ligands in specific tissues. Because profiles of chemokine receptor expression in human melanoma metastases are unknown, we performed a study investigating the expression of eighteen chemokine receptors in almost pure paraffin embedded metastatic melanoma tissue on the mRNA level by real-time PCR, employing a semiquantitative approach. Expression of CCR9, CXCR6 and XCR1 was studied by immunohistochemistry. Ten cutaneous-subcutaneous metastases, twelve lymph node metastases, three intestinal metastases, two lung metastases, two brain metastases, two kidney metastases and one metastasis to the bone, liver and testicle, respectively, were analysed. Additionally, normal tissue of intestine, brain and kidney of the same patients was also studied. Transcripts for CXCR5 and CXCR6, which were absent in nevi and melanoma cell lines, were found inconsistently in metastatic lesions. Strong expression of CXCR6 was observed in intestinal metastases, followed by lymph node metastases, brain and kidney metastases, this expression pattern was partially mirrored by the positive staining observed by immunohistochemistry. CXCR4 and CCR1 were consistently expressed in all metastatic lesions. Relative levels of both receptors were highest in visceral metastases followed by lymph node and cutaneous-subcutaneous metastases. CCR9 and XCR1 were expressed in all lesions at different levels, most abundantly in lymph nodes. Immunohistochemistry confirmed the occurrence of both receptors in lymph node metastases.CCR10 was not expressed in any of the lesions. Our results suggest that the upregulation of certain chemokine receptors, like CXCR5 and CXCR6, which are not regularly expressed by normal tissue and an increase in expression of receptors, which are consistently expressed, may contribute to melanoma progression.

P107

Perforin is not involved in the control of basal total IgE serum level in vivo

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In atopic patients, perforin(Pf)-expression of peripheral blood lymphocytes is significantly reduced and serum levels of total IgE are significantly elevated as compared to healthy control individuals. To elucidate if Pf is involved in IgE-regulation in vivo, Pf-knockout (-/-) mice were backcrossed from a C57BL/6 background (known low IgE responder, purchased from The Jackson Laboratory, Bar Harbor, Maine, USA; C57BL/6-Prf1tm1Sdz/J) onto a Balb/cJ background (good IgE responder, The Jackson Laboratory, BALB/cJ). Total serum IgE-levels were measured with a commercially available ELISA in triplets (Becton Dickinson, Heidelberg, Germany) in Pf -/-, Pf +/- and wt-mice (10 female and 16 male of each group) at 7, 12 and 20 weeks of age. Siblings of generation F8 were compared and results were analysed using the SPSS-software. Male mice showed significant lower total IgE levels as compared to female mice. There was no significant difference detectable between Pf -/-, Pf +/- and wt-mice. This indicates that Pf is not involved in the control of basal serum IgE levels.

P108

The differential effects of prednisolone and non-steroidal selective GR-agonist (SEGRA) tool compounds on thymocyte apoptosis

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Glucocorticoids (GCs) are the most effective therapeutic agents for the treatment of acute and chronic inflammatory diseases despite their potential to induce severe side effects, such as thymocyte apoptosis resulting in thymus atrophy. The pro-apoptotic effect of classical GCs is especially notable for immature CD4+CD8+ double-positive thymocytes. GC-mediated apoptosis of thymocytes is mediated via the transactivation (TA) mechanism and requires DNA binding of the glucocorticoid receptor (GR). We examined the impact of the classical GC prednisolone and non-steroidal selective GR-agonist (SEGRA) tool compounds being agonists in transrepression (TR) and either agonists or antagonists in TA on thymocyte apoptosis. BALB/c mice were treated daily with the respective agents for up to 5 days. The expression kinetics of pro-apoptotic genes in thymocytes was monitored by quantitative real-time PCR (RT-PCR). The effect on thymic output was investigated in genomic DNA samples isolated from peripheral blood by determining the levels of TCR rearrangement excision circles (TRECs) using RT-PCR. TRECs are extrachromosomal double-stranded DNA fragments of the TCR rearrangement process in the naive recent thymic emigrants (RTE) population and are not replicated during mitosis. Therefore, TREC levels reflect RTE numbers. We demonstrated an upregulation of the pro-apoptotic gene GILZ (glucocorticoid-induced leucine zipper) in thymi of prednisolone or TA-agonist treated mice. In contrast, thymocytes of TA-antagonist-treated mice did not show upregulation of GILZ. Furthermore TREC content in peripheral blood of TA-antagonist treated mice was less reduced than after treatment with prednisolone or the TA-agonist.Taken together, SEGRA with sustained TR but less TA-activity have a lower potential to induce thymocyte apoptosis than classical GCs as monitored in situ and by TREC determination in peripheral blood. Non-steroidal SEGRA may therefore represent a new class of drugs for the treatment of inflammatory disorders exhibiting an improved therapeutic index in comparison to classical GCs.

P109 (V36)

Immune responses and clinical outcome of immunotherapy with peptide-loaded dendritic cells in 60 patients with metastasized malignant melanoma

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Immunotherapeutic approaches are a promising option in melanoma treatment. Expansion of tumour-specific T-cells and tumour regression has been observed upon dendritic cell (DC) vaccination but the variability of the DC vaccines used in various trials is a major problem. In a recent trial ($n = 60$ patients; Stage III and IV metastasized melanoma) we investigated the induction of T cell responses and clinical outcome following vaccination with a highly standardized DC vaccine consisting of mature monocyte-derived dendritic cells (DC) loaded with eight MHC class I (HLA-A1 or HLA-A2 restricted) and six class II tumor-associated antigen peptide sequences. The first series of four vaccinations administered over 3 months induced or expanded tumor-antigen-specific T cells measured by ex vivo and stimulated ELISPOT or mixed lymphocyte peptide culture (MLPC). Precursor frequencies of tumor-antigen specific CD8+ T-cells in MLPC increased in most patients, in part of them from 10 to 800-fold. Such remarkably strong elevations in the precursor frequencies correlated with progression free survival and overall survival. Adverse events included erythema and induration at injection sites in >80% of patients, and less often type IV allergy (including rash) to human serum albumin used for freezing, vitiligo and fatigue. No WHO class III or IV severe adverse events were observed. This is the largest DC vaccination study ever performed using a standardized DC vaccine and serially valuated by several accepted quantitative immunomonitoring assays. The reliable induction of tumor-antigen-specific immune responses allows now randomized phase II trials for further optimization before clinical efficacy can be meaningfully addressed in a phase III trial.

P110

PRISM (Pictorial Representation of Illness and Self Measure) is a reliable and valid method to measure perception of the impact of illness in hospitalised dermatological patients

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Perception of the burden of illness in hospitalised patients is difficult to assess and is thought to be an important measure in order to provide and improve individual therapy. There is no #gold standard measure# for suffering. Recently PRISM gained attention as a reliable and valid non-verbal method to measure burden of illness. Tested mainly in non-dermatological subjects our aim was to validate PRISM for hospitalised dermatological patients. 171 dermatological patients performed the PRISM task at the beginning of hospitalisation and later every week until demission. At the same occasions patients were asked to fill out two standardised health quality of life questionnaires (DLQI (Dermatology Life Quality Index) and skindex-29). PRISM showed good correlation with DLQI and skindex-29 (Spearman#s rho = 0.42, 0.43 respectively, $P < 0.005$). PRISM scores correlated also well with changes in skin status during hospitalisation. We conclude that PRISM is a valid method to measure perception of the impact of illness in hospitalised dermatological patients. It is very acceptable to patients and takes less than 5 min to complete.

P111 (V16)

Psoriasis shifts the metabolic state towards insulin resistance

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Psoriasis is characterized by chronic recurrent inflammation and associated with increased cardiovascular mortality. Recent observations

suggest that psoriasis may be an independent risk factor for coronary artery calcification. To further clarify a possible pathogenetic link between psoriasis and atherosclerosis we studied 40 consecutive patients with moderate-to-severe plaque-type psoriasis. Detailed informations were obtained on the patients# clinical picture and history of psoriasis as well as smoking habits and medication. Body mass indices (BMI) were calculated. Laboratory investigations focussed on parameters for inflammation, lipid profile, and multiple cytokines. Besides, intima-media thickness of the carotid artery was measured based on ultrasound ('leading edge' method). Finally, an oral glucose tolerance test was performed, and relevant parameters were recorded for calculation of the homeostasis model assessment of insulin resistance (HOMA). Statistical analyses were performed using linear regression, multiple regression with PASI as a response variable and, where adequate, Wilcoxon–Mann–Whitney's *U*-test. Initially, the technique to measure vessel wall thickness was successfully validated. Subsequent analyses of this comprehensive data set confirmed numerous well-recognized correlations such as BMI and HOMA ($P < 0.02$) as well as BMI and vessel wall thickness ($P < 0.05$). Furthermore, we observed a significant correlation between the PASI and insulin secretion. Moreover, the PASI was significantly correlated with serum resistin levels – a cytokine known to be increased in insulin resistance. Finally, there was a trend ($P = 0.051$) towards a correlation between vessel wall thickness and serum TNF-α. Taken together, several parameters indicative for insulin resistance were found to be either significantly correlated with the PASI, or showed a trend close to statistical significance. The concept of insulin resistance as a consequence of chronic inflammation and possible pathogenetic cause for co-morbidities known to be associated with psoriasis is supported by these data. Our findings validate further studies on larger cohorts as well as interventional studies.

P112

Prospective randomized investigator-blind therapy study on the early treatment of hemangiomas: PDL versus cryotherapy versus observation

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The aim of this study was to assess the efficacy and safety of early treatment of low-risk hemangiomas to prevent further proliferation and to induce involution. We included infants with superficial hemangiomas in the early proliferative phase without any previous treatment and no signs of spontaneous involution. Children with systemic steroid or interferon therapy were excluded. The patients were randomly assigned to one of three groups: 1. pulsed dye laser treatment (PDL), 2. cryotherapy, 3. observation. The lesions in the two treatment groups were treated up to six times in monthly intervals by the interventional study team (cryotherapy: freezing time = 2x10 s, T = -32°C/PDL therapy: lambda = 585 nm, radiation dose for lesions on head, neck and genitals = 5.5 J/m^2, dose for all other locations = 6.5 J/m^2) after pretreatment with EMLA® cream. An independent investigator team assessed the results as complete remission (CR), partial remission (PR), stop of growth (SG) and progression (PRO) after 12 months. All lesions were evaluated for scars, change of pigmentation, change of skin texture, vascular and stromal residuals. 132 out of a total of 246 patients completed the 12-month follow-up period by February 2006. CR was seen significantly more often ($P < 0.05$) in both treatment groups [PDL: 40.4%/cryotherapy: 42.1%/observation: 10.5%]. Only PDL-treatment, however, lead to a significant reduction of both height and area of the lesion ($P < 0.05$). The risk of adverse effects was significantly higher in both treatment groups compared to the observation group ($P < 0.05$). There were more complications (hyper-/hypo-pigmentation, shallow scars) in the group with PDL treatment than in the group with cryotherapy (n.s.). Our study clearly shows that early treatment of proliferating, low-risk hemangiomas successfully stops proliferation and induces involution with an acceptable rate of side effects. Both treatment modalities are easy to perform and can take place in an out-patient setting.

P113

Lipid parameters in psoriasis vulgaris

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In the past, an increased atherogenic risk with severe cardiovascular complications has been described for patients with chronic inflammatory disorders. However, psoriasis has not been examined in depth recently. Consequently, in this study highly sensitive CRP, triglycerides, LDL, HDL, VDL and lipoprotein A were evaluated in 104 psoriatic patients and related to the clinical activity as detected by PASI as well as biographic data. Whereas PASI and CRP values correlated significantly, none of the lipid parameters reflected the severity of the psoriasis. However, stratification by the concentration of LDL showed a higher percentage of individuals within the high-risk group than in the control group. Whereas the mean of LDL concentration of all psoriatic patients as a group was only slightly elevated when compared to normal controls, the distribution of stratified groups was distinctly different. The increased cardiovascular risk among psoriatics may be mediated by both inflammatory factors and lipid parameters. Consequently, inflammation should be treated early and effectively to help decrease the atherogenic risk of psoriatic patients.

P114

Depletion of regulatory T cells (Treg) in humans: Analysis of the time course of Treg depletion and assessment of immune functions

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The capacity of regulatory T cells (Tregs) to suppress the proliferation of effector T cells in vitro and in vivo is well described. In healthy individuals this function is responsible for controlling immune responses and for suppression of autoimmunity. However, these suppressive functions may prevent induction of potent immune reactions during cancer treatment. We therefore attempted to remove Tregs in vivo by ONTAK® (a fusion protein of IL-2 and diphtheria toxin) infusions and to evaluate its effects on immunization with tumor specific peptides. Two groups of patients were injected with different doses of ONTAK®. Patients injected with a dose of 5 μg/kg bodyweight showed a reduction of CD25+/FoxP3+ Tregs in peripheral blood over a period of 13 days after application. However, Treg numbers recovered to normal levels there-

after. The second group of patients was treated with a dose of 18 μg/Kg bodyweight ONTAK®. Here a similar reduction of Treg numbers was recorded as compared to the low dose treatment.During treatment with ONTAK®, all patients were vaccinated three times with MelanA/gp100 peptides injected into an area of skin that had been sensitized with DCP. After every vaccination blood samples were taken and analysed for the presence of MelanA/gp100 specific T lymphocytes by tetramer staining and IFNγ ?ELISPOT. Here we could show that during vaccination the population of MelanA/gp100 specific T lymphocytes increased in the majority of the patients and these cells also displayed effector functions as indicated by in vitro cytotoxicity assays. Furthermore we noticed that individuals that were resistant to DCP-induced hypersensitivity reactions before ONTAK® treatment, developed vast eczemas and generated DCP-specific T cells after the ONTAK® application. In aggregate, our data indicate that ONTAK® depletes Tregs in vivo, resulting in enhanced immune functions and substantial development of antigen specific CD8+ T cells in vaccinated individuals.

P115

HLA-A1 restricted peptides derived from metastasin 1 induce specific T cell responses in PBMC of melanoma patients

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Metastasin 1 (mts1, S100A4) belongs to the S100 family of Ca^{2+} binding proteins. It is not expressed on most differentiated adult tissues. However, mts1 is upregulated in the tumor micromilieu, where it can primarily be detected on tumor-associated macrophages and fibroblasts, but also on tumor endothelial cells and cancer cells themselves. Mts1 upregulation in the tumor microenvironment is associated with tumor cell proliferation, invasion, angiogenesis and metastasis. Due to its strong induction in tumors, for example in melanoma, mts1 is a promising target for cancer immunotherapy. By reverse immunology, using epitope prediction programs, we have identified three HLA-A1 restricted peptides (mts1 A1-1, mts1 A1-2, mts1 A1-3). These mts1-derived peptides induce human T-cell responses in PBMC of melanoma patients, measured by IFN-γ ELISPOT and cytotoxicity assays. In addition, IFN-γ responses to mts1 A1-2 can not only be detected after stimulation of PBMC with peptide-loaded DC but also after stimulation with mts1 protein-loaded DC, indicating that this peptide is also generated by processing of endogenously expressed mts1. In consequence, this peptide is a candidate for immunotherapeutical approaches targeting mts1-expressing cells in the tumor stroma.

P116

Analyses of a new antagonist of TGF-ß/BMP signalling cascade which seems to play a role in skin sclerosis

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Fibrotic diseases are characterized by excessive scarring due to an increased production, deposition and contraction of extracellular matrix. Examples of fibrotic diseases include scleroderma, keloid, fibrosarcomas, liver cirrhosis and arteriosclerosis. To understand the molecular basis of fibrotic disease, it is essential to appreciate how matrix deposition is normally controlled and how this process is dysregulated in fibrogenesis. Transforming growth factor (TGF-ß) is known to play a central role in fibrotic diseases. Expression of TGF-ß and TGF-ß receptors are elevated in keloid-derived fibroblasts, and in dermal fibrotic lesions of scleroderma patients, elevated TGF-ß levels are also found at the leading edge of the forming scar tissue. Bone morphogenetic proteins (BMPs) are implicated in a variety of pathobiologic processes in skin, including wound healing and carcinogenesis. Thus, BMPs represent new important players in the molecular network regulating homeostasis in normal and diseased skin.In our present study we focused on TGF-ß/BMP signalling especially in wound healing. We were interested in expression and regulation of TGF-ß and BMP signalling in normal human fibroblast in contrast to fibrotic diseases like keloid or scleroderma.We used a collagen contraction model to determine contraction capacity and gene expression of BMP and TGF-ß of normal fibroblasts and fibroblasts stimulated with TGF-ß or BMP and in addition we used this collagen contraction model to analyse contraction and gene expression in fibroblasts derived from patients with keloids or localized scleroderma. Interestingly, we found differences in gene expression of both TGF-ß and BMP molecules in normal fibroblasts compared to those stimulated with TGF-ß or BMP and we found also differences between normal fibroblasts and those of keloid and scleroderma patients. Additionally, we identified a novel antagonist of BMP/TGF-ß signalling which seems to play an important role in wound healing processes. When we used keloid derived human dermal fibroblasts or fibroblasts isolated from patients with localized scleroderma in a collagen contraction model, we found expression differences of the new antagonist over a time course of 8 h between those cells and normal human fibroblast.

P117

Electroporation of ELUTRA generated dendritic cells with RNA – Towards a closed system

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Electroporation of monocyte-derived dendritic cells (moDC) with RNA, to load them with antigen, or to manipulate their function, has become a widely used method, also in clinical applications. Since the production of moDC is increasingly performed under GMP conditions, the development of a closed system should prove very beneficial, which is hampered by the standard isolation method of monocytes via plastic adherence from density-gradient purified peripheral blood mononuclear cells. An alternative way to isolate monocytes is cell elutriation via ELUTRA cell separator, which is a functionally closed system. Here we examined the question whether ELUTRA-generated moDC can be electroporated with equal efficiency and display equal functionality compared to moDC obtained via plastic adherence. Both DC populations were compared with respect to several features relevant for production and effectivity of a DC vaccine after transfection of RNA encoding a tumor antigen. The ELUTRA-derived DC were clearly superior in yield, i.e. a higher amount of DC could be generated from the same amount of leukapheresis product, while the plastic-adherence generated DC were marginally better in expression levels of the transfected tumor antigen. Both populations could be electroporated with similar, high efficiency (>90%), and appeared equal in antigen expression kinetics, T cell stimulation capacity,

CCR7-mediated migration, and expression of surface maturation markers. The data obtained allow for the use of ELUTRA-generated DC for RNA-electroporation in clinical settings and pave the way for a future fully closed system.

P118

High frequency of secondary haematological neoplasias in patients with primary cutaneous lymphomas

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The occurrence of secondary malignancies of various origins in patients suffering from primary cutaneous lymphomas is well known. However, the frequency of secondary neoplasm deriving from the lymphoid lineage is still a matter of debate and maybe underestimated. We screened our patients with primary cutaneous lymphomas for a coexisting second lymphoproliferative disorder. 82 patients with primary cutaneous lymphomas had attended our department within six consecutive years. 62 with primary cutaneous T-cell lymphomas, 18 with primary cutaneous B-cell lymphomas and two with CD4+/CD56+ hematodermic neoplasm/blastic NK cell lymphomas.In this cohort we identified seven patients with a coexisting lymphoma of a different lineage. Four patients with Sézary syndrome suffered from systemic B-cell lymphoma. Three patients with various types of skin lymphoma (Sézary syndrome, Mycosis fungoides and primary cutaneous marginal zone lymphoma) developed Hodgkin disease.Two of the patients developed Sézary syndrome after chemotherapy treatment for hairy cell leucemia and B-CLL respectively. Our data indicate that patients with primary cutaneous lymphomas have an elevated risk for the development of a secondary lymphoproliferative disorder even without previous chemotherapy. Possible explanations for this association comprise a genetic predisposition for the development of lymphoproliferative disorders, alterations in early progenitor cells and underlying viral infections. Stimulation of a B-cell clone by the malignant helper T-cells of the primary T-cell lymphoma might also be relevant for the occurrence of coexisting lymphomas. Further cases have to be analysed combining clinical data, morphology, immunophenotyping and molecular biology in order to clarify whether the sequential occurrence of lymphoma entities follows a regular pattern.

P119

Pimecrolimus but not betamethasone treatment normalizes the structure of the skin barrier in atopic dermatitis

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Impaired barrier in atopic dermatitis (AD) allows for penetration of allergens (e.g. house dust mite or grass pollen) into the skin which results in immunological reactions and inflammation. Here we investigated whether treatment with pimecrolimus or betamethasone improves the skin barrier in AD. Fifteen patients with mild to moderate AD were treated with pimecrolimus 1% or betamethasone cream 0.1% twice daily for 3 weeks. Both pimecrolimus and betamethasone improved AD regarding the clinical score. Also, transepidermal water loss (TEWL), a marker of the inside-out barrier, decreased in both groups. However, electron microscopy (EM) studies of the barrier structure at the stratum granulosum/corneum (SG/SC) interface showed much more regular lamellar bodies and regular SC lipid bilayers after treatment with pimecrolimus compared with betamethasone. Quantification of EM pictures revealed that in skin of healthy volunteers about 91% of lamellar bodies (LBs) were filled with regular lamellar sheets, whereas in lesional skin of AD only 13% of LBs were filled. After pimecrolimus treatment 82% of LBs were filled, whereas after betamethasone treatment the number of filled LBs did not improve (9% of LBs filled). The reason for the absence of repair of the barrier function after betamethasone treatment may be related to the antiproliferative effect and to the suppression of protein and lipid synthesis by this potent corticosteroid. Betamethasone treatment resulted in a 74% reduction in epidermal proliferation in AD to values below normal which led to epidermal thinning. After pimecrolimus treatment epidermal proliferation was reduced by 49% and no epidermal thinning occurred. The reduced TEWL in AD after treatment with betamethasone may be due to the well known vasoconstrictive effect which reduces the influx of water into the skin. This means that betamethasone mimics an improvement of skin barrier function in AD if only biophysical measurements of the skin barrier were taken. In contrast, a reduction in TEWL and a normalization of SC barrier structure occurred after pimecrolimus treatment. Repair of the skin barrier hinders the entry of irritants and allergens into the skin and may prevent relapse of AD.

P120

Tumor necrosis factor alpha is essential for monocyte survival and differentiation into immature dendritic cells

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Immature dendritic cells (DC) can be generated from peripheral blood monocytes by addition of GM-CSF and IL-4 to the media. This procedure is robust and a 6-day culture of adherent monocytes has become a worldwide standard for laboratory use and clinical application of DC. However, regardless of the media used, e.g. RPMI + 1% autologous plasma, X-Vivo 15 or CellGro, the yield of DC from the starting monocyte population is highly variable between donors, and sometimes the procedure even completely fails.In an attempt to optimize generation of monocyte-derived DC (MoDC), we examined the role of TNFα during culture. Interestingly, TNFα appeared early on during culture and stayed constant at around 100 pg. This was also true when pure monocyte populations obtained by magnetic bead separation were used, ruling out a role of contaminating cells and pinpointing at TNFα ?production by the monocytes themselves. The crucial role of this early TNFα ?became apparent by blocking experiments: addition of either an anti-TNFα monoclonal antibody (MAb11, Biolegend) or a recombinant TNFα-receptor fusion protein (Etanercept, Wyeth), during the first day of the monocyte culture was sufficient to reduce cell yields at day 6 down to 20%. Surviving cells partially expressed class II

and CD86 and did not form clusters. Moreover, a subpopulation of these cells were small and of round shape, thus lacking typical DC morphology. Addition of recombinant TNFα at onset of monocyte culture not only prevented the detrimental effects of the blocking TNFα reagents on cell yield and morphology, but generally enhanced the yield of immature DC up to 1.5 fold. MoDC generated with addition of recombinant TNFα did not differ in phenotype and could be properly matured using the standard cytokine cocktail (IL-1, IL-6, TNFα, and PGE2).In aggregate, we report on a hitherto unrecognized role of TNFα as crucial factor for the viability of monocytes and their differentiation into immature DCs. Addition of TNFα, besides GM-CSF and IL-4, at onset of monocyte culture will not only harmonize this procedure between donors, but also optimize the yield of MoDC needed for various applications such as DC-based vaccines.

P121

Systemic sclerosis involves the right heart

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Background: Heart involvement in scleroderma occurs in up to 50%, and may confer a higher mortality. Available data addresses left ventricular disease. Recent research has assessed the occurrence of pulmonary arterial hypertension (PAH) in SSc.

Method: As part of the German scleroderma network and as a cooperation between our dermatology-based scleroderma-clinic and our local department of cardiology, we designed a prospective observational study to evaluate a standardized cardiological diagnostic pathway in SSc, to assess incidence and outcome of heart involvement in SSc, and to detect predictive factors for the occurrence of major cardiac events in SSc. We present data of the baseline evaluation as the total observation period will be 5 years and is still ongoing. After approval by our local ethics committee, 60 patients (♀ 53, ♂ 7; average age 56.2 years) with SSc (40 lSSc, 12 dSSc, eight overlap-syndrome) were recruited.

Results: Echocardiographic study of our patients (pts) revealed left atrial dilation in nine pts, right atrial dilation in three, and right ventricular dilation in three pts. No left ventricular abnormalities could be demonstrated. LVEF was <50% in none, <60% in only three pts, thereby confirming the results of Anvari (1992). 20/62 pts suffered from diastolic relaxation abnormalities (45% of ACA-positive pts, 43% of SCL 70-positive pts). In only three of those patients a coexisting CAD could be demonstrated. So far 9/20 pts with diastolic relaxation abnormalities were diagnosed as suffering from pulmonary arterial hypertension (RVSP > 30 mmHg). Only six of those 20 pts are currently under ACE-inhibiting medication suggesting under-treatment. 37% of the total study population suffered from pulmonary arterial hypertension (RVSP > 30 mmHg). A clear inverse correlation could be demonstrated between RVSP and DLCO as published by Mukerjee (2004). Furthermore, a correlation was found between age and RVSP. This might, however, rather reflect a correlation between duration of SSc and RVSP and will be subject to further statistical evaluation. RVSP also correlated with symptoms, i.e. NYHA grade II or more, confirming prior studies.

Conclusions: As SSc patients benefit from modern treatment and display less end-stage renal disease, cardiac involvement is directed at the right heart.

P122

Adalimumab therapy augments Langerhans cells and diminishes activated CD83+ dendritic cells in psoriatic skin lesions

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Expression of CD83 is the most specific marker of activated and mature dendritic cells (DCs). We compared CD83+ DCs before and after treatment in psoriatic skin lesions of 15 patients treated with Adalimumab, a humanized monoclonal antibody against TNF-alpha. Other DCs, expressing CD1, i.e. Langerhans cells, and S-100 protein in the suprabasal layers of the epidermis (non-melanocytes) were also examined. five patients with eczematous dermatitis and five with lichen planus served as controls. At week 0, patients received a loading dose of 80 mg Adalimumab, then 40 mg every other week starting at week 1 until week 11 by subcutaneous injection. Week 12 was the end of observation. Skin biopsies for staining with H&E, anti-CD1, S-100, and anti-CD83 were taken at weeks 0 and 12. Epidermal thickness was measured with a microscopic micrometer grid. Numbers of epidermal DCs were determined as the means in three high-power fields. We found a diminution of the psoriasis area and severity index (PASI) from 23.6 ± 2.1 at week 0 to 2.5 ± 1.0 (mean ±SE) at week 12. The epidermal thickness of non-lesional skin was: 0.1 ± 0.03, of lesional skin at week 0: 0.44 ± 0.03, at week 12: 0.19 ± 0.02 mm. DC counts changed as follows [pre- vs posttreatment (uninvolved skin)]: CD1 increased from 3.8 ± 2.8 to 11.1 ± 5.1 ($P < 0.01$, Wilcoxon test) (7.7 ± 1.1), S-100 from 5.11 ± 1.0 to 9.1 ± 1.1 ($P < 0.01$) (5.99 ± 0.5); by contrast CD83 decreased from 3.27 ± 0.42 to 1.1 ± 0.36 ($P < 0.01$) (0.33 ± 0.16). Epidermal thickness correlated directly with CD83+ DC counts ($P < 0.01$). Patients with eczema or lichen planus all expressed more CD1+ and S-100+ but significantly less CD83+ DCs compared to psoriasis ($P < 0.01$). Our results demonstrate that successful treatment with Adalimumab on one hand increases diminished epidermal Langerhans cell density, and on the other, down-regulates activated and mature CD83+ dendritic cells in psoriatic skin lesions. This suggests that CDCs may play an important role in pathogenesis of psoriasis.

P123

Pustular psoriasis of the palms and soles effectively treated by Efalizumab

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Therapeutic management of pustular psoriasis of the palms and soles (PPP) is difficult. Efalizumab has not been used. We report the successful treatment of a 61-year-old man suffering from PPP for 6 years. Efalizumab was administered subcutaneously once weekly by subcutaneous injection for 12 weeks. Topical treatment consisted of emollients only. Skin biopsies from palmar sites were taken before treatment and at week 13. Histological sections were stained with hematoxylin & eosin and with antibodies specific for neutrophil elastase, lymphocyte markers CD3, CD4, CD8, CD45 RO (memory T-cells), HLA-DR, dendritic cell (DC) markers CD1, S100, CD83 (activated DCs), differentiation markers cytokeratin 16 (K16), Filaggrin, Involucrin and the proliferation marker MIB (Ki67), all visualized by peroxidase. Clinical photographs were also taken. We found that the number of pustules of the palms and soles cleared at week 13, beginning at week 3. The stratum corneum normalized. Staining for neutrophil elastase, CD3, CD4, CD8, CD45 RO all

decreased. CD1 and S100 in the epidermis increased, by contrast, CD83 decreased, K16 and MIB decreased after treatment. Filaggrin and involucrin normalized. We conclude that efalizumab cleared pustulation and normalized the skin in pustular psoriasis of the palms and soles. The increase of CD1+ and decrease of activated CD83+ cells in the epidermis after therapy suggests that activated DCs may be important in pathogenesis. Efalizumab may be of value in management of pustular psoriasis of the palms and soles.

P124

A cold-response index for the quantification of Raynaud's phenomenon

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Quantification of Raynaud's phenomenon (RP) is a prerequisite in the evaluation of novel therapeutic strategies. Fingertip rewarming in response to local cold provocation has been used in many studies but not been systematically validated. We previously described the time elapsed before 63% of precooling temperature is reached as RP activity index. Here, we describe a cold response index (CRI) defined as the log transformation of the 63% rewarming time upon fingertip cold challenge. The CRI shows excellent intra-individual reproducibility (coefficient of variation <15% for measurements on separate days) and good discrimination of control versus RP patient cohorts (mean CRI values were: 2.4 ± 0.3 in controls ($n = 53$) vs 2.7 ± 0.3 in RP ($n = 50$, $P < 0.0001$), as well as control versus scleroderma-associated RP (2.7 ± 0.3; $n = 46$, $P < 0.0001$). In addition, baseline fingertip temperature was also found to be significantly reduced both in primary as well as scleroderma-associated RP. Kinetic analysis of rewarming temperature curves demonstrate that the CRI is independent of individual rewarming patterns. Finally, the CRI decreases significantly upon a single low-level systemic hyperthermia treatment in scleroderma patients (2.68 ± 0.28 before vs 2.45 ± 0.33 after, $P = 0.0003$), while the extent of cooling remained unchanged, thus demonstrating sensitivity to change. Our results provide a solid basis for using the cold response assay as an endpoint in RP treatment trials. The data further support the concept that deficient vasodilation, but not exaggerated vasoconstriction, is central to the pathogenesis of RP.

P125

Neuropeptide release and mast cell degranulation in murine skin after topical administration of pimecrolimus and tacrolimus

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The topical calcineurin inhibitors pimecrolimus and tacrolimus have been demonstrated to be an effective anti-inflammatory therapy. The only clinical relevant side effect refers to transient application site burning and stinging itch at the beginning of topical therapy. In order to understand the underlying mechanism of this effect, we examined whether or not the compounds are able to stimulate neuropeptide release in normal murine skin as well as in a mouse model of experimentally-induced irritant contact dermatitis. Balb/c mice were treated with 1% pimecrolimus cream or 0.1% tacrolimus ointment. Untreated and corresponding vehicle-treated mice served as controls. Skin specimens were investigated by light, immunofluorescence, electron microscopy as well as ELISA and PCR. Topical application of pimecrolimus and tacrolimus is followed by an initial release of Substance P and Calcitonin gene-related peptide from primary afferent nerve fibres as well as mast cell degranulation. Neither pimecrolimus cream nor tacrolimus ointment did affect NK1R mRNA levels. These results suggest that application of pimecrolimus as well as tacrolimus may lead to a neurogenic inflammation by release of neuropeptides from afferent nerve fibres and to mast cell degranulation. Mediators of mast cells such as histamine and tryptase may induce pruritus and burning by binding to the corresponding receptors (H1, PAR-2) on sensory nerve fibres which explains the initial side-effects during a therapy with calcineurin inhibitors. Finally, it may be speculated that calcineurin inhibitors directly stimulate intracellular signalling pathways or bind to ion channels such as TRPV1 or receptors involved in nociception.

P126

Effects of Endothelin-1 on human skin mast cell mediator secretion

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Endothelin-1 (ET-1) plays a crucial role in neurogenic inflammation, sepsis and tissue repair. Although the peptide causes mast cell dependent inflammation following UVB irradiation of murine skin previous studies with this peptide on human skin suggested mast cell independent activities. In the present study, we therefore investigated whether ET-1 can effect human skin mast cell mediator secretion via specific endothelin receptor triggering. Human skin mast cells were isolated from healthy skin by enzymatic dispersion and enriched by Percoll-density centrifugation. Stimulation of mast cells with ET-1 (0.01–10 μM) gave rise to a dose-dependent release of histamine, which exceeded 10% of total histamine content above spontaneous levels at maximum concentrations. Although these amounts were generally lower than anti-IgE-induced histamine secretions, statistically they were highly significant. Preliminary data also showed that ET-1 triggered mast cell TNFα ?release and substantial PGD2 synthesis. Furthermore, pretreatment of mast cells with the ET-1-receptor antagonist PD142893 (0.01–10 μM) dose-dependently inhibited histamine release caused by ET-1 (1 μM) demonstrating exclusive ETA/ETB receptor involvement. Although ET-1 induces significant histamine release from human skin mast cells at high concentrations, in comparison, it is more potent at activating rodent mast cells, where one nM ET-1 sufficed to cause the same levels of degranulation in isolated mouse skin mast cells than their human counterparts. Despite these differences in potency, our data suggests that ET-1 mediated mast cell activation in human skin may play a more prominent role in ET-1-induced flare responses than previously appreciated.

P127 (V17)

Activators of peroxisome proliferator-activated receptor-delta strongly induce IL-8 expression by endothelial cells via NF-kappaB activation

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Peroxisome proliferators 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 in part by inhibition of distinct pro-inflammtory genes (e.g. IL-1beta, IFN-gamma). Interleukin-8 (IL-8) is an important member of the pro-inflammatory CXC chemokine family, known to mediate neutrophilic leukocyte activation and eosinophil adhesion to endothelial cells. Especially PPAR-alpha and PPAR-gamma activators have been demonstrated to effectively inhibit the expression of IL-8 in various cell types. The impact of PPAR-delta activators on endothelial IL-8 expression, however, had yet to be explored. We therefore determined the influence of PPAR-delta activators on IL-8 expression by cultured endothelial cells (HUVEC). PPAR-delta agonists (e.g. L165,041) are herein shown to potently induce IL-8 expression by HUVEC in a concentration- and time-dependant manner. The induction is demonstrated both at the level of protein and mRNA IL-8 expression. As mRNA stability of IL-8 is left unchanged in response to PPAR-delta activation, PPAR-delta agonist likely mediate IL-8 induction by transcriptional mechanisms. Our promoter activation studies with deletional reporter gene constructs indeed revealed that PPAR-delta agonists convey their inducing effects through a critical NF-kappaB binding site. In addition, a profound increase in NF-kappaB and IkappaB phosphorylation was detected in response to PPAR-delta activation. Together, our data provide evidence that PPAR-delta, as opposed to PPAR-gamma and -alpha activators, strongly induce IL-8 expression via NF-kappaB-dependant mechanisms, and may therefore function as a counterpart to other PPAR agonists in the regulation of inflammatory responses.

P128

Epidermal stem cells differ in their response to proinflammatory signals from other proliferative keratinocytes

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The epidermis has a pool of adult stem cells which guarantees homeostatic epidermal tissue renewal. Although the existence of epidermal stem cells (ESC) has been known for some decades, we lack a clear understanding of the role of stem versus progenitor cells in perturbed conditions (e.g. inflammation). Despite some efforts to identify a universal ESC marker, no such markers have been commonly accepted. It has been proposed that human ESC can be identified by the expression of high levels of β1-integrin and their capacity of rapid adherence. In this study we isolated two population of keratinocytes according to their adherence ability. ESC enriched by adherence showed a higher CD29 and α6-integrin (CD49f) expression compared to other keratinocytes. Surface expression of CD54 was increased to a higher degree in ESC compared to other keratinocytes upon IFNγ ?stimulation. Furthermore, adherence enriched ESC differed from other keratinocytes by reduced IFNγ induced mRNA expression for IL-18, CCL2 and CCL22, reduced basal production of CCL2 and a markedly weaker secretion of IL-18 upon stimulation with IFNγ. No differences in IFNγ? induced IL-10, CXCL10, CCL22 or TGFβ1 secretion was observable between the two keratinocyte subpopulation. In conclusion, the results suggest that ESC are less prone to express proinflammatory cytokines. They can, however, produce chemokines upon activation and do not build up an anti-inflammatory microenvironment by means of TGFβ ?or IL-10. Activated ESC possess the capability to interact with infiltrating lymphocytes via CD54 which is highly inducible by IFNγ. Further studies are needed to understand the precise role of ESC in inflammatory conditions.

P129

Acne inversa: structural basis for the pathogenic action of nicotine

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Acne inversa is a chronic inflammatory, nicotine-dependent disease of unknown pathogenesis emerging from the pilosebaceous unit of the intertriginous areas. In previous works, we have demonstrated a crucial role of the non-neuronal cholinergic system in terminal differentiation and barrier formation. To clarify the role of nicotine in the pathogenesis of acne inversa, we studied the expression of nicotinic AChR in acne inversa epidermis, infiltrating inflammatory cells and blood monocytes isolated from acne inversa and control donors using double-label immunofluorescence and RT-PCR. In acne inversa specimens, the infundibulum showed the strongest immunoreactivity (IR) for all AChR present and the strongest endogenous ACh production. Alpha 5 and alpha 7 AChR IR was stronger than in control, detectable in all living layers, while alpha 3 and alpha 9-AChR showed the same pattern as control. For the first time, we could demonstrate the presence of alpha 3, alpha 5 and alpha 7 nAChR IR in variable amounts on macrophages in lesional skin. In accord with published data, we detected the expression of alpha 7 AChR mRNA in all populations of isolated blood monocytes. In addition, for the first time, we found both the alpha 5 and the alpha 10 subunit in ~90% of donors examined, however, without apparent differences between healthy donors and acne inversa patients, indicating that qualitative differences in AChR expression do not seem to influence acne inversa pathogenesis. Rather, high levels of endogenously produced ACh especially in the hair follicle infundibulum indicate a possible synergy with tobacco-delivered nicotine in aggravating infundibular epithelial hyperplasia and thus follicular plugging. Altogether we provide first hints for a causative role of the non-neuronal cholinergic system in the pathogenesis of acne inversa. The role of AChR on tissue-bound inflammatory cells in acne inversa remains to be clarified.

P130

A potential role of IL-20 in psoriasis vulgaris

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Interleukin (IL)-20 is a recently discovered immune mediator that belongs to the IL-10-interferon family of cytokines. Transgenic

overexpression of IL-20 in mice leads to psoriasis-like skin alterations. However the role of IL-20 in the pathogenesis of psoriasis vulgaris in men has not been elucidated so far.Here, we show that psoriasis patients had both elevated cutaneous IL-20 mRNA levels and increased IL-20 serum levels.In vitro, IL-20 was produced by activated monocytic cells as well as by activated keratinocytes. Corresponding to the IL-20 receptor expression pattern, IL-20 can act on keratinocytes but not on immune cells. In primary human keratinocytes IL-20 selectively activated STAT-3. Moreover IL-20 induced the expression of S100 proteins and reduced the expression of differentiation-associated proteins, but did not affect the chemokine levels in these cells. These data suggest that IL-20 is a distal mediator in the pathogenesis of psoriasis.

P131

The M4 muscarinic acetylcholine receptor play a key role in the control of murine hair follicle cycling and pigmentation

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Cholinergic receptors of the muscarinic class (M1-M5) are expressed in epidermal keratinocytes and melanocytes as well as in the hair follicle. Knockout (KO) mice of all five receptors have been created and resulted in different phenotypes. KO mice with a deletion of the M4 muscarinic acetylcholine receptor (M4R) present a striking hair phenotype, which we have analysed here in greater detail by quantitative histomorphometry. Earlier studies revealed a retarded hair follicle morphogenesis in M4R KO mice, compared to age-matched wild type controls. On day 17, when mice enter the first hair growth cycle, the KO mice still showed a slightly retarded catagen phase. Subsequently, hair follicles of the KO mice stayed in a highly significantly prolonged telogen phase, while wild type mice had already far progressed in the hair cycle by entry into anagen. Most strikingly, the M4R KO mice did not engage in follicular melanogenesis and failed to produce pigmented hair shafts. The current pilot study suggests that the M4R plays a fundamental role in the control of the murine hair follicle cycling and is an essential signalling element in the control of hair follicle pigmentation.

P132

Silent phagocytosis of *Leishmania major* by murine polymorphonuclear neutrophils (PMN) and subsequent transfer to inflammatory dendritic cells (DC)

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In murine cutaneous leishmaniasis, resistance and susceptibility critically depends on the interaction of DC and macrophages (MΦ) with T-cells. MΦ are the first cells to phagocytose infectious stage metacyclic promastigote of *L. major* transmitted into the skin by sand flies. Later on, immigration and infection of DC with obligate intracellular amastigotes released from lysed MΦ leads to IL-12 release and induction of protective Th1-dependent immunity. Although PMN are professional phagocytes and among the first to arrive at the site of infection, their contribution to resulting adaptive immune responses is still not fully understood. First, we performed uptake studies with PMN and both life forms of Leishmania in vitro. Surprisingly, we found that promastigotes were poorly phagocytosed ($12 \pm 12\%$ infected cells, parasite/cell ratio 1:3, 18 h, $n \geq 4$), whereas amastigotes were taken up much more efficiently ($80 \pm 2\%$). Leishmania uptake by PMN was dose- and time dependent, maximal infection rates were obtained after 48 h of co-culture ($n \geq 8$). Parasite internalization was confirmed by light microscopy and FACS analysis of cells incubated with CFSE-labelled parasites. Inside of PMN, intracellular promastigotes transformed into amastigotes as expected. Each PMN contained maximally ~1–3 parasites, independent of the time point indicating that parasites did not replicate or get killed within PMN. No release of IL-6, IL-10, IL-12p40 or TNF-α was detected from infected PMN within 18 h after infection. Finally, we investigated if infected PMN are able to pass on their intracellular organisms to other cells such as DC recruited during inflammation. We co-incubated infected PMN with bone marrow-derived DC at a ratio of 1:1 and found $30 \pm 4\%$ infected CD11c+ DC after 48 h. Interestingly, transfer of amastigotes was in part dependent on cell-to-cell contact since the percentage of infected DC was decreased in transwell assays allowing for passage of only amastigotes through 3 μm pores ($15 \pm 4\%$, $P \leq 0.05$ $n = 3$). In summary, our data indicate that PMN contribute to resulting protective immune responses by silent phagocytosis of amastigote *L. major*. Parasites may then be transferred to immigrated, inflammatory adjacent DC which ultimately results in IL-12 release and induction of Th1 immunity.

P133

Enhanced in vivo secretion of the antimicrobial proteins Psoriasin (S100A7) and RNase 7 after experimental disruption of the skin barrier

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Recently we identified the S100 protein Psoriasin as well the ribonuclease RNase7 as potent antimicrobial proteins (AMP) from healthy human stratum corneum extracts. The antimicrobial activity of Psoriasin is mainly directed against *E. coli*, whereas RNase7 reveals broad spectrum antimicrobial activity including vancomycin-resistant *Enterococcus faecium*. Disruption of the epithelial barrier (e.g. by micro-wounds) very seldom results in skin infection. Therefore we hypothesized that the release of potent AMP like Psorasin and RNase7 may be upregulated after superficial skin injuries.Healthy volunteers ($n = 7$) were included in this pilot study. Tape-stripping of a standardized area of the forearm skin was performed until a transepidermal waterloss (TEWL) of 40 g/m^2/h was reached. In vivo washing fluids (10 mM sodium phosphate buffer, pH 7.4) derived from the untreated forearm and the pretreated area were collected at different timepoints and the amount of both AMP was determined by ELISA.In 6/7 persons Psoriasin secretion above the detection limit (1.25 ng/ml) could be determined in the samples derived from the untreated forearms, whereas RNase7 was identified in 4/7 persons (detection limit 0.3 ng/ml). One hour after tape-stripping all washing fluids contained measurable amounts of both AMP and an increased secretion of AMP could be identified up to 7 days after tape stripping in individual persons.In summary, this study demonstrates for the first time that enhanced secretion of the antimicrobial proteins Psoriasin and RNase7

after barrier disruption may contribute to the low infection rate observed after skin injuries.

P134 (V32)

CJ9-gD, a novel replication-defective and dominant-negative recombinant herpes simplex virus type 1 (HSV-1), can serve as vaccine against genital HSV-1 or HSV-2 disease in mice

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The best option to prevent primary genital infection with herpes simplex viruses (HSV) and subsequent recurrences would be a safe and efficient prophylactic vaccine. We previously generated an HSV-1 recombinant, CJ83193, capable of inhibiting its own replication as well as that of wild-type HSV-1 and HSV-2. In addition, we found that CJ83193 served as an effective vaccine against wild-type HSV-1 infection in a mouse ocular model (J. Virol 2004: 78: 5756–5765). To ensure its safety and augment its efficacy as a vaccine against HSV infection, we developed a much improved CJ83193-like HSV-1 recombinant CJ9-gD, which contains a deletion in an additional HSV-1 essential gene and an extra copy of the gene encoding HSV-1 glycoprotein D (gD) under the control of the tetO-bearing hCMV immediate-early promoter. Thus, CJ9-gD is completely replication-defective and expresses significantly higher levels of the major antigen gD immediately following infection. Mice immunized with CJ9-gD produce higher titres of anti-HSV-1-specific neutralization antibody which correlates directly with increased levels of anti-gD-specific antibody. Moreover, no CJ9-gD genome can be detected in mouse trigeminal ganglia following ocular inoculation. The efficacy of CJ9-gD as a vaccine against wild-type HSV-1 and HSV-2 infection was then tested in a mouse model of genital infection. Immunization with CJ9-gD induced immune responses both against HSV-1 and in a lesser extent against HSV-2. After challenge with HSV-1 or HSV-2, the amount and duration of viral shedding from the vagina was significantly reduced in mice immunized with CJ9-gD compared to mock vaccinated mice. Immunized mice were completely protected from development of local genital lesions and exhibited no signs of systemic disease after challenge with HSV-1 or HSV-2. In contrast, all mock-vaccinated mice developed severe genital lesions associated with high mortality. Collectively, we have generated a gD-expressing replication-defective and dominant-negative HSV-1 recombinant CJ9-gD that can elicit strong and effective protective immune responses against primary genital HSV-1 and HSV-2 infection in mice.

P135 (V03)

Progressive disease in *L. major*-infected IL-1 receptor antagonist (RA) deficient mice

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We have shown previously that dendritic cell (DC)-derived IL-1α/β via signalling through IL-1R type I plays a critical role (together with IL-12) for induction of Th1-dependent protective immunity from Th0 cells. Later on in established Th responses, IL-1 promotes expansion of differentiated Th2 cells and worsens disease outcome. Thus, the timing of IL-1 release is critical for resulting T cell immunity. DC from Leishmania-susceptible BALB/c mice produce less IL-1 as compared to resistant C57BL/6 mice resulting in aberrant Th2 development and ultimate death of infected mice. We now investigated the role of IL-1 in more detail using IL-1α/β ?double-deficient C57BL/6 and IL-1RA-/- BALB/c mice. IL-1RA-/- mice are characterized by excessive IL-1 signalling due to lack of its antagonist. IL-1α/β-/- mice infected with standard high dose (HD; 2x10E5) inocula showed slightly improved disease outcome as compared to wild-type C57BL/6 mice, no phenotype in physiological low dose (LD; 1000 parasites) infections was observed. These findings are consistent with those previously obtained in IL-1RI-/- C57BL/6 mice demonstrating that continuous defects in IL-1 signalling suppress Th2 expansion. Surprisingly, IL-1RA-/- BALB/c mice infected with HD or LD inocula displayed significantly more progressive lesion development from week3 (HD) and week6 (LD) on, respectively, and were euthanized in week6 and week10, whereas controls progressed more slowly. Lesion sizes were paralleled by significantly higher lesional parasite burdens and decreased IFNγ production of antigen-specifically restimulated lymph node (LN) cells. Levels of IL-4/IL-10 were unaltered in IL-1RA-deficient. Interestingly, both in LN cultures of infected mice and in vitro we identified a markedly impaired production of IL-12 by DC from IL-1RA-/- mice. In summary, in addition to effects on Th0 and Th2 cells, dysregulated IL-1 signalling also leads to downmodulation of IL-12 synthesis in DC as observed in IL-1RA-/- mice resulting in impaired Th1 development and worsened disease outcome. Thus, the complex regulation of various members of the IL-1 cytokine family and effects on both DC and T cells critically contributes to disease outcome against this important human pathogen.

P136

Macrophages (MΦ) and dendritic cells (DC) use different strategies for killing of intracellular *Leishmania major*

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In murine experimental leishmaniasis, MΦ represent the main target cells for *Leishmania major*. Following silent entry of MΦ via CR3, Leishmania parasites progressively replicate until the cells get lysed and release the parasites into the tissue. Once activated by Th1-derived IFNγ and TNF-α, infected MΦ eliminate intracellular Leishmania by means of NO. In contrast, DC phagocytose Leishmania via FcγR and infection is associated with DC activation (upregulation of MHC/costimulatory molecules, IL-12 release) and control of intracellular parasite replication. However, DC do not eliminate the parasites. We now studied the mechanisms underlying the differences in the control of parasite replication between DC and MΦ. Using bone marrow-derived DC and time kinetic analyses, we first confirmed that no significant increase in the percentage of infected DC ($29 \pm 2\%$/18 h, $35 \pm 5\%$/120 h, $n \geq 5$; parasite:cell ratio 5:1) or the number of intracellular amastigotes (AM) per DC (5 ± 1/18h, 4 ± 1 120 h, $n \geq 5$) was observed in DC from C57BL/6 mice. Since IFNγ is essential for parasite elimination in MΦ, we incubated infected DC with varying doses of recombinant IFNγ. There was no significant difference between IFNγ-treated and untreated DC with regard to infection rate and number of intracellular AM. In parallel,

IFNγ-/- DC controlled parasite replication comparable to wild-type cells, suggesting that endogenously produced IFNγ is also not necessary for this effect. Addition of IFNγ to infected MΦ induces NO release, which was not observed in DC ± infection with *L. major*. Utilization of iNOS/DC confirmed that NO production of infected DC is dispensable for the confinement of parasite replication in DC. We next investigated the role of O^{2-} for control of parasite growth using DC from gp91phox-/-mice. Surprisingly, DC deficient for production of O^{2-} also controlled parasite replication comparable to wild-types. In summary, control of parasite replication in infected DC may not be mediated by classical reactive oxygen or nitrogen intermediates. However, since DC are critical for the initiation of protective immunity and long-term parasite persistence in DC is essential for the maintenance of anti-Leishmania memory responses, DC may have evolved to develop specialized strategies for the growth control of intracellular organisms.

P137

Effects of Miltefosine on Leishmania infected and uninfected dendritic cells (DC)

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The first orally active agent approved for the treatment of leishmaniasis is the alkylphospholipid Miltefosine (Hexadecylphosphocholine, HePC). HePC mainly interacts with the parasite lipid metabolism and directly kills parasites. In prior studies it was shown that HePC also activates infected macrophages (MΦ) to eliminate intracellular amastigotes via oxidative radicals (NO^- and O^{2-}), whereas the effect of HePC was independent of TNF-α from MΦ or IFNγ secretion from T-cells. Infected DC are critically involved in the initiation and maintenance of protective, Th1-dependent immunity against *L. major* infection via IL-12 release. We now attempted to investigate the effect of HePC on parasite elimination from DC and resulting alterations of immune functions of infected DC. First, we determined that the toxicity of HePC on bone marrow-derived DC was limited (below 50% PI+ cells) at concentrations below 50 μM. In contrast to control stimulation with LPS, HePC-treatment did not activate the DC as determined by cytokine release (ELISA for IL-12p40, IL12p70, TNF-α, IL-10) or significant upregulation of MHC II or costimulatory molecules (CD40, CD54, CD86). Interestingly, HePC-treatment of infected DC induced elimination of intracellular amastigotes in a time-dependent fashion, reaching statistical significance after 96 h (72.6 ± 7.4% killing as compared to untreated controls, $n = 3$, $P \leq 0.05$) and 120 h (68.2 ± 7.2%, $n = 4$, $P \leq 0.005$), respectively. Infection of DC with *L. major* led to induction of several inflammatory cytokines including TNF-α and IL-12 (untreated DC: 33 ± 18 pg vs infected DC: 211 ± 61 pg IL-12p40/ml, $n = 5$, $P \leq 0.05$). Cytokine release from infected DC was not altered by HePC treatment (e.g. 211 ± 61 pg vs 210 ± 61 pg IL12p40/ml from infected and infected/HePC-treated cells, $n = 6$). Surprisingly, antigen-specific proliferation of CD4+ and CD8+ T-cells from infected C57BL/6 mice induced with infected DC was not impaired when infected, HePC-treated DC (which had eliminated most of their viable intracellular parasites) were used. In conclusion, HePC has proven to be an efficacious treatment of persisting cutaneous and visceral leishmaniasis. Immunologically, HePC leads to elimination of intracellular parasites from DC without altering their important function as inducers of adaptive, protective Th1 immune responses against this important pathogen.

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Study on frequency and localisation of Th1/Th2 cells in lesions of human cutaneous leishmaniasis

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In murine cutaneous leishmaniasis (CL), healing is conferred by IL-12 release from epidermal Langerhans cells (LC) inducing IFNγ+ Th1/Tc1 cells, while Th2 responses facilitate parasite growth and lesion progression. Polarization of Th responses in human CL is not as clear cut probably reflecting differences between experimental and naturally occurring infections. The cytokine response of peripheral T cells in patients with CL revealed mixed Th1/Th2 immunity. However, studies recently examining local skin immune responses showed that in localized CL IFNγ predominates, whereas IL-4 was detected only in cases of diffuse, non-healing mucocutaneous leishmaniasis. Cure was regularly associated with IFNγ production. We now assessed the frequency and localisation of Th1/Tc1 cells as well as APC in 24 patients which acquired cutaneous leishmaniasis while travelling. Infection was verified by Giemsa stain and/or PCR. The distribution of APC [macrophages (MΦ, CD68+), LC (CD1a+), B-cells (CD20+)] and T-cells (CD4+/CD8+) was determined using immunohistology. In addition, Th1/Tc1 and Th2/Tc2 cells were detected by two-colour staining using anti-CD4/CD8 and mAb against chemokine receptors CXCR3 (Th1) and CCR4 (Th2). As expected, most of the specimens showed a strong granulomatous inflammation in which parasitized MΦ were encircled by CD4+ T-cells. We detected fewer CD8+ T-cells and B-cells. No significant alteration was found with regard to the number/distribution of LC. We found predominant expression of CXCR3 and no CCR4, double staining showed that most CXCR3+ were also CD4+, only some of them were CD8+. When available, data was correlated to clinical information (age, duration of infection). Younger patients (≤40 years) showed a stronger lesion infiltration with MΦ and B-cells ($P \leq 0.05$, $n \geq 5$), whereas the number of CD4+ T-cells was higher in patients >40 years ($P \leq 0.05$, $n \geq 5$). Interestingly, the number of CD4+, CD8+ T-cells and B-cells decreased significantly the longer the infection persisted (≤6 months vs >6 months, $P \leq 0.002$, $n \geq 3$). In summary, histopathological data about the inflammatory infiltrate of human lesions of CL underline the relevance and importance of findings obtained in the experimental murine mouse model. Both Th1 as well as Tc1 cells appear to be critical for lesion resolution in human CL.

P139

The role of singlet oxygen and oxygen concentration in photodynamic inactivation of bacteria

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New strategies are required in light of the increasing resistance of bacteria to antibiotics. A promising new technique is the photodynamic inactivation of bacteria. Under exposure to light, a photosensitizer in bacteria can generate singlet oxygen, which oxidizes

proteins or lipids leading to bacteria killing. Basic research is necessary to elucidate the oxidative processes during bacteria killing. In our investigations, *S. aureus* was incubated with a standard photosensitizer and the generation and decay of singlet oxygen could be directly detected by its luminescence at 1270 nm. At low bacteria concentrations (0.15 mg/ml), the time resolved luminescence showed a decay time of 6 $\mu s \pm 2$ μs. This value is an intermediate time of singlet oxygen decaying in phospholipids (14 $\mu s \pm 2$ μs) of membranes and in the surrounding water (3.5 $\mu s \pm 0.5$ μs). Obviously, singlet oxygen had sufficient access to the water outside the bacteria by diffusion. Thus, singlet oxygen seems to be generated in the outer cell wall areas or adjacent cytoplasma membranes of *S. aureus*. When measuring luminescence at high bacteria concentrations (1.5 mg/ml), the decay time significantly increased up to about 40 μs, which is partially due to an interchange of rise and decay time of luminescence. This is a clear sign of oxygen depletion during singlet oxygen generation at high bacteria concentrations. Both effects, depletion and diffusion of oxygen, seems to contribute regarding the difference of luminescence decay times at low and high bacteria concentrations. The results show that the photodynamic inactivation of bacteria is affected by oxygen consumption in large bacteria agglomerates. This may affect the efficacy of oxidative processes and therefore photodynamic inactivation of bacteria in vivo.

P140

Investigation of antifungal activity of Chitosan Flake 1130 against *Candida albicans*, *Candida krusei* and *Candida glabrata* by means of a microplate nephelometer

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Objectives: Chitosan is a water insoluble polyaminosaccharide with antimicrobial activity. It is obtained by alkaline deacetylation from chitin. Therefore more than 50% of beta-(1→4)-linked 2-acetamido-2-deoxy-D-glucopyranose of chitin have to be deacetylated into 2-amino-2-deoxy-D-glucopyranose. Chitosan Flake 1130 (ChiPro GmbH), which is chemically modified, was used in the investigation.Chitosan Flake 1130 has a molecular weight of 120 kDa and a degree of deacetylation of 85.2%.

Methods: Different solutions of Chitosan Flake 1130 (1%, 0.5%; 0.25%; 0.1%, 0.05%, 0.025%, 0.01%, 0.005%, 0.0025%) were prepared and their influence on *Candida albicans* (DSM 11225), *Candida krusei* (ATCC 6258) and *Candida glabrata* (DSM 11226) was investigated. Yeasts (3×10^5 cells/ml) were incubated with Sabouraud liquid medium at 30°C. Measurements were carried out by means of a microplate nephelometer (NEPHELOstar Galaxy, BMG LABTECH GmbH, Germany) for 24 h. High values of opacity correlate with strong cultural growth. Results were shown in a special curve, corresponding to microbial growth over the period of 24 h. Then values after 1 h and 24 h were compared using the Wilcoxon's test. Significant differences were interpreted as missing growth.

Results: *C. albicans* showed missing differences for 0.05%, *C. krusei* between 0.05% and 1%. *C.glabrata* showed difference for each concentration.

Conclusions: Complete antifungal activity of Chitosan Flake 1130 could be verified for *C. krusei*. This yeast was inhibited in a wide range between 0.05% and 1%. There is no complete inhibition for *C. albicans* and *C. glabrata*.

P141

Mechanism of antimicrobial activity of Dermcidin-derived peptides

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Dermcidin (DCD) is an antimicrobial peptide, which is constitutively expressed in eccrine sweat glands and transported via sweat to the epidermal surface. By postsecretory proteolytic processing in sweat the dermcidin protein gives rise to a whole group of truncated DCD-peptides with different biochemical properties and yet unknown functional activities. In this study we analysed the mechanism of antimicrobial activity of several naturally occurring DCD-peptides which differ in length and charge. We could show that DCD-peptides do not act by membrane desorption or pore formation of the bacterial membrane, however, they are able to change the bacterial membrane potential over time. Furthermore, DCD-peptides are able to inhibit DNA-, RNA- and protein synthesis in *S. aureus*. Using gel retardation experiments we show that the inhibition of macromolecular synthesis in bacteria is not due to binding of DCD-peptides to bacterial DNA or yeast RNA. Further studies will indicate whether DCD-peptides are able to penetrate the bacterial cell membrane or whether DCD-peptides bind to components in the bacterial membrane.

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Expression of lactonases in human skin

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The human paraoxonase (PON) family contains three genes (PON1/2/3) that are believed to be involved in the protection against oxidative stress. However, it has been shown that these paraoxonases exhibit lactonase activity. Many pathogenic bacteria use N-acylhomoserine lactone (AHL) signals to regulate genes controlling virulence and biofilm formation. The lactonase activity of PON1/2/3 hydrolyse AHLs into inactive products thereby representing a novel anti-infective mechanism to modulate bacterial virulence. It has been shown that among PON1/2/3 PON2 exhibits highest lactonase activity.Since human skin is well protected against biofilms, we addressed the question whether skin is able to produce the lactonases PON1/2/3. Real-time PCR with primers specific for PON1/2/3 was performed to analyse gene expression of PON1/2/3 in primary keratinocytes. We detected no gene expression of PON1 and only moderate gene expression of PON3. In contrast, PON2 showed intense gene expression in primary keratinocytes. Expression of PON2 in human skin was further confirmed by immunohistochemistry using PON2 antibodies. PON2 immunoreactivity was detected in the keratinocytes of the outer layers of human epidermis, where the first contact with bacteria takes place. In addition the sebaceous glands showed distinct PON2 immunoreactivity. We conclude that human skin is able to produce lactonase activity which may help to keep human skin free of bacterial infection.

P143

Biofilm-forming *Pseudomonas aeruginosa* secretes a novel IL-8-inducing pathogen-associated molecule

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The CXC-chemokine IL-8 is a potent chemoattractant for neutrophils and is always found in high concentrations in epithelial infections. A wide range of acute and chronic infections like in patients with cystic fibrosis or burns, is caused by the opportunistic pathogen *Pseudomonas aeruginosa* (PA). It is well known that PA can induce the production of IL-8 in epithelial cells.Biofilm formation is common in PA-infection and is dependent on the intercellular signal-mechanism called quorum sensing. Thus we were interested to know, whether biofilm-forming PA secrete proinflammatory cytokine-inducing pathogen-associated molecules (PAMs) and whether also other microbes secrete similar PAMs. Therefore PA laboratory strains were cultured under biofilm-forming conditions in minimal-medium. Biofilm formation was quantified in different culture media by measuring the adherent cells with crystal violet.As a result, we found supernatants of different strains of PA as well as other bacteria like *P. stutzeri* or *E. coli* to induce IL-8-secretion in primary keratinocytes and other human epithelial cells in vitro. In previous studies we have shown that PA produces a proinflammatory cytokine-inducing PAM that is distinct from a defensin-inducing PAM. We therefore aimed to further characterize the IL-8-inducing PAM in the supernatant of PA. As a result we found the IL-8-inducer to resist freezing and boiling. Partial purification experiments of this inducer by treatment with methanol and chloroform and ion exchange chromatography indicate that this product is neither a lipid nor a protein. Separation by size exclusion chromatography indicated a small molecular mass, which is in contrast to known PAMs. These results indicate this IL-8-inducer to be a novel PAM, distinct from known Toll-like-receptor activating PAMs such as LPS, LTA or flagellin. Further analyses will help to identify the chemical structure of this biofilm-dependent low molecular mass Pseudomonas-PAM.

P144

The *Pseudomonas aeruginosa* Quorum sensing system is involved in the induction of hBD2 in human epithelial cells

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The opportunistic human pathogen *Pseudomonas aeruginosa* (P. a.) causes a wide variety of infections especially in immunocompromised individuals. The bacterial Quorum sensing system resembles a cell to cell communication system with cell density-dependent signalling mechanism that is made up by mainly two interlinked N-acylhomoserine lactone (AHL) dependent regulatory circuits. The Quorum sensing system plays a prominent role in virulence gene regulation of the pathogen. Moreover the Quorum sensing system contributes to adherence and biofilm formation of P.a. on medical devices. In earlier studies we could demonstrate that adherent growing P. a. release a human-beta-defensin-2 (hBD2)-inducing activity in human keratinocytes. hBD2 is a small antimicrobial protein that is known to be induced by either endogenous proinflammatory cytokines or exogenous pathogen-associated molecules (PAMs). Due to this we were interested to elucidate the contribution of the two AHL-dependent circuits lasI/lasR and rhlI/rhlR in P. a. in release of hBD2-inducing activity. P. a. and respective knock out P. a. mutants of the two AHL circuits were cultivated under adherent growing conditions and supernatants were taken. hBD2-inducing activity was determined by stimulation of human epithelial cells and following quantification of hBD2, released within 16 h, by ELISA.As a result we found that supernatants of P. a. with a knock out in the lasI/lasR circuit showed an increased hBD2-induction, whereas for the second circuit rhlI/rhlR a decrease of hBD2 induction could be determined. It is known, that the rhlI/rhlR circuit enhances the synthesis of surfactant-like rhamnolipids in P. a. Interestingly, we recently found that rhamnolipids enhance hBD-2-induction by a poorly characterized hBD-2-inducing ''Pathogen-associated molecule, PAM'' of P.a., which occurs by a yet unknown mechanism. But it is known that rhamnolipids are incorporated into the host cell membrane where it causes selectively alterations by yet unknown mechanisms. Therefore, our results indicate that the release of hBD2-inducing activity in P. a. is linked with rhamnolipid-synthesis, although the exact mechanism how this biosurfactant enhances hBD-2-induction is yet unknown. Further investigations will enlighten the function of rhamnolipids in the epithelial hBD2-induction caused by PAM(s) of P. a.

P145

Disturbed skin barrier function and changes in epidermal differentiation including induction of human beta-defensin 2 expression in tinea corporis

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Tinea corporis is a superficial mycotic infection resulting in substantial epidermal changes. In the present study we determined skin barrier function, epidermal differentiation and human-beta-defensin 2 protein expression in ten patients with tinea corporis caused by *Trichophyton rubrum*. We found disturbed skin barrier function as shown by a significant increase in transepidermal water loss and ultrastructurally by a disturbed formation of extracellular lipid bilayers and the deposition unprocessed lamellar bodies. Epidermal proliferation in lesional skin increased several fold and accordingly, proliferation and inflammation associated keratins K6, K16, and K17 were expressed. Expression of basal keratins K5 and K14 extended to suprabasal layers and expression of the suprabasal differentiation associated K10 was reduced in tinea. Also, there was a reduction of the cornified envelope proteins involucrin, loricrin and filaggrin. Reduced filaggrin expression correlated with a reduced skin hydration; protein break down products of filaggrin have been shown to be important for water binding. Surprisingly, we found pronounced epidermal protein expression of human beta-defensin-2 (hBD-2), which may be related to the disturbed epidermal differentiation and to inflammation. In areas directly underneath fungal hyphae in the stratum corneum the hBD-2 expression was somewhat less pronounced and in the microdilution assay this defensin did not show antifungal activity. Together we describe profound changes in skin barrier function, epidermal proliferation and differentiation including pronounced protein expression of hBD-2 in tinea corporis.

P146

Establishing in vitro models to determine atrophogenic potential

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Glucocorticoids (GCs) are the most frequently used anti-inflammatory drugs at all. Their main draw back, however, is the induction of side effects. Skin atrophy remains one of the most prominent undesired effects of topically administered GCs. One interesting approach to avoid the limitation of classical GCs is to identify novel compounds acting via the glucocorticoid receptor with similar anti-inflammatory effects as known GCs but reduced skin atrophy potential. So far, compounds can be tested regarding their risk for skin atrophy in animal models only. Thus, it is important to develop new test systems which can be performed easily and allow a higher throughput. The aim of the present study is to establish an in vitro test system for predictive determination of skin atrophy potential. Such test system will consist of two components, the assay itself and the read out parameter to be determined. As first candidates for the assay skin cell cultures were investigated. Using HaCaT (human keratinocytes) and 3T3 mouse fibroblast cultures, no reliable effects of GCs with heterogeneous atrophogenic potential was seen on cell proliferation. In contrast dexamethasone and clobetasol induced a dose-dependent decrease of type I and type III collagen mRNA expression in 3T3 fibroblasts. To identify further candidate genes to be used as read out parameters, we determined gene expression profile of skin biopsies from rats and mice after short- (0, 6, 24, 48, 96, 192 h) and long-term (14 or 19 days) topical treatment with reference GCs. Both, positive and negative regulation on gene expression level of several genes were found, but need further validation. Further studies are planned to investigate potential roles of genes identified in GC induced skin atrophy and their suitability for early detection of skin atrophy and for measurement in in vitro skin atrophy models.

P147

Effect of the sterilization method on the binding capacity of bovine collagen for IL-1β and MMP-2

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Introduction: The healing of acute and chronic wounds differs significantly. As several studies have shown, exudates from non-healing wounds contain elevated levels of proteolytic enzymes, like matrix metalloproteinases (MMPs), and increased concentrations of inflammatory immune modulators, e.g. the cytokine IL-1β. Therefore, the binding of these mediators to reduce the overall concentration seems a suitable way to support the healing process. As sterilization is essential for medically applied products, the aim of this study was to investigate whether the sterilization of the wound dressing Suprasorb® C containing native bovine collagen type 1 by treatment with ethylene oxide or γ- and β-radiation (maximum doses of 20 kGy) has an influence on the binding capacity for MMP-2 and IL-1β compared to the native collagen.

Materials & Methods: The wound dressing samples were cut into equal pieces. Each specimen was taken in a final volume of 1 ml of protein solution (100 pg/ml IL-1β ?and 4000 pg/ml MMP-2). Samples were incubated up to 24 h at 37 C on a plate mixer. Supernatants were collected, immediately frozen and stored at -20 C until testing. The concentration of unbound proteins was determined by means of specific ELISAs (IL-1β ELISA, milenia biotec, Bad Nauheim, Germany; Quantikine Immunoassay for MMP-2 from R&D Systems, Minneapolis, USA).

Results: Suprasorb® C is able to decrease the concentration of IL-1β ?in solution significantly. γ- and β-irradiated collagen is also capable of binding significant amounts of IL-1β. EO-sterilized collagen can reduce the IL-1β concentration in the same manner as native collagen. Moreover, Suprasorb® C possesses binding capacity for MMP-2. Already after 1 h a highly significant reduction of the MMP-2 concentration was observed. The different sterilized wound dressings were also able to bind significant amounts of MMP-2 over the examined period.

Conclusions: Suprasorb® C is able to bind interleukins and proteases in different rates. The decrease of excessive inflammatory IL-1β and MMP-2 concentrations by Suprasorb® C in chronic wounds should improve the healing outcome. As the presented data show, treatment of bovine collagen with γ- as well as β-radiation of 20 kGy or EO gas sterilization does not change the binding affinity for IL-1β and MMP-2 compared to native collagen.

P148

Influence of alginate and silver containing alginate on elastase and ROS activity in vitro

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The exudates of chronic wounds contain elevated levels of proteolytic enzymes, like neutrophil elastase, and reactive oxygen and nitrogen species (ROS/RNS). This overproduction results in an elongated inflammatory phase and severe tissue damage. Therefore the reduction of these active species seems to be a suitable way to promote normal wound-healing. Within the present study we investigated the influence of the alginate wound dressing Suprasorb® A, Lohmann & Rauscher, on the binding capacity for elastase and ROS/RNS. As well as the effect of two alginate wound dressings containing ionic silver (Suprasorb® A+Ag, Lohmann & Rauscher) and nanocrystalline silver (Acticoat® Absorbent, Smith & Nephew) respectively. The wound dressing samples were cut into equal pieces. Each specimen was taken in a final volume of 1 ml of elastase solution (0.1 U/ml). Samples were incubated up to 24 h at 37°C on a plate mixer. Supernatants were collected and stored at –20°C until testing. The activity of unbound proteases in the supernatants was determined by means of substrate digestion (EnzChek Elastase® Assay Kit from Molecular Probes, Eugene OR, USA). Antioxidant potential was measured using the chemiluminescent ABEL® Antioxidant Test Kits containing Pholasin® specific for superoxide and peroxynitrite (Knight Scientific Limited, UK).Suprasorb® A as alginate alone is able to bind elastase. Already after 1 h a highly significant decrease of the elastase activity was observed. The wound dressings of alginate containing ionic or nanocrystalline silver were also able to reduce the activity of elastase significantly over the examined period. Furthermore, all tested samples showed antioxidant capacity. The binding of ROS/RNS was significantly higher for the silver containing products in contrast to alginate alone. Suprasorb® A possesses a high binding capacity for the pro-inflammatory protease elastase and inhibits the formation of free radicals. As our studies have shown, alginate wound dressings with ionic or nanocrystalline silver

as anti-microbial agent improved the effect of the wound dressings on reduction of elastase activity and ROS scavanging in vitro. The alginate wound dressing containing nanocrystalline silver exhibited slightly better results upon binding of elastase. The reduction of superoxide and peroxynitrite anions by both silver alginates were equivalent.

P149

Dissection of the signal transduction pathways in sulphur mustard exposed keratinocytes. Involvement of NF-κB, JNK and MAPK

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Cutaneous exposure to sulphur mustard [bis(2-chloroethyl) sulfide] produces a delayed inflammatory skin response and severe tissue injury. The NF-κB signal transduction pathway is involved in many cellular processes. It takes part in the immune response, inflammatory processes and is placed at the interface between inflammation and malignancy. NF-κB is a complex formed by homo- and heterodimerization of the NF-κB/Rel family members p50 (NF-κB1), p52 (NF-κB2), RelA (p65), RelB and c-Rel. Their cytoplasmic receptors are members of the IκB family, with IκBα being the most important in the cellular stress response. Activation of cells by various stimuli results in serine-phosphorylation of IκBα by the IκB kinase (IKK)-signalosom, followed by degradation of IκBα and subsequent translocation of NF-κB to the nucleus. The aim of this study was to analyse the function of NF-κB in the inflammatory skin response to sulphur mustard. In mouse keratinocytes we could show, that NF-κB is activated in a biphasic response following stimulation by sulphur mustard. The activation was preceded by phosphorylation of IKKβ and IκBα and resulted in a phosphorylation of RelA. The activation of genes coding for IL-1β and TNF, two NF-κB regulated genes, proved the functional relevance sulphur mustard induced NF-κB activation. These findings strongly support the idea of NF-κB as key modulator in the cellular response to sulphur mustard. In addition to NF-κB, we can show that MAPK signalling pathways involving c-Raf, ERK1/2, MSK1 and p38, MSK1, as well as JNK signalling cascades involving MLK3, MKK7, JNK1/2 and ATF-2 participate in the keratinocyte response.

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Usnea barbata extract inhibits ultraviolet-B induced prostaglandin E2 synthesis and cell proliferation of HaCaT keratinocytes

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Background: *Usnea barbata* and its major compound usnic acid display potent antimicrobial effects against *Propionibacterium acnes* and *Malassezia furfur*. Here we have investigated the anti-inflammatory and antiproliferative potential of an *Usnea barbata* extract containing 4% usnic acid.
Methods: *Usnea barbata* extract (UBE) was prepared by high pressure extraction with liquid carbon dioxide and isopropylic alcohol. The extract contained 4% (+)-usnic acid as determined by HPLC analysis. HaCaT keratinocytes were irradiated with 30 mJ/cm^2 ultraviolet-B (UVB) and were incubated in the presence or absence of various concentrations of UBE for 24 h. Subsequently, prostaglandin E2 (PGE2) production was measured in the supernatant by ELISA, and COX-2 expression in cellular lysates was determined by western blot analysis. Cell proliferation was determined by incorporation of 3H thymidine (3H-Td) into cellular DNA, and cell viability was assessed by luminometric measurement of intracellular ATP.
Results: UVB-induced PGE2 production was dose-dependently inhibited by UBE with a half maximal inhibitory concentration (IC50) of 50 μg/ml. This concentration did not affect the UVB-induced upregulation of COX-2, suggesting an effect of UBE on enzyme activity rather than on protein expression. UBE also dose-dependently inhibited the proliferation of HaCaT cells with an IC50 of about 100 μg/ml. The anti-inflammatory and antiproliferative concentrations were below the toxic threshold concentration of UBE (625 μg/ml).
Conclusion: Besides its known antimicrobial properties, UBE with a defined content of usnic acid displays specific anti-inflammatory and antiproliferative effects. Therefore, UBE might be useful in the topical treatment of acne vulgaris.

P151

Pharmaceutical and biological characterization of a new betulin-rich plant extract from Betulae cortex

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Background: Triterpenes are biologically active secondary plant substances that display antimicrobial, anti-inflammatory and hepatoprotective effects. However, the poor solubility of triterpenes in both polar and non-polar solvents as well as expensive purification procedures have prevented the large scale isolation of these compounds for medicinal purposes.
Methods: We describe a novel quantitative extraction method of triterpenes from the outer bark of birch (Betula species) in which betulin, a lupan triterpene, predominates. The physical and chemical characterisation of the triterpene extract (TE) was done by rheology, laser diffraction, gas adsorption and gas chromatography analysis. Furthermore, we have investigated the TE in comparison to its main isolated compounds in cell culture experiments with human immortalized keratinocytes (HaCaT) and skin cancer cells (A431) using commercially available cell proliferation, cytotoxicity and apoptosis assays.
Results: The highly purified TE has the form of a dry powder and contains betulin as the major compound (80% w/w), but also betulinic acid, lupeol, erythrodiol and oleanolic acid. We have found that the TE is able to form an oleogel, thus providing an opportunity for the topical application of pharmacologically relevant amounts of triterpenes. In cell culture experiments, we could demonstrate dose-dependent cytotoxic and apoptosis-inducing effects of TE and betulin.
Conclusion: These experimental data support the notion from a previous clinical pilot study that TE from the outer bark of birch might represent a new tool for the topical treatment of skin cancer precursors like actinic keratoses.

P152

Response of lymphatic vessels and cultured lymphatic endothelial cells to NO

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The lymphatic system has several functions particular the removal of interstitial fluid and proteins. This is accompanied by specific structures, which can be influenced. Lymphatic endothelial cells were isolated by immunomagnetic separation from HDMEC. For identification with flow cytometry cells were labelled with podoplanin and D2-40 antibodies. The cell surface glycoprotein Podoplanin is a capable marker for native LEC's. By adding L-arginine in different concentrations a potential effect of the generated NO were analysed. For the cellular response proliferation and cytotoxicity parameters were examined with the help of BrdU/anti-BrdU method and Annexin V-PI analysis. Another aspect of work was the response of lymphatic vessels/endothelial cells exposed to VEGF subtypes in HET-CAM model to the same L-arginine conditions. The investigations answer the question if there is any response in cellular research as well as in HET-CAM model to L-arginine.

P153

Epidermal stem cells as drug target

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Homeostatic epidermal tissue renewal is the result of the combined activity of rare but potent stem cells, and a large pool of short-lived progenitor cells termed transit amplifying cells. A characteristic of epidermal stem cells, with that they can be distinguished from other proliferative cells in the basal layer, is their greater overall proliferative capacity during the whole lifetime. Originating from neonatal foreskin, we isolated epidermal stem cells as rapidly adhering cells and transit amplifying cells as slowly adhering cells on collagen IV. By investigating the colony forming efficiency and long-term proliferation we were able to show the higher proliferative potential of the rapidly adherent cells compared to the slowly adherent cells in vitro. The control of the proliferation potential of these two populations is an important way to achieve long-term curative effects in the therapy of epidermis-based skin diseases such as psoriasis. However, stem cells and transit amplifying cells may respond differently to active agents. Therefore, we choose established drugs, glucocorticoids and vitamin D analogues, for hyperproliferative skin diseases to research for potential differences between the drug effects on epidermal stem cells and transit amplifying cells in vitro.

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Histone deacetylase inhibitors suppress dermal inflammation in murine contact hypersensitivity models

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Although histone deacetylase (HDAC) inhibitors are in clinical development for cancer, they have shown potent anti-inflammatory properties in a number of in vitro and in vivo model systems. In the studies described here, we have examined the effects of HDAC inhibitors on the inflammation induced by contact hypersensitivity reactions in mice. The models involve sensitization with DNFB and either a single challenge with the same sensitizing agent on the ear, or repeated challenge once per day for 3 or 4 days. The primary readout for inflammation in all models is ear oedema, as measured by an increase in ear weight. Several HDAC inhibitors were compared and topical treatment with MS-275, SAHA, or CI-994 reduced ear oedema in a dose-dependent manner (EC50 = 0.3–0.5% w/v for all compounds). At the highest concentration tested (3% w/v), the HDAC inhibitors completely suppressed the antigen-induced oedema. While the reduction in oedema occurred in the absence of any effect on neutrophil or eosinophil infiltration into the skin, we observed a dose-dependent reduction in the percentage of T cells that paralleled the reduction in oedema. Treatment with the HDAC inhibitor MS-275 (3% w/v) also led to a reduction in TNF and IFN-? levels in the skin, by 75% and 60% respectively, suggesting a possible link between the release of these pro-inflammatory cytokines and antigen-induced ear oedema. The suppression of TNF and IFN-? release by HDAC inhibitors was confirmed with in vitro studies using human whole blood stimulated with LPS and purified human T cells stimulated with anti-CD3 and anti-CD28 antibodies (for TNF) and the mixed lymphocyte reaction (for IFN-?). These studies reveal a novel means of suppressing skin inflammation in murine contact hypersensitivity models and support exploration of HDAC inhibitors as potential therapeutics for the treatment of inflammatory skin diseases.

P155 (V28)

Inhibition of 5alpha-reductase type 1 leads to suppression of DHT production but does not abrogate lipid synthesis in the SZ95 human sebocyte line.

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Acne is a common disease of the sebaceous glands affecting particularly adolescents. Its pathogenesis is complex, yet disturbed androgen production or androgen sensitivity might contribute to increased sebum production and acne since treatment of patients with antiandrogens ameliorates the disease. Consistently, individuals with androgen receptor (AR) deficiency produce less sebum and are protected from acne. These findings imply that 5alpha reductase (5aR), which metabolizes testosterone (T) to the more bioactive androgen dyhydrotestosterone (DHT) provides a target for acne treatment. This refers particularly to 5aR type I (5aRI), which is predominantly expressed in the skin. We therefore asked whether AR antagonists or selective 5aR inhibitors exert inhibitory effects on the lipid synthesis of human sebocytes and may thereby interfere with acne.The human sebocyte line SZ95 was treated with T in the presence of novel or established 5aRI-inhibitors (ZKA, ZKB, MK-386), the 5aRII-inhibitor finasteride, the 5aRI/II dual inhibitor dutasteride, AR antagonist cyproteronacetate (CPA), or dexamethasone. Cellular

DHT and neutral lipid production was assessed. As expected, CPA and finasteride showed no or weak suppression of DHT production while MK-386, ZKA, ZKB, and dutasteride led to 70–90% inhibition of DHT production. IC50s of MK-386, ZKA, ZKB, and dutasteride revealed potencies in the low nanomolar range. Notably, neither 5aR inhibitors nor CPA exerted any inhibitory effect on lipid production. In contrast, dexamethasone blocked lipid synthesis in a RU-486-sensitive, i.e. glucocorticoid receptor-specific manner.This study demonstrates that SZ95 sebocytes produce DHT in a 5aRI dependent fashion which can efficiently and potently be blocked by selective inhibitors. Yet, neutral lipid formation in SZ95 sebocytes does not depend on DHT, while it is susceptible to GR agonism. This study does therefore not support the concept that antiandrogens directly interfere with skin lipid production and does not provide evidence for 5aRI being a valid target for control of skin lipid synthesis and thus treatment of acne.

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Reevaluation of the hamster flank organ as a model for anti-seborrhoeic effects of oral anti-androgens

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Androgens might underlie acne since treatment with anti-androgens ameliorates the disease. Consistently, androgen receptor (AR) deficient individuals are protected from acne. Thus, both AR and 5alpha reductase (5aR), which metabolizes testosterone (T) to the more bioactive androgen dihydrotestosterone (DHT), provide targets for acne therapy. This refers particularly to 5aR type I (5aRI), which is predominantly expressed in human skin. Yet, drug discovery for acne is still hampered by the lack of animal models. Nevertheless, the hamster flank organ (FO), two androgen dependent, lipid containing, dark coloured skin appendices have been used to assess topical anti-androgens for acne therapy. We here evaluate the FO model for profiling oral anti-androgens for acne treatment. The clinically effective AR antagonist cyproterone acetate (CPA) was administered p.o. over up to 42 days at 0.3 to 30 mg/kg/d. To test whether CPA effects could be recapitulated by separately inhibiting 5aRI or 5aR type II (5aRII), known and novel 5aRI inhibitors MK-386, ZKA and ZKB, the 5aRII inhibitor finasteride, or the dual type dutasteride was applied. Change of FO area was measured over time. FO weight, thickness, lipid content, DHT serum levels, prostate and adrenal gland weight were assessed at the end of the study. Only minor effects of CPA or 5aR inhibitors on FO weight occurred in TP-substituted castrated hamsters. Normal hamsters showed partially significant but highly variable FO weight reduction under CPA or 5aR inhibitor treatment. FO area was the most sensitive and robust marker for anti-androgenicity. Area reduction was visible after 3 weeks but reached its maximum after 6 weeks. Reduced FO areas correlated with diminished skin lipid staining. Prostates were generally not affected by 5aRI inhibitors but lost weight under CPA, finasteride or dutasteride. Surprisingly, FO area was decreased similarly by CPA and all types of 5aR inhibitors.The hamster FO is an androgen-dependent organ that can in principle be used for profiling oral anti-androgens. However, the model shows limitations since long-term treatment may be necessary. Caution is required regarding 5aR inhibition since no isozyme selectivity was observed in the skin possibly due to 5aR isozyme distribution unlike in humans or due to cross-reaction of inhibitors optimized for human 5aR isozymes.

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Response of human keratinocytes to low micromolar dithranol as analysed by an inflammation-related cytokine and receptor cDNA-Microarray

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The application of dithranol, a highly efficacious topical antipsoriatic drug, is hampered by its skin-irritative potential.To further clarify the underlying mechanisms, hyperproliferative HaCaT keratinocytes and normal human epidermal cells (NHEC) were incubated with dithranol concentrations from 0.1 to 10 μM for 2 h. Immediately thereafter and also 4, 24 and 48 h later, attached cells were harvested and counted. HaCaT keratinocytes were tested for parameters of apoptosis by flow cytometry, and IL-1α mRNA expression was quantified by RT-PCR. In parallel the culture supernatants were analysed for IL-1α release by ELISA. In front of these control experiments, NHEC were analysed by a cytokine and receptor cDNA-microarray.Overall, dithranol at a concentration as low as 1 μM led to a substantial, time-dependent increase of IL-1α mRNA expression and release. In parallel the drug resulted in a decrease of cell number and an induction of apoptosis. At 4 h after dithranol treatment the microarray analysis confirmed a markedly upregulation of the mRNA of IL-1α in comparison to the untreated control.Furthermore, at 24 h after drug treatment the mRNA of the competetive IL1R antagonist IL1RN was down-regulated. Remarkably, mRNA of chemokines CCL13 and CXCL14, both, decreased with time.Thus the study shows (i) the dithranol-dependent pro-inflammatory event of an increased IL-1α synthesis and secretion as well as a synergistic IL1RN decrease coupled to (ii) antiproliferative and pro-apoptotic phenomena coinciding with down-regulation of distinct chemokines.

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Pathogenesis and management of Erlotinib (Tarceva®) – induced cutaneous side effects

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Inhibitors of the epidermal-growth-factor receptor (EGFR), like Erlotinib (Tarceva®), a specific inhibitor of the EGF receptor tyrosine kinase, have been proven to be effective novel therapeutics in patients suffering from non-small cell lung cancer (NSCLC) and a variety of other neoplasias. Cutaneous side effects are the most common adverse effect associated with the use of Erlotinib (Tarceva®) occurring in more than 50% of the patients. Over the past 2 years we have attended a collective of 33 patients who developed cutaneous side effects under treatment with Erlotinib (Tarceva®). In our patients we observed dermal signs of inflammation, infection, atrophy and angiogenesis. Distinct dermatological side effects include acneiform eruptions, xerosis cutis, telangiectasia, hair changes and paronychia. So far, the underlying mechanisms causing cutaneous side-effects remain illusive. The management of skin eruptions is challenging and effective therapy regimens are still to be defined. Yet, left untreated these side-effects represent a serious threat to

patient compliance.Based upon clinical observations, diagnostic parameters (e.g. microbial swaps and skin surface biopsies by cyano-acrylate technique) and evaluation of various treatment options in our patients we were able to design an effective treatment algorithm for the management of Erlotinib (Tarceva®) – induced cutaneous side effects.Moreover, to elucidate the pathogenesis of Erlotinib (Tarceva®) – induced cutaneous side effects, we performed in vitro analyses in human primary keratinocytes applying the model EGFR inhibitors PD168393 and AG1478. Quantitative real-time PCR analyses demonstrated that inhibition of the EGF receptor tyrosine kinase differentially regulates the expression of chemokines and antimicrobial peptides. Our results present interesting new insights into the pathogenesis of Erlotinib (Tarceva®) – induced cutaneous side effects and link in vitro and clinical observations. Moreover, results might help to develop more effective, rational-based therapy regimens.

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Activation of the transcription factor PPAR delta in psoriasis

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Analysis of altered gene expression in lesional psoriatic skin indicated that 90% of dysregulated genes were concordant to transcripts previously reported to be dysregulated, indicating that the vast majority of transcriptional changes in psoriasis do not represent noise, but form of a highly reproducible disease-associated pattern. The single largest group of dysregulated genes unexpectedly were related to fatty-acid signalling, adipocyte differentiation, and redox activity. All of these functions are critically dependent on the acitivity of PPAR delta, a transcription factor regulating keratinocyte differentiation, which was itself one of the most highly upregulated transcripts. PPAR delta was constitutively expressed and localized to the nucleus in primary adult epidermal keratinocytes in vitro. Ligand-specific activation of PPAR delta enhanced, while lentivirus – mediated knock-down of PPAR delta inhibited proliferation in primary keratinocytes. Furthermore, activation of PPAR delta enhanced keratinocyte differentiation in non-differentiated keratinocytes whereas it blocked differentiation in differenciated cells, consistent with abnormal distribution of differentiation states in psoriatic epidermis. Finally, activation of PPAR in primary keratinocytes reproduced numerous transcriptional changes seen in vivo and identified putative novel target genes of relevance to psoriasis. As a transcriptional target of TFN alpha, PPAR delta appears to be a major factor inducing and/or maintaining the regenerative phenotype characteristic for psoriasis.

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Heat shock and UVA-induced cell death: tissue dependence

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In previous studies we have shown that heat shock protects the human squamous cell carcinoma cell line A431 from UVA-induced cell death. The mechanism seemed not related to an antioxidative effect. In the present study we were interested whether this protection occurs also in other tissues, and if so which mechanisms were underlying this effect.-Normal human keratinocytes (NHEK), A431, normal human dermal fibroblasts (NHDF), XPA- and XPV-fibroblasts, the human fibrosarcoma cell line HT1080, peripheral blood mononuclear cells (PBMC), EBV-transformed lymphocytes, XPAand XPV-lymphocytes, the human leukaemia cell lines Jurkat and U937 were exposed to heat shock (3 h, 42°C) and subsequently to UVA from a metal halide source (315–390 nm). Cell viability was measured by an MTT assay. The levels of the 72 kDa heat shock protein (Hsp72) and haeme oxygenase-1 (HO-1) were determined by enzyme-linked immunosorbent assay. Oxidative stress was determined by measurement of TBARS and glutathione.In addition to A431, heat pretreatment increased the survival rate after UVA in PBMC, EBV-lymphocytes, XPA-lymphocytes and Jurkat compared to controls. However, no induction of Hsp72 could be found in these cells after heat exposure. Furthermore, UVA-induced TBARS formation and glutathione depletion did not differ between heat treated and control cells. On the other hand, PBMC, XPA- and EBV-lymphocytes showed a baseline and heat-inducible expression of HO-1.These results show that heat shock inhibits UVA-induced cell death depending on the tissue type with protection mainly in lymphoid cells. In contrast to UVB-induced cell death Hsp72 seemed not to play a role in this protective effect. The mechanism is not due to an antioxidative effect of heat shock, but might be related to HO-1 expression.

P161

Regulation of the expression of CYP24A1 (25-hydroxyvitamin D-24-hydroxylase) on the transcriptional level by UVB-induced synthesis of 1α,25-dihydroxyvitamin D3 (calcitriol) in human keratinocytes

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UVB radiation initiates the conversion of 7-dehydrocholesterol (7-DHC) to the secosteroid vitamin D3 (cholecalciferol) in basal and suprabasal keratinocytes of the skin. Keratinocytes have the complete array of enzymes (CYP27A1 and CYP27B1) to hydroxylate vitamin D3 to hormonally active 1α,25-dihydroxyvitamin D3 (calcitriol) and, finally, to catabolize the latter (CYP24A1). Both, synthesis of vitamin D3 and formation of calcitriol depend in vitro in a very similar manner on the wavelength of UVB radiation. The synthesis rate of both metabolites in cultured keratinocytes enriched with 25 μM 7-DHC shows a maximum at 302 nm. By contrast, both metabolites are not detectable at 320 nm. However, the physiological function of UVB-triggered synthesis of calcitriol in the skin remains to be clarified. Interestingly, both UVB and biologically active vitamin D analogs are used singly or in combination in the treatment of psoriasis. The catabolic enzyme CYP24A1 which initiates degradation of calcitriol is sensitively and quickly induced by calcitriol in a genomic manner. We have studied (i) whether UVB-triggered production of calcitriol in cultured keratinocytes supplemented with 25 μM 7-DHC upregulates the CYP24A1 mRNA and (ii) if the synthesis of CYP24A1mRNA follows the wavelength-dependent synthesis of calcitriol. Using real time PCR we found that regulation of CYP24A1 on the transcriptional level excellently correlates with the synthesis rate of calcitriol in the wavelength range between 290 and 310 nm. Data for the expression of CYP24A1 mRNA were normalized by using the housekeeping gene GAPDHD. The synthesis rate of CYP24A1 mRNA was maximum 24 h after irradiation at 300 nm (36-fold elevation versus determination

immediately after irradiation).On the other hand, we found only a moderate upregulation of the CYP24A1 mRNA (7-fold elevation versus determination immediately after irradiation) in the control experiment without addition of exogenous 7-DHC. We assume from our findings that further genes beside the CYP24A1 gene might be regulated by calcitriol produced within keratinocytes. We focus our attention on such genes which appear particularly relevant for controlling of growth and differentiation of keratinocytes.

P162 (V08)

Langerhans cells but not bone marrow-derived dendritic cells direct UV-induced regulatory T cells into the skin

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We could recently show that UV-induced regulatory T cells (UV-Treg) inhibit the sensitization but not the elicitation of contact hypersensitivity (CHS) when injected intravenously (i.v.). This is due to the fact that UV-Treg migrate primarily into the lymph nodes but not into the skin, since they express the lymph node homing receptor CD62L but not the ligands for the skin homing receptors. To utilize UV-Treg not only in a prophylactic but also in a therapeutic fashion, emigration into the skin is crucial. Coincubation of UV-Treg with Langerhans cells (LC) downregulated the expression of CD62L and induced the expression of the skin homing receptor PSGL-1. Accordingly, i.v. injection of UV-Treg pretreated in such a way inhibited the elicitation but not the sensitization of CHS. In contrast, coincubation with bone marrow-derived dendritic cells (BM-DC) did neither alter the migratory behaviour nor the expression of homing receptors on UV-Treg. To finally prove, that UV-Treg upon coincubation with LC migrate into the skin, UV-Treg were labelled with CFSE. Upon i.v. injection CFSE-labelled cells could only be observed in the skin, when the Treg were preincubated with LC but not with BM-DC. In contrast upon coincubation with BM-DC the vast majority of labelled cells were found in the lymph nodes and the spleen upon i.v. injection. Double staining with CD25 confirmed that the CFSE-labelled cells are UV-Treg which are known to express CD4 and CD25. To exclude that the suppressive activity of UV-Treg is altered by the coincubation with BM-DC and thereby do not suppress the elicitation of CHS, UV-Treg which were coincubated with BM-DC were injected intracutaneously into the ears of sensitized mice which resulted in a pronounced inhibition of the ear challenge. This confirms that the coincubation with DC alters the tissue homing but not the activity of UV-Treg. Taken together, these data show that the migratory behaviour of UV-Treg can be altered by tissue-specific DC. This may have input on strategies trying to utilize Treg not only for the prevention but also for the treatment of immune-mediated disorders.

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Further characterization of regulatory T cells induced by experimental extracorporeal photopheresis

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Since extracorporeal photopheresis (ECP) is effective not only in the treatment of cutaneous T cell lymphoma but also of autoimmune diseases, solid organ transplant rejection and GvHD, it was postulated that ECP might exert immunosuppressive properties. Utilizing an experimental in vivo model for ECP, we could demonstrate that ECP might induce regulatory T cells (Treg). Infusion of leukocytes obtained from dinitrofluorobenzene (DNFB)-sensitized mice, which were exposed to psoralen plus UVA (PUVA) in vitro, rendered the recipient mice unresponsive to DNFB. This unresponsiveness was due to the induction of Treg in the recipients since transfer of lymphocytes into a second generation of naïve recipients inhibited sensitization against DNFB. This study was performed to further characterize the phenotype and function of ECP-induced Treg (ECP-Treg). ECP-Treg express CD4 and CD25 since depletion for these markers before injection resulted in loss of transfer of suppression. Upon coincubation of ECP-Treg with antigen presenting cells in the presence of DNBS induced the release of the immunosuppressive cytokine interleukin-10. Since we previously could show that the vast majority of in vitro PUVA-treated leukocytes migrate into the spleens of the recipients, PUVA-exposed leukocytes were injected into splenectomized mice. In contrast to untreated controls, splenectomized mice were not suppressed in their sensitization to DNFB. In addition, Treg did not develop in splenectomized recipients, indicating that the spleen is crucial for the generation of ECP-Treg. So far ECP-Treg were shown to inhibit the sensitization since they were injected into naïve recipients that were subsequently sensitized. To check whether ECP-Treg also inhibit the elicitation, ECP-Treg were injected into already sensitized recipients. Upon injection of ECP-Treg, ear challenge was significantly suppressed, indicating that these cells do not only inhibit the induction but also the effector phase of contact hypersensitivity. Taken together, these data suggest that PUVA-treated leukocytes induce antigen-specific Treg, presumably via contact with CD4+CD25+ cells in the spleen and that ECP-induced Treg have the potency to inhibit not only the induction but also the elicitation of immune responses.

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Repetitive UVA irradiation induces the release of cathepsin L but not cathepsin B in fibroblasts

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UVA-therapy is a recently introduced therapeutic modality to treat fibrotic skin disorders as e.g. systemic scleroderma. The mechanisms by which UVA exerts its anti-fibrotic effects are poorly understood.-Cathepsin B and L are lysosomal cysteine proteases participating in intracellular protein turnover. Recent studies indicate that these enzymes act also extracellularly and are able to degrade a number of extracellular matrix proteins as collagen telopeptides or elastin. In our present study, we investigated whether single or repetitive irradiation with UVA regulates the expression and release of Cathepsin B and L.Human dermal fibroblasts were irradiated with increasing doses of UVA (0–40 J/cm^2) as well as on consecutive days (2–4) with a single dose each (20 J/cm^2). Thereafter supernatants, cell extracts and RNA were analysed. Whereas no alteration of protein synthesis and release of cathepsin B and L was found after single irradiation, a clear extracellular release of pro-cathepsin L and two mature forms of cathepsin L was found after repeated irradiation using western blot and zymographic analysis. This release correlated with the number of consecutive irradiations. Detection of extracellular cathepsin L was associated with a significant reduction of levels detected intracellularly indicating a mobilization from preformed intracellular pools.This is the first study which demonstrates the extracellular release of a cysteine protease by repetitive UVA irradiation having important implications for the understanding of UVA induced

connective tissue turnover as e.g. in UVA therapy of fibrotic diseases or skin aging.

P165

25-Hydroxyvitamin D3 1alpha-hydroxylase splice variants in human skin

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1,25-Dihydroxyvitamin D3 (1,25(OH)2D3), the biologically active metabolite of vitamin D, has been shown to regulate the growth of various cell types, including human keratinocytes. There are two principal enzymes involved in the formation of circulating 1,25(OH)2D3 from vitamin D, the hepatic microsomal or mitochondrial vitamin D 25-hydroxylase (25-OHase) and the renal mitochondrial enzyme 1α-hydroxylase (1α-OHase) for vitamin D and 25(OH)D3, respectively. 25-hydroxyvitamin D3-1α-hydroxylase catalyses the synthesis of the active form of vitamin D, 1,25-dihydroxyvitamin D3, in the kidney. Recently, extrarenal activity of 1α-OHase has been reported in various cell types including macrophages, keratinocytes, prostate and colon cancer cells. Local production of calcitriol has been postulated to play an autocrine or paracrine role in vitamin D-mediated growth control.As already described for other cytochrome **P450** genes, alternative splicing can play a role in regulating the enzyme level and may cause tissue-specific variations in healthy cells. Recently 16 splice variants (Hyd-V1 -Hyd-V16) of 1α-hydroxylase mRNA have been described in glioblastoma and melanoma cell lines. We did western blot analysis to identify splice variants in cultured human keratinocytes (HaCaT) after treatment with UV-B (7.5–50 mJ/cm^2). Antibody used, identified up to six proteins between 45 kDa and 75 kDa likely representing splice variants Hyd-V3 (46 kDa), Hyd-V4 (59 kDa), Hyd-V5 (46 kDa), the normal enzyme (56 kDa) and additional variants that have not been identified so far. Twelve hours after treatment with UV-B we identified at higher dosages (20–50 mJ/cm^2) a change in the splice pattern of 1α-hydroxylase. The variant Hyd-V4 and the normal enzyme decrease, whereas variants Hyd-V3 or Hyd-V5 were increased. This effect could not been shown at lower dosages (7.5–10 mJ/cm^2). We suggest that reduction of active enzyme will influence the level of 1,25(OH)2D3 and therefore will modulate keratinocyte differentiation after UV-B treatment.

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The human hair follicle as a model for exploring the effects of ultraviolet radiation in a complex tissue interaction system in situ

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The life-long impact of ultraviolet radiation (UVR) on skin results in predominantly harmful effects such as skin aging and photocarcinogenesis. Even though human scalp hair follicles (HFs) are exposed to UVR throughout life, the impact of UVR on HFs has not yet been systematically investigated. Here, we show that, compared to sham-irradiated controls, microdissected, organ-cultured HFs in anagen VI, irradiated with UVB 280 nm at 20–50 mJ/cm^2 prematurely enter into HF regression (catagen), accompanied by significantly reduced hair shaft elongation and hair matrix proliferation (Ki67), and a significant upregulation of hair matrix keratinocyte apoptosis (TUNEL). As biochemical indicator of both necrosis and apoptosis, LDH-activity increases significantly in supernatants of UVB-irradiated HFs. Quantitative Masson-Fontana histochemistry reveals significant reduction of the follicular melanin content, while immunohistology shows a shift of immunoreactivity for a key hair growth-inhibitory cytokine (TGFβ2) from the outer root sheath towards the inner root sheath cuticle. Unexpectedly, ACTH immunoreactivity declined, while that of α-MSH remained unaffected. As an indication of oxidative DNA damage, 8-OHdG immunoreactivity increased in UVB-irradiated HFs. Strikingly, the number of Giemsa-stainable mast cells in the HF connective tissue sheath increased significantly, while the total number of c-Kit+ mast cells remained essentially unaltered - indicating maturation of resident mast cell precursors in response to experimental UVR. The current findings identify the HF as a target of phototoxicity. Even though human HFs are unlikely to be exposed to such high doses of UVB 280 nm in vivo, the current organ culture assay offers a novel, instructive assay for studying the molecular and cellular pathology of UV-induced damage in a complex, prototypic human epithelial-mesenchymal tissue interaction unit in situ.

P167 (V15)

Oxidized phospholipids that are generated in UVA-1 irradiated dermal fibroblasts mediate biological responses to UVA-1 via the redox – sensitive transcription factor Nrf2.

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We have previously shown that the common membrane phospholipid 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (PAPC) is prone to UVA-1 oxidation. One or more of the oxidation products can mediate expression of the UVA-1 stress-response gene haeme oxygenase-1 in skin fibroblasts (FB) and can thus act as mediators of UVA-1 induced gene expression. We could demonstrate, using HPLC-MS/MS analysis, that oxidation products able to induce HO-1 are formed after in vitro irradiation of PAPC with UVA-1 but not UVB and that these products accumulate in lipid extracts of FB immediately after UVA-1 irradiation. We thus hypothesized that UVA-1 mediated phospholipid oxidation could be responsible for the transcriptional regulation of other UVA-1 regulated response genes.To test this hypothesis, we carried out a comparative microarray chip expression analysis of dermal fibroblasts that were either untreated, UVA-1 irradiated or treated with oxidized phospholipids. As much as 10% of the genes that are regulated 4 h after UVA-1 treatment are co-regulated in the cells treated with oxidized phospholipids. Among these co-regulated genes are, besides HO-1 and the glutathion synthesis gene GCLM, also other Phase II antioxidant response genes and the cytokine IL8. Bioinformatic analysis revealed that many of the known genes contain an antioxidant response element (ARE) in their promoter and are thus prone to regulation with the redox sensitive transcription factor Nrf-2. SiRNA mediated knockdown of this transcription factor left most of the co-regulated genes less responsive to oxidized

PAPC or UVA-1, while proinflammatory genes like IL6 and COX2 exclusively regulated by UVA-1 still responded to the stimulus. Our data thus suggest that a substantial part of transcriptional regulation upon UVA-1 treatment can be mediated by phospholipid oxidation and that the redox sensitive transcription factor Nrf2 is involved in this process.

P168

Variable pulsed light less painful than light emitting diodes for topical photodynamic therapy of actinic keratoses: a prospective randomized controlled trial

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Background: Photodynamic therapy (PDT) of actinic keratoses (AK) using methyl-aminolevulinate (MAL) is an effective and safe treatment option.

Objective: To evaluate the painfulness and efficacy of variable pulsed light (VPL), a prospective, randomized, controlled split-face study was performed.

Patients/Methods: Topical MAL-PDT was conducted in 25 patients with AK ($n = 238$) that were suitable for two-side comparison. After incubation with MAL, irradiation was performed with a light emitting diode (LED) (50 mW/cm^2; 37 J/cm^2) versus VPL (80 J/cm^2, double pulsed at 40 J/cm^2, pulse train of 15 impulses each with a duration of 5 ms, 610–950 nm filtered hand piece) followed by re-evaluation up to 3 months.

Results: The pain during and after therapy was significantly lower with VPL irradiation [$t(df = 24) = 4.42$, $P < 0.001$]. The overall infiltration and keratoses score 3 months after treatment was 0.86 ± 0.71 (LED-system) vs 1.05 ± 0.74 (VPLTM device) (no statistically significant difference; $P = 0.292$). Patient satisfaction following both treatment modalities did not significantly vary during the 3 months follow-up ($P = 0.425$).

Conclusions: VPL use for MAL-PDT is an efficient alternative for the treatment of AK that results in complete remission and cosmesis equivalent to LED irradiation but causes significantly less pain.

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Evaluation of different 5-Ala/5-Ala methylester formulations regarding fluorescence induction and penetration using an ex vivo porcine skin model

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Topical photodynamic therapy is a quite new modality for treatment of localised non-melanoma skin cancer. Of particular interest is the suitable application of 5-aminolevulinic acid (5-Ala), which is metabolized to protoporphyrin IX (PpIX) in the tissue. Therefore, the purpose of the study was to investigate skin penetration of different 20% 5-Ala/5-Ala-methylester formulations as well as fluorescence induction of PpIX using a new ex vivo porcine skin model. Fluorescence signals of PpIX were detected first at 4 and 6 h after application of a 5-ALA cream (+40% DMSO), on damaged and intact porcine skin respectively. Fluorescence signal intensities of PpIX were decreased in order of 5-Ala cream (+40% DMSO) >5-Ala cream (-DMSO) >5-Ala-methylester cream >5-Ala in lipophilic ointment >5-Ala hydrophilic ointment. In case of the 5-Ala hydrophilic ointment fluorescence intensity was ~10x times lower as compared to the PpIX signal intensity of the 5-ALA cream containing 40% DMSO. In all cases the maxima of PpIX fluorescence intensity was achieved between 18–24 h after application. Penetration of 5-Ala within the lower parts of the epidermis were increased in order of 5-Ala hydrophilic ointment <5-Ala lipophilic and 5-Ala methylester cream <5-Ala – DMSO <5-Ala + DMSO. Penetration of all tested formulations was restricted to the basal cell membrane. No fluorescence signals were detected within the dermis. Histological evaluations of untreated skin areas (±5-Ala, without light) showed no significant degree of necrosis and apoptosis determined by NBTC staining and TUNEL-Assay indicating the porcine skin is still vital up to 24 h. In contrast, illumination (λem = 550–700 nm; 40 mW/cm^2, 24 J/cm^2) of skin areas incubated with the different 5-Ala formulations did show a marked increase of death cells (10–45% ± 5) of the epidermis depending in order of the penetration depth of the 5-Ala formulations.In the present study it has been demonstrated that an ex vivo porcine skin model is able to discriminate penetration depth and PpIX fluorescence intensities of different 5-Ala formulations. Furthermore, this porcine skin model can be used to study phototoxicity efficacy of new photosensitizer selected for a possible clinical application.

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Direct detection of singlet oxygen generated by UVA irradiation in phospholipids, human cells and skin

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UVA light produces deleterious biological effects in which singlet oxygen plays a major role. These effects comprise a significant risk of carcinogenesis in the skin and the cataract formation of the eye lens. Singlet oxygen is generated by UVA light absorption in endogenous molecules present in the cells. To elucidate the primary processes and sources of singlet oxygen in tissue, it is a major goal to uncover the hidden process of singlet oxygen generation, in particular in living tissue.Singlet oxygen can be directly detected by its luminescence at 1269 nm. When exposing keratinocytes, HT29 cells, pig skin (ex vivo) or human skin (in vivo) to UVA laser light (355 nm, 6 J/cm^2), we measured a clear luminescence signal of singlet oxygen. This is a positive and direct proof of singlet oxygen generation in cells and skin by UVA light. Moreover, when exposing pure phosphatidylcholine in an aqueous suspension to 355 nm laser light, singlet oxygen is clearly detected by its luminescence. This provides evidence that phosphatidylcholine can contribute to the generation of singlet oxygen when irradiated by UVA light. This is a very striking result in light of the oxidative damage and gene regulations in cells caused by singlet oxygen in vitro and in vivo. Additionally, to compare with UVA broadband experiments, the key cytokine IL-12 was measured and found up regulated to a similar extent when using our monochromatic UVA irradiation at 355 nm.

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Direct detection of oxygen depletion induced by singlet oxygen

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Singlet oxygen is a highly reactive oxygen species. It can readily react with biological molecules such as lipids and proteins leading to oxidative damage in cells. The direct detection of singlet oxygen can be performed by measuring time-resolved its luminescence. The determination of the lifetime of singlet oxygen can be used to detect the site of singlet oxygen generation and decay especially in living cells. However, the lifetime measured depends also critically on the local oxygen concentration, which can decrease in case oxygen is consumed due to oxidative reactions with lipid and proteins.In our study, singlet oxygen was generated by exciting a photosensitizer (TmPyP) and detected by its luminescence at 1269 nm. The photosensitizer (100 μM) was added to 3 ml aqueous solution containing 30 μM of bovine serum albumin. The results show that the luminescence signal changed its rise and decay time during irradiation. The longer the exposure to laser light the shorter the rise time and the longer the decay time. When measuring the oxygen concentration in the aqueous albumin solution during irradiation, the oxygen concentration rapidly decreased with irradiation time (oxygen depletion). The extent and time course of oxygen depletion in aqueous albumin solution depends on the amount of light energy as well as on the concentration of albumin and photosensitizer. The time span to achieve maximal oxygen depletion in our experimental setup varied from a few seconds to a few minutes. After irradiation, the albumin was investigated by chromatography (HPLC), which showed a clear alteration of albumin caused by oxidation. Thus, one must be careful when evaluating the course of singlet oxygen luminescence in case oxygen is consumed. Moreover, the luminescence signal of singlet oxygen acts as a sensor for oxygen concentration in the solution and shows clearly oxygen depletion during irradiation, which is due to the oxidation of proteins by singlet oxygen. In clinical practice of photodynamic therapy and photodynamic inactivation of bacteria, oxygen depletion is a known effect, which can be handled by using breaks during irradiation.

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Ultraviolet-B irradiation induces vascular hyperpermeability in the skin of SKH1 mice

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The biologic effects of ultraviolet B radiation (UVB) on the skin are of particular interest because of the importance of UVB-induced injury in the process of photoaging and skin cancer and the role of UVB radiation in the therapy of skin diseases. The vascular endothelium is an important component of the inflammatory process and manifests striking changes during inflammation following UVB radiation. Recent data have highlighted the involvement of angiogenesis factors in the pathophysiology of acute photodamage. It was reported that a single exposure to UVB radiation caused an upregulation of the proangiogenic protein vascular endothelial growth factor (VEGF)-A in human and murine skin. To investigate the vascular permeabilizing effect of UVB radiation, back skin of hairless SKH1 mice was exposed to graded doses of a single UVB irradiation, ranging from 50 to 120 mJ/cm^2. Twenty-four hours thereafter Evans Blue dye (EBD) was injected into the retroorbital venous plexus of irradiated and non-irradiated control mice. Thirty minutes later, leakage of protein-bound dye was detected as bluish colouration in the skin that was exposed to UVB radiation. Spectrophotometric quantitation of the extravasated dye showed a 2.6-fold increase in the dye concentration in mice irradiated with 120 mJ/cm^2 (65.3 ± 6.2 ng EBD/mg tissue, mean ± SD.), when compared to non-irradiated control mice (25.3 ± 2.3 ng EBD/mg tissue, mean ± SD). The alterations in vascular permeability were dependent on the UVB-dose. We observed an increase in the amount of exuded dye when the exposure was raised from 90 mJ/cm^2 to 120 mJ/cm^2 and only one mouse reacted with a very feeble response to UVB irradiation with 50 mJ/cm^2. Histological analysis of back skin stained with the pan-endothlial cell marker CD31 revealed a marked induction of vascular remodelling in UV-irradiated back skin, when compared to skin of non-irradiated control mice. These histological findings raise the possibility that a proangiogenic environment could, at least in part, provide an explanation for the observed increased vascular leakage upon UVB exposure.

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Chk1 kinase executes ATR-signalling for premature senescence of psoralen plus UVA-treated human fibroblasts

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PUVA (psoralen plus UVA) is widely used for the treatment of hyperproliferative and inflammatory skin diseases. Accelerated aging of irradiated skin is a common side effect of long-term PUVA therapy. Cellular senescence, the loss of proliferative capacity of a single cell, is nowadays regarded as a persistent DNA damage response and is likely linked with aging. We could previously show that DNA interstrand crosslink (ICL) formation by photoactivated psoralens causes a senescent phenotype of human dermal fibroblasts that is mediated by ATR kinase signalling from telomere-localized DNA-damage foci. ATR transduces the damage signal by phosphorylation of effector proteins. Now, we could identify Chk1 kinase as an effector downstream of ATR in PUVA-induced senescence. The kinase Chk1 is a substrate of ATR that is activated by phosphorylation. Chk1 itself then mediates cell cycle arrest by inhibitory phosphorylation of Cdc25-phosphatases which are key regulators of cell cycle progression. We find that Chk1 phosphorylation in response to PUVA persists from 2 h until at least 28 days after irradiation. Monitoring of the subcellular distribution of Chk1 by immunofluorescence analysis initially reveals a diffuse localisation pattern which is then followed by an accumulation of Chk1 at DNA-damage foci, as determined at day 12 after PUVA. Inhibition of Chk1 function by siRNA prior to psoralen photoactivation resulted in increased cell death. Depletion of Chk1 in already PUVA-senesced fibroblasts led to restart of DNA-synthesis in 17% of the cells, as determined by increased BrdU-incorporation. In summary, we have identified Chk1 as an effector kinase downstream of ATR in the PUVA-induced DNA damage response that mediates the senescent phenotype of human fibroblasts after psoralen photoactivation.

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Variable pulsed light (VPL) reduces treatment induced pain in patients undergoing photodynamic therapy for actinic keratosis

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Background: Photodynamic therapy (PDT) is a well documented and established treatment alternative for epithelial skin cancer like basal cell carcinoma and actinic keratoses (AK). PDT induces selective tissue necrosis that occurs upon illumination with red light; this based upon the induction of reactive oxygen species upon activation of synthesized porphyrins. Unfortunately, free nerve endings are co-stimulated during this process thus inducing pain which sometimes leads to treatment interruption. The purpose of this study was to investigate a modification of the illumination process using a variable pulse light source (VPL(TM), Energist Ultra, Energist, U.K.) with spectral characteristics matching the absorption spectrum of the photosensitizer.
Method: A randomized parallel-group trial was conducted. A total of 25 patients (eight female, 17 male, mean age 73 years) were included suffering from actinic keratoses (AK) on the skin and the scalp. Methyl aminolevulinate (MAL, Metvix, Galderma, France) was applied on the targeted area for 3 h, subsequently one side received an illumination with a LED light source (37 J/cm^2, duration 12 min), and the contra lateral side received 80 J/cm^2 (double pulsed at 40 J/cm^2) with VPL (TM), with a pulse train of 15 impulses each with duration of 5 ms utilising a 610–950 nm filtered handpiece. Therapeutic outcome was evaluated using a lesion score after 2 weeks and 3 months; pain assessment was made after each treatment side with the use of a visual analogue scale (VAS).
Results: At beginning of the treatment patients showed an overall of 238 AK on face and scalp. After 2 weeks and 3 months, there was no significant difference between the therapeutic outcomes using the different illumination systems. However, pain assessment immediately after PDT revealed a significant lower pain level (4.3 vs 6.4) for the VPL (TM) treated side.
Conclusions: The use of short pulsed light (variable pulsed light) is thus an efficient and useful alternative in the photodynamic treatment of AK where otherwise pain development can be a limiting factor for the performance of PDT.

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Selection against large scale mtDNA deletions occurs on the cellular level in normal and CS cells circumventing apoptotic pathways.

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Mutations of mitochondrial DNA are involved in pathological processes such as cancer and aging. One of the best described mutations in the mitochondrial genome is the common deletion, a 4977 bp large deletion which can be induced in vitro by UVA irradiation. In normal fibroblasts and in fibroblasts from patients suffering from Cockayne syndrome (CS) a rare autosomal recessive disease with progeroid symptoms and sun sensitivity these mutations have been shown to increase up to a maximum followed by their disappearance whereas in vivo these mutations persist for up to 1.5 years. In order to determine the mechanism by which these mutations disappear, we induced mitochondrial common deletion in healthy fibroblasts and fibroblasts of CS patients by repetitive UVA radiation at a dose of 8 J/m^2. and measured the common deletion by real time PCR in viable adherent as well as in non-viable cells in the supernatant. In both cell lines the common deletion could be induced by UVA irradiation up to a maximum and followed by subsequent disappearance in adherent viable cells. Disappearance of the common deletion in viable cells from normal and CS individuals closely correlated with an increase of the common deletion in non-viable cells in the supernatant. This shift from deletion rich viable cells to deletion rich non-viable cells was not mediated by apoptosis as measured by FACS analysis of annexin V. These data indicate that reduction of possibly metabolically relevant large scale deletions reaching pathological levels are controlled on the cellular level mainly in an apoptotic independent way.

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Chemokine and chemokine receptor profiles in keratinocytes, fibroblasts and melanocytic cells after UVB irradiation

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Chemokines and chemokine receptors are key determinants of immune responses, thus contributing to skin tumorigenesis. Because changes in expression levels after UV irradiation are unknown, we investigated on the expression profiles in human fibroblasts, keratinocytes, melanocytes and two melanoma cell lines (451Lu, SBcl2). Irradiation was performed with a Waldmann UV21 at an UVB dose of 12.5 or 25 mJ/cm^2. Semiquantitative real-time PCR was performed on a GeneAmp 5700 Sequence Detector. Expression of 18 chemokine receptors and seven ligands was determined in triplicate and measured before irradiation, after 10 min, 24, 48 and 72 h.UVB irradiation led to significant changes in expression levels of some receptors and their ligands. As exemplified, at 12,5 mJ/cm^2 CCR2, CCR4, CCR9, CXCR3 was downregulated in 451Lu, CXCR1 in melanocytes and CXCR4 in keratinocytes. Chemokine receptors and ligands, which were upregulated: CCR3 in 451Lu, CXCR1 at 25 mJ/cm^2 in fibroblasts, and CCR3, CCR5, CCR9, CCL25 and CXCL13 at 12,5 mJ/cm^2 in keratinocytes. We also observed de novo synthesis, like CCR10 and CXCL12 in keratinocytes, CXCR4 in melanocytes, CCL25 and CCL20 in fibroblasts. Comparisons of dose-response-relationships revealed huge differences in expression levels within the five cell lines. Importantly, alterations in ligand-receptor pairs for paracrine stimulation, like CXCL12-CXCR4 expression in keratinocytes and melanocytes, respectively, were determined.This study shows that UVB is a strong inducer and modulator of chemokine expression, with direct etiopathogenic implications.

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Epigenetic silencing of the PTEN gene in melanoma

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Phosphatase and tensin homologue deleted from chromosome 10 (PTEN) seems to be an important tumor suppressor gene in melanoma.

As it is only infrequently deleted or mutated and as the PTEN protein is low to absent in a significant number of melanomas, we investigated alternative methods of epigenetic silencing. We performed positional methylation analysis in corresponding sera and frozen as well as paraffin-fixed melanoma specimens in 21 patients by pyrosequencing and TaqMan® RT-PCR, respectively.We report significant PTEN methylation in sera of patients with melanoma and melanoma metastases. The percentage of methylation of a selected CpG island in blood showed a correlation with methylation levels in corresponding melanoma tissue. In corresponding frozen melanoma specimens and corresponding patients sera, we found a correlation of the rate of methylation with PTEN expression. In summary, epigenetic PTEN silencing seems to be a relevant mechanism of inactivating this tumor suppressor gene in melanoma patients that may promote melanoma development by de-repression of the AKT pathway.

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Molecular profiling of cutaneous melanoma and functional characterization of ASK/Dbf4 upregulation

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The molecular and genetic events that contribute to the development and progression of malignant melanoma are only partly understood. To identify novel determinants for tumor development and progression, we performed oligonucleotide microarray-based comparison of gene expression profiles of a series of nevi ($n = 11$), primary cutaneous melanomas ($n = 10$), cutaneous melanoma metastases ($n = 11$) and melanoma cell lines ($n = 5$) with normal human melanocytes (NHM) as calibrator. Multi class significance analysis identified two novel genes, activator of S-phase kinase (ASK/Dbf4) and translocated promoter region (Tpr) that were confirmed in 51 additional samples by quantitative real-time RT-PCR. We also show that a 4-gene signature of ASK/Dbf4 and Tpr in combination with the established markers melanoma cell adhesion molecule (MCAM/MUC18) and hepatocyte growth factor receptor (c-MET) can distinguish benign nevi from malignant melanoma. In addition, Western blot analysis detected strong ASK/Dbf4 staining in cutaneous melanoma specimens. In keeping with its expected role as a cyclin-like regulatory subunit of mammalian Cdc7, our data suggest that upregulated ASK binds to human Cdc7 to form Cdc7-ASK/DBF4 complexes in several analysed melanoma cell lines. Further, we demonstrate that ASK has an essential function in cell growth by showing that siRNA-mediated depletion of ASK retarded cell survival and proliferation. In summary, we suggest that upregulation of ASK/Dbf4 is a novel molecular determinant conferring a proliferative advantage in cutaneous melanoma.

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UVB irradiation enhances melanoma cell motility via induction of autocrine IL-8 secretion

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Ultraviolet radiation (UVR) is known to be involved in the initiation and progression of malignant melanoma. Many studies have focused on the initiation of melanoma, but less is known about the effect of UVR on established tumor cells. Here we show that after ultraviolet-B (UVB) irradiation, melanoma cells (MM) are able to secrete autocrine factors that enhance their motility. Time-lapse videomicroscopy of UVB irradiated (15 or 30 mJ/scm) MM showed an initial decrease in MM cell motility 1 h after irradiation, with subsequent increase 24 h after UV-B treatment. Conditioned media harvested from MM 24 h following UV-B irradiation specifically enhanced the motility of un-irradiated MM, suggesting that a newly synthesized soluble factor released by UVB MM is involved. Since interleukin 8 (IL-8) is known to be upregulated by different cell types after UV-B irradiation, we investigated IL-8 expression after UVB exposure. Quantitative rt-PCR and ELISA demonstrated an induction of IL-8 in MM by UVB (15 or 30 mJ/scm) and addition of recombinant IL-8 to cell cultures enhanced cell motility to a similar degree than UVB. Importantly, blocking IL-8 activity by a neutralizing anti IL-8 antibody inhibited the up-regulation of MM motility after UVB treatment. We conclude that UVB enhances MM motility and that this effect is mediated at least in part by IL-8 released by MM in an autocrine fashion. Our findings are consistent with the hypothesis that UVB is not only involved in the initiation of melanoma, but may also be important for some aspects of tumor progression.

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The CCR5Δ32 gene polymorphism is a predictor of survival in melanoma patients receiving immunotherapy

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Chemokines are supposed to influence both tumor progression and anti-tumoral immune responses. In this regard, a 32-bp-deletion polymorphism in the chemokine receptor 5 gene (CCR5Δ32) resulting in a non-functional receptor protein allows to study the potential impact of the CCR5 system on the clinical course and treatment outcome of malignant diseases. CCR5 genotyping was performed by PCR on DNA extracted from serum samples of 782 cutaneous melanoma patients with known disease history and long-term clinical follow-up. Genotypes were correlated with patient survival in consideration of different therapeutic modalities. Out of 782 patients, 90 (11.5%) were heterozygous and 12 (1.5%) were homozygous for CCR5Δ32. Disease-specific survival starting from primary diagnosis was not influenced by CCR5 status. Equally, no impact of the CCR5 status could be detected on treatment outcomes of stage III patients. In 139 stage IV patients treated with immunotherapeutics, however, CCR5Δ32 was associated with a diminished survival compared to patients not carrying the Δ32 allele ($P = 0.029$). Multivariate analysis revealed the CCR5 genotype as an independent factor impacting disease-specific survival in this patient group ($P = 0.0084$), followed by gender ($P = 0.017$), and type of primary ($P = 0.029$). Vice versa, stage IV patients without immunotherapy revealed a trend towards a favorable survival when harboring CCR5Δ32 ($P = 0.12$). The disadvantage of CCR5Δ32-bearing stage IV melanoma patients with respect to outcome of immunotherapy indicates a strong impact of the CCR5 mediator system on anti-tumoral immune responses, and should be taken into account when choosing therapeutic modalities for these patients.

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Endothelium-specific anti-angiogenic tumor therapy by a gene-directed enzyme prodrug strategy

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The growth of tumors beyond a minimal size requires the formation of new blood vessels to ensure the supply of the tumor tissue with oxygen and nutrients. Anti-angiogenic strategies are therefore promising therapeutical approaches to prevent solid tumor growth.The vascular endothelial growth factor (VEGF)/VEGF-receptor system is crucially involved in the process of tumor angiogenesis. The expression of endothelial receptor tyrosine kinase VEGFR-2 is downregulated in most adult vascular beds, but is strongly and specifically upregulated in the tumor endothelium. Therefore, gene regulatory elements of the VEGFR-2 gene are ideally suited to deliver therapeutic genes specifically to the tumor vasculature, minimizing the risk of systemic side effects. In transgenic mice expressing the LacZ reporter gene under the control of distinct promoter/enhancer sequences of the murine VEGFR-2 (Flk1) gene, we observed a strong reporter gene expression specifically in the tumor endothelium in different experimental tumors. These sequences were used to develop novel approaches for an endothelium-specific anti-angiogenic tumor therapy. One approach involves the gene-directed enzyme prodrug therapy (GDEPT), in which the transgenes used encode enzymes that activate specific non-toxic prodrugs to cytotoxic products. We generated transgenic mice, expressing the potent chimeric suicide gene yeast super-cytosine deaminase (super-yCD) under the control of the Flk1 promoter/enhancer sequences (Flk1/super-yCD transgene). Conversion of non-toxic 5′-Fluorocytosine to cytotoxic 5′-Fluorouracil by super-yCD will lead to cell death of super-yCD expressing endothelial cells and, due to the robust bystander effect, also of neighbouring tumor cells. Endothelial expression and functionality of the Flk1/super-yCD transgene was proven in vitro. Super-yCD expression in vivo was verified by RT-PCR of transgenic embryos and subcutaneous tumors induced in adult transgenic mice. Experimental tumor growth will be analysed in Flk1/super-yCD transgenic mice after 5′-Fluorocytosine treatment. The generated transgenic mice represent a powerful tool to investigate the efficacy of endothelium-specific gene-directed prodrug strategies for tumor therapy in vivo.

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Dendritic cell immunization to induce tissue-targeting of cytotoxic T cells for melanoma defence

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We have recently shown that different routes of 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. In our current study, we have begun to investigate the role of homing receptor polarization by DC vaccination for anti-tumor immunity in the mouse model of B16.F10 melanoma given subcutaneously 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 (LCMV).We examined the role of DC immunization routes 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 i.c., i.v. or i.p. route. After s.c. 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 immunisation 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 DC are injected i.v. or i.p., respectively, allow efficient tumor defence in this setting.Currently, we analyse 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. Furthermore, we examine the reprogramming ability of effector T cells following in vitro imprinting of a gut homing phenotype and adoptive transfer into recipient B6 mice, previously injected subcutaneously with both tumor cell lines. P14 T cells in vitro primed with pulsed splenocytes are used as negative control. In both settings the ability of tumor growth is measured. We also investigate the role of the DC vaccination route for defence against established tumors. Our findings are relevant for the biological therapy of human cancers.

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cFLIP regulates death receptor sensitivity to TRAIL- or CD95L-mediated apoptosis in human melanoma cells

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The prognosis of metastasized melanoma is poor and novel treatment modalities are urgently needed. Induction of apoptosis by death ligands like TRAIL or CD95L could represent an attractive therapeutic option. However, detailed knowledge about the regulation of death receptor sensitivity in melanoma is missing. In this report we have analysed those signalling pathways in detail. CD95 and in part TRAIL-R1, but not TRAIL-R2 surface expression, which was found in all cell lines examined, largely correlated with apoptosis sensitivity. In contrast, the expression of proteins of the apical apoptosis signalling cascade (FADD, initiator caspases 8/10 and their inhibitor cFLIP) did not predict apoptosis sensitivity. Since both TRAIL-R1 and -R2 are able to signal for apoptosis, we asked if cFLIP, highly expressed in several of the cell lines tested, is sufficient to maintain resistance to TRAIL-R2-mediated apoptosis. We thus transduced cFLIP-expressing IGR cells (a TRAIL-resistant melanoma line expressing only TRAIL-R2) with cFLIP-specific retroviral siRNA. Downregulation of cFLIP was associated with a dramatic

increase in TRAIL sensitivity as well as caspase-8 and -3 activation. Conversely, retroviral transduction of highly TRAIL-sensitive, only TRAIL-R2-expressing EP melanoma cells with different cFLIP isoforms resulted in inhibition of TRAIL- and CD95L-mediated apoptosis as well as blockade of caspase-8 and -3 activation.We thus functionally demonstrate that downregulation of cFLIP is necessary for sensitization of TRAIL-R2-expressing melanoma cells, while cFLIP upregulation is sufficient for confer resistance to these death ligands. Moreover our data indicate that TRAIL-R1 negative tumor cells contain all proteins necessary for death receptor-mediated apoptosis and indicate that intracellular regulation of cFLIP expression may decide sensitivity or resistance to death ligands in melanoma. Therefore cFLIP may represent an attractive therapeutic target for melanoma treatments when combined with death receptor agonists.

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Selectively targeting IKKbeta by the novel small-molecule inhibitor Bay 65-5811 enhances susceptibility of melanoma cells to antitumoral treatment

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Constitutive activation of the transcription factor NF-κB has been implicated in regulation of cell proliferation, apoptosis, angiogenesis, local invasion, metastasis and inducible chemoresistance to anthracyclins of various tumor types.We have studied Bay-65-5811, a novel highly selective (*Ki*-value = 2 nM) small-molecule inhibitor of I-κB kinase-β (IKK-β). It prevents phosphorylation and degradation of I-κB, thereby diminishing activation and nuclear translocation of NF-κB. Suppression of NF-κB activity by Bay 65-5811 in melanoma cells resulted in downregulation of anti-apoptotic gene products, such as inhibitors of apoptosis proteins (IAPs) including XIAP and Apollon. Furthermore, when NF-κB-regulated gene products involved in proliferation and metastasis were studied, Bay 65-5811 significantly repressed expression of cyclin D1, MMP-9, MCP-1, COX-2 and others. Of note, inhibition of NF-κB activation through Bay 65-5811 alone did not affect proliferation or apoptosis in melanoma cells. However, when Bay 65-5811 was combined with doxorubicin at low concentrations, significant synergistic inhibition of proliferation, as assesed by MTS-assays, and increased apoptosis, as shown by DNA-fragmentation, was observed.When pulmonary metastasis in C57BL6 mice intravenously injected with B16Bl6 melanoma cells was studied, Bay 65-5811 alone at doses as high as 10 mg/kg had no therapeutic effect. Doxorubicin was not efficacious at 1 mg/kg, and was toxic at 4 mg/kg. Interestingly, a combination of low-dose Bay 65-5811 (3 mg/kg) and low-dose doxorubicin (1 mg/kg) significantly diminished the development of pulmonary metastases as compared to either treatment alone ($P < 0.01$). Manifest metastases also showed significant responses to the combinatorial treatment as determined by proliferative index and size of metastases ($P < 0.001$ in either case as compared to monotherapy). Moreover, given that cultures re-established from pulmonary metastases of treated mice showed similar response to cytostatic treatment as compared to the parent cells, Bay 65-5811 did not induce chemoresistance.Thus, the novel therapeutic principle of selectively inhibiting IKKβ may enhance the efficacy of antitumoral therapies and prevent NF-κB-related chemoresistance.

P185

Selectin-dependent interactions of melanoma cells with endothelial cells and their antibody- or small-molecule-mediated modulation

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Metastasis of malignant tumors is pivotally influenced by adhesive interactions of tumor cells with vascular endothelial cells. Although endothelial adhesion molecules of the selectin family have been implicated previously in tumor metastasis, the respective data are not conclusive.We have shown here that some human and murine melanoma cells induced marked expression of E-selectin on cultured endothelial cells. This selectin expression could be achieved through direct cellular contact or through conditioned media from melanoma cells, implying that some melanoma cells released soluble factors which were responsible for selectin induction on endothelial cells.In vivo, pulmonary metastasis of melanoma cells was tested under conditions of selectin inhibition. Murine B16BL6 melanoma cells were injected intravenously into C57BL6 mice. The number of pulmonary metastases and lung masses were determined after 3 weeks. After treatment with function-blocking antibodies directed against E-selectin, P- selectin or both, metastasis was significantly reduced by 50% to more than 80% when the mice received the function-blocking antibodies prior to melanoma cell injection ($n = 7$/group; $P < 0.05$). In contrast, selectin blockade after tumor cell dissemination did not result in diminished metastasis. These findings were corroborated by P-selectin-deficient mice which showed significantly diminished melanoma metastasis as compared to wildtype mice ($n = 7$/group; $P < 0.05$).In another series of experiments, selectin inhibition was performed using a small-molecule inhibitor of selectin functions (efomycine M) in different regimes: vehicle or efomycin M were injected intraperitoneally either before or after melanoma cell injection. We could show that the number of metastases in the efomycine-treated mice could be significantly diminished (by approximately 60%) as compared to the vehicle-treated mice when the compound was present before injection of the melanoma cells ($n = 5$/group; $P < 0.01$).Based on these results, we propose that blocking selectin functions may contribute to the reduction of tumor metastasis in some cases. However, the potential inclusion into therapeutic and/or preventive regimens remains to be determined.

P186

CLU expression in malignant melanoma

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Clusterin (CLU) is a glycoprotein that has been implicated in various cell functions involved in carcinogenesis and tumor progression. There are two known CLU protein isoforms generated in human cells. The nuclear form (nCLU) is proapoptotic, whereas the secretary form (sCLU) has pro-survival functions. CLU expression is modulated by many factors that are involved in carcinogenesis or that regulate tumor growth and/or apoptosis, including 1,25-dihydroxyvitamin D3 [1,25(OH)2D3] and ultraviolet radiation. First, CLU expression was

studied immunohistochemically in paraffin sections of primary cutaneous malignant melanomas ($n = 18$), metastases of malignant melanoma ($n = 25$), and acquired melanocytic nevi ($n = 30$). We also investigated CLU expression along with 1,25(OH)2D3, in vitamin D-sensitive (MeWo, SK-MEL-28) and -resistant melanoma cell lines (SK-MEL-5, -25), as well as in normal human melanocytes (NHM). In contrast to acquired melanocytic nevi, CLU immunoreactivity was detected in human primary cutaneous malignant melanomas (five cases out of 18) and metastases of malignant melanomas (3 cases out of 20) in situ. Both the CLU protein and the corresponding mRNA were detected in all melanoma cell lines and NHM investigated, with varying expression levels. Real-time RT-PCR showed that vitamin D-resistant human melanoma cell lines had a higher basal level of CLU expression (7.5 x 10-3 of normalized ratio) as compared to -sensitive human melanoma cell lines. NHM had an expression level similar to that of vitamin D-sensitive melanoma cell lines. In western blot analyses, only sCLU was detected in all melanoma cell lines and NHM analysed. This sCLU protein expression was comparable to that of the mRNA where vitamin D-resistant melanoma cell lines showed stronger bands compared to -sensitive melanoma cell lines. Following 1,25(OH)2D3 treatment, one out of the two vitamin D-sensitive human melanoma cell lines analysed (MeWo), showed an up-regulation in CLU mRNA expression after 96 h. In contrast, the vitamin D-resistant melanoma cell line investigated (SK-MEL-5) showed no regulation of CLU expression along with 1,25(OH)2D3 treatment. Our findings indicate that CLU may be of importance for the pathogenesis, progression and therapeutic outcome in these malignancies.

P187

Expression of nm23 homologues nm23 -H4, -H6, and -H7 in human melanoma cell lines in vitro

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Nm23 is a tumor metastasis suppressor gene that has eight different genes, named nm23-H1 to -H8. These genes are linked to suppression of tumor metastasis, differentiation, apoptosis, and proliferation. The nm23 proteins are multifunctional, and they can be divided into two groups. The first group includes nm23-H1 to -H4, and the second group includes nm23-H5 to -H8. Research has been done on the first group to examine the effects in various tumors, mainly nm23-H1 and -H2. Nm23 expression was found to be reduced with the progression of oral melanoma and it has been reported that the expression of nm23 in primary cutaneous melanoma was significantly associated with poor survival. Human malignant melanoma is a highly aggressive skin cancer with a high metastatic potential, compromising only 10% of all skin cancers. Very few studies have analysed the expression and function of nm23-H4, -H6, and -H7 in malignancies, and it was the aim of this study to characterize nm23-H4, -H6, and -H7 expression in malignant melanoma tissues and cell lines. We analysed immunohistochemically paraffin sections of primary cutaneous malignant melanomas ($n = 5$), metastases of malignant melanomas ($n = 5$) and acquired melanocytic nevi ($n = 5$) for nm23 expression. Also, we analysed the mRNA from the mentioned tissues and from various human melanoma and keratinocyte cell lines using real-time RT-PCR for the nm23 genes. There was no nm23 staining observed in the immunohistochemistry (IHC) in any of the tissues analysed. Real-time RT-PCR revealed low expression levels for nm23-H4, and -H6 in the tissues. However, there was a significant increase in nm23-H7 mRNA levels in the malignant melanoma tissues. The real-time RT-PCR results for the melanoma cell lines were corresponding to the IHC, with no significant differences between the cell lines.We conclude that nm23-H7 could be of importance for the pathogenesis of malignant melanoma. However further experiments are needed to confirm any more roles for nm23-H4, -H6, and -H7 genes in malignant melanoma.

P188

MMP-induction in the tumor stroma does not depend on CD147 expression in murine B16 melanoma

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Degradation of extracellular matrix (ECM) components and basement membranes by matrix metalloproteinases (MMPs) are regarded as essential mechanisms for angiogenesis, tumor growth, tumor invasion and subsequent metastasis. It was conclusively demonstrated that the cell surface glycoprotein CD147 on tumor cells mediates induction of MMPs by stromal cells in humans. However, for murine models such evidence remains elusive. To address the impact of CD147 on MMP expression in the murine B16 melanoma model, we consequently stably knocked down CD147 expression in two B16 sublines. The CD147 knock down remained stable under in vivo conditions as confirmed by immunohistochemistry. However, no differences in MMP-2, MMP-9 and MT1-MMP expression by stromal and tumor cells were detectable, in CD147+ and CD147- tumors. Since the tumor microenvironment is a complex system, involving several cell types, the extracellular matrix and plethora soluble factors, we subsequently studied the role of murine CD147 in vitro. Co-culture of melanoma cells with different fibroblast cell lines demonstrated that neither CD147+ nor CD147- B16 tumor cells altered the expression of MMP-2 or MMP-9 by the fibroblasts, although we could confirm the susceptibility of these fibroblasts for MMP induction. In summary, at least for the murine B16 melanoma model, CD147 expression on tumor cells seems not to be crucial for MMP-2, MMP-9 and MT1-MMP induction on tumor associated stromal cells.

P189 (V35)

Role of bone morphogenetic proteins in migration, invasion and angiogenesis of malignant melanoma

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Malignant melanoma cells are known to have altered expression of growth factors compared with normal human melanocytes. These changes favor tumor growth and progression and influence the tumor environment. The induction of transforming growth factor β1 (TGFβ1), TGFβ2, and TGFβ3 expression in malignant melanoma has been reported before. We could show that BMP4 and BMP7, members of the TGFβ superfamily are strongly expressed in melanoma cell lines, as well as in primary and metastatic melanomas, but not in nevi. Cell

clones with reduced BMP activity, generated by either overexpression of the BMP inhibitor chordin or stable antisense BMP4 transfection, displayed a strong reduction of migratory and invasive properties, suggesting that BMPs promote cell migration and invasion. Beside these autocrine effects, paracrine effects on the vascular network were analysed. BMP2 and BMP4, both induce endothelial cell migration in Boyden chamber models and induce tube formation on matrigel. Moreover, melanoma cell clones with impaired BMP activity attracted less endothelial cells in matrigel coated invasion chambers compared to the control melanoma cell clones. In vivo analyses of these melanoma cells clones in athymic nude mice revealed significantly smaller tumors derived from chordin over-expressing cell clones compared to the mock transfected control cells. Tumors from antisense BMP4 melanoma cell clones did not significantly differ in size from the controls, but further analyses displayed large apoptotic and necrotic areas, which are due to a reduced number of CD31 positive vessels in the antisense BMP4 tumors.In summary we revealed that BMPs are not only essential factors to enhance migration and invasion of melanoma cells but also have a strong and important influence on the tumor microenvironment. BMPs act as a chemo-attractant for endothelial cells migrating to the tumor site and are necessary factors to establish a vascular network for tumor nutrition.

P190

Comparative gene expression profiling: deep penetrating nevus vs. metastatic melanoma

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The deep penetrating nevus (DPN) is a rare variant of benign melanocytic nevus with clinical and histologic features that are mistaken for nodular malignant melanoma (NMM) in up to 40% of the cases. Here, we intend to clarify the infiltrative, but non-metastatic character of DPN and target on potentially differentiating diagnostic markers. Microdissections from 17 paraffin-embedded DPNs and 16 NMMs served as starting material. To overcome the problem of formalin-induced RNA degeneration, we used the Paradise® Reagent System which has been established for extraction and amplification of fragmented RNA from FFPE-samples. Samples with high RNA quality (DPN $n = 4$, NMM $n = 4$) were singly spotted on GeneChip® Human X3P Arrays. Out of all significantly regulated transcripts (180 genes, $P < 0.05$), we selected five differentially expressed candidate markers with particular relevance to melanoma biology: MITF, TIMP-2, BCL-2, TPD52 and ATM. According to the transcriptional regulations seen by microarrays, the upregulation of MITF, TIMP-2 and BCL-2 in DPNs and the upregulation of TPD52 and ATM in NMMs could be immunohistochemically confirmed (DPN $n = 17$, NMM $n = 16$). Regarding the subcellular immunolocalization of all markers, at the invasive tumor portions, NMMs mostly reached higher expression scores. However, when we additionally considered the tumor cores representing the main part of the tumors, MITF, TIMP-2 and BCL-2 expression of DPNs exceeded that of NMMs. ATM and TPD52 expression of NMMs a priori outreached that of DPNs in all regions. Particularly in case of MITF staining, we additionally observed an intracellular signal heterogeneity. We found a predominantly nuclear staining pattern at the tumor cores of DPNs and a nuclear plus cytoplasmic pattern at the invasive rims. NMMs mostly preferred a pure cytoplasmic pattern suggesting a different activity status of MITF. In contrast to all other markers, particularly ATM might serve as a melanoma discriminator because only ATM revealed a reliable staining consistency within all tumor samples. Since UV-induced induction of ATM has been recently suggested to be linked to onset of melanoma, the clear transcriptional and translational overexpression of ATM in NMMs might contribute to the differential metastatic potential of both entities.

P191 (V31)

Activation of the MAP kinase pathway induces apoptosis in Merkel cell carcinoma

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Merkel cell carcinoma (MCC) is a rare aggressive tumor of the skin. Recently we have shown that MCC cells in situ are characterized by a complete absence of MAPK pathway signalling accompanied by a high expression level of the Raf kinase inhibitor protein (RKIP), a pattern which is preserved in the MCC cell line UISO. Here we show that silencing of the MAPK pathway is essential for survival of MCC cells but that it is independent of RKIP since siRNA knock down of RKIP in UISO cells did not induce MAPK phosphorylation. However, by transducing a fusion protein consisting of a modified estrogen receptor and the c-Raf-1 kinase domain which is activated upon addition of 4 hydroxytamoxifen (OHT), signal transduction from Raf to MEK and ERK was induced in UISO cells. Activation of Raf led to morphological changes, loss of actin stress fibres and induction of apoptosis. The morphological changes as well as cell death could be prevented by the MEK inhibitor U0126, consequently these effects are mediated by signal transduction through MEK and ERK. Furthermore, MAPK activation, morphological changes and cell death could be induced by the c-Raf-1 activating compound ZM336372. Although activation of the MAPK pathway contributes to oncogenesis in many other cancers it seems to be a negative selection factor for MCC cells. These results provide new perspectives for potential therapeutics for this highly aggressive and difficult to treat tumor.

P192

A new technique unravels mRNAs coding for plasma membrane associated proteins in CTCL

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Cutaneous T cell lymphoma (CTCL) is characterized by clonally expanded, neoplastic CD4+ T cells. Antibody therapy and small inhibitory molecules are presently discussed as promising alternatives to conventional therapy. Thus, tumor-specific antigens preferentially located at the plasma membrane are needed, which may also serve as tumor markers. We used a new combination of detergent fractionation of cells and subtractive suppression hybridization (SSH) to gain mRNA coding for tumor-specific membrane-associated proteins. Fractionation of cells with digitonin allows sequestering mRNA of the rough endoplasmatic reticulum. This mRNA is supposed to code preferable for membrane-bound or secreted proteins and is assumed to be in the very process of translation. Fractionated mRNAs from the CTCL

cell line HuT78 and from normal peripheral blood monocytes were used for SSH in order to enrich tumor-specific sequences. We identified some 21 overexpressed genes, among them GPR137B, FAM62A, NOMO1, Hsp90, Slit-1, IBP-2, CLIF, IRAK, and Arc. For these and related genes (PAI-1, uPA), we accomplished RT-PCR to gain their expression pattern in CTCL specimen and control tissues. Most of the detected sequences are clearly related to cancer, but have not yet been shown to be associated with CTCL. Especially Slit-1, CLIF, TIC2 and Arc might be new targets for CTCL. Furthermore, the newly developed combination of digitonin treatment and SSH is a promising technique for the detection mRNAs coding for membrane-associated overexpressed proteins.

P193

UVB irradiation inhibits and UVA irradiation promotes melanoma cell growth in human skin reconstructs

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Background: Ultraviolet radiation (UV) is considered to be an important risk factor for the development of cutaneous melanoma. However, once melanoma is induced, it is not known whether UV has any further effects on tumor progression.
Objective: We investigated if and to what extent growth of melanoma cells in human skin reconstructs can be influenced by UVA or UVB radiation.
Methods: A total of 195 skin reconstructs with five different melanoma cell lines from three different tumor progression stages were irradiated with 10–20 J/cm^2 UVA or 10–100 mJ/cm^2 UVB up to four times. Histological analysis was performed after 7–20 days of cultivation.
Results: In all progression stages melanoma cells formed tumors in the epidermis with invasion in the dermis mimicking the natural growth patterns in vivo. UVA irradiation led to enhanced melanoma cell growth (up to 37%) when compared to the non-irradiated control group. On the contrary, UVB irradiation decreased the number (up to 50%) and size of tumors in the skin reconstructs. Invasion of the melanoma cells into the dermis was increased by UVA and decreased by UVB.
Conclusion: Both UVA and UVB had an influence on melanoma cell growth in skin reconstructs, but in a different way. Our results suggest that UVA can increase melanoma cell growth and invasion possibly by effects on the extracellular matrix, whereas UVB can have inhibitory effects on melanoma cells. This means that UV exposure of skin does not only play a role in initiation, but also in progression of melanoma.

P194

High degree of intra-tumoral heterogeneity of aberrant promoter hypermethylation in malignant melanoma

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Background: In malignant melanoma, aberrant promoter hypermethylation (APH) of tumor suppressor genes has a crucial pathogenetic role. The aim of our study was to investigate intra-tumoral coexistence and heterogeneity of APH of different genes.
Methods: We analysed the intra-tumoral distribution of APH of p16, Rb, DAPK, RASSF1A, and MGMT in 339 assays of 34 tumors (15 melanoma primaries, 19 metastases) by methylation specific PCR, correlation to histopathological architecture and RASSF1A expression.
Results: We detected APH of at least one gene in 76% of the tumors (58, 40, 33, 30, and 20% for p16, Rb, DAPK, RASSF1A, and MGMT, respectively). 65% of the cases exhibited an inhomogeneous APH pattern (48, 40, 33, 20, and 13%). Samples from the core of the tumors represented the APH state of the whole tumors more accurately than samples from the edges. Local intra-tumoral correlation between the APH state of p16 and Rb and of p16 and DAPK was found. Moreover, a correlation between epitheloid tumor cell type and RASSF1A and p16 APH was detected. Mitosis rate and female sex was correlated with APH state of RASSF1A. Histological results confirmed that APH state of RASSF1A led to an aberrant expression pattern in distinct sections.
Conclusions: This is the first report on the degree of intra-tumoral inhomogeneity of APH. The frequent heterogenous patterns suggest that APH occur late in tumorigenesis and support the hypothesis of clonal instability during progression of melanoma. Extrapolation from a limited topographical tumor region to the total tumor biological status seems to be non-recommendable.

P195

Search for molecular modulators of ß-catenin activity in malignant melanoma

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The Wnt/ß-catenin pathway is involved in various differentiation events during embryonic development and it participates in tumor formation when aberrantly activated. Molecular studies concentrating on colorectal cancer revealed the activating mutations of adenomatous polyposi coli, CTNNBI (ß-catenin), BTRC and lymphoid enhancing factor genes to be causal for development of this kind of cancer. However, such mutations are rarely found during the development of malignant melanoma. Because of this, we analysed the Wnt/ß-catenin signaling pathway and molecular modulators of ß-catenin activity in this type of skin cancer. The accumulation of ß-catenin in the cytoplasm of melanoma cells and lack of a nuclear localization was the first significant difference between colon carcinoma and malignant melanoma, we found in western blots and immunohistochemistry. Exogenous change of ß-catenin protein amounts or shifting of this molecule into the nucleus of malignant melanoma cell lines lead not to induction of TCF transcriptional activity analysed in TOPflash/FOPflash luciferase-reporter assays. Additionally, the co-transcriptional activity of ß-catenin via the transcription factors LEF/TCF comprising the upregulated expression of the classical ß-catenin target genes c-myc, cyclinD1 and MMP-7 (matrixmetalloproteinase-7) can not be found in malignant melanoma cell lines.Our recent data identified a new signaling pathway in malignant melanoma which challenges the meaningfulness of nuclear ß-catenin and emphasizes the importance of ß-catenin distribution in the cytoplasm and the phosphorylation of cytoplasmic protein. The target molecule of phosphorylated ß-catenin is the transcription factor NFkappaB and after activation of NFκB the expression of the cell-cell adhesion molecule N-cadherin is upregulated.In summary, the alternative pathway of ß-catenin signaling in malignant melanoma is dependent on cytoplasmic ß-catenin and

leads to activation of the transcription factor NFκB. The constitutive expression of N-cadherin, already described for melanoma development, is regulated through cytoplasmic ß-catenin and NFκB. The knowledge of new carcinogenic cytoplasmic events which are within the ß-catenin signalling pathway and the inhibition of involved cytoplasmic molecules is important for the development of potential therapeutic targets.

P196

Relapse of cutaneous B-cell lymphoma after treatment with rituximab: role of bcl-2

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Background: Rituximab has been established as an effective and safe therapy for cutaneous B-cell lymphoma (CBCL). Apart from antibody dependent cytotoxicity and complement mediated lysis, different signal transduction pathways regulate rituximab efficacy. The downregulation of survival pathways, i.e. the Raf/MEK/Erk- or the p38MAPK-cascade, has been suggested as one major intracellular signalling property of rituximab. Relapse, however, is frequently observed and remains an unsolved issue.
Materials and methods: In order to delineate possible factors being associated with recurrence, eleven biopsies from four CBCL patients were obtained at various time points of relapse during or after therapy with 375 mg rituximab per m^2 of body surface area. Specimen were analysed by immunohistochemistry for the expression of the target molecule CD20, as well as signal transduction molecules, i.e. proapoptotic raf kinase inhibitor protein (RKIP) and antiapoptotic bcl-2.
Results: No CD20-loss variants, i.e. the suggested main tumor escape mechanism to rituximab therapy, were observed in any specimen of relapsing CBCL. Notably, the expression of proapoptotic RKIP remained increased in these tumor samples indicating at least that one of the proposed antitumor effects of rituximab was still operational. However, relapsing CBCL exhibited a strong upregulation of the antiapoptotic molecule bcl-2 in comparison to pretherapeutic levels.
Conclusion: Our data suggest that upregulation of bcl-2 is supposed to play a central role for rituximab refractory CBCL. The balance between pro- and antiapoptotic factors may predict the clinical outcome of CBCL treated with rituximab.

P197

Coculture of dendritic cells and fibroblasts convert mature dendritic cells into immature dendritic cells and influences matrixmetalloproteinase-secretion in fibroblasts

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Tumor growth evolves through complex interactions of cellular and non-cellular components of the tumor microenvironment. Tumor cells and stroma cells (fibroblasts, vascular and immune cells like dendritic cells (DCs) and macrophages) interact through adhesion molecules, enzymes, cytokines and chemokines. Proteolytic enzymes like matrix metalloproteases secreted by tumor and stroma cells are known to play an important role in tumor invasion and migration of immune cells (i.e. DC). However little is known about interactions between stromal fibroblasts and DC.To address this notion, murine bone marrow derived DCs, bone marrow derived macrophages, fibroblasts and B78-D14 melanoma cells were analysed by zymography for MMP-2 and MMP-9 expression. Fibroblasts and DCs showed high levels of MMP-2 and MMP-9 secretion, whereas B78-D14 cells and macrophages secreted small amounts of MMP-2. Changing the maturation state of DC by cytokines (LPS, TNF, TGF) influenced their expression of MMP-2 and MMP-9.To investigate the influence of cell interactions between fibroblast and DC on MMP expression and one DC-function, respective cells were cocultured in vitro. After 24 h of coculture both MMPs increased dramatically. Not only direct cell-cell contact but culture supernatant of fibroblast induced remarkable MMP-2 and -9 expression. Macrophages exerted no obvious influence on MMP expression in the other cells.Analysis of the DCs after coculture showed a significant reduction of costimulatory molecules like MHC class II, CD80, CD86 in FACS analysis and in the MLR a reduced stimulatory capacity.In summary, our data demonstrate that DCs on one hand strongly affect secretion of MMP-2 and MMP-9 in fibroblast and vice versa possibly contributing to a modified environment facilitating tumor invasion and on the other hand are affected themselves in their maturation state after coculture.

P198

Lymphatic vessel architecture in A431 and A549 tumors

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High tumor interstitial fluid pressure (TIFP) is a characteristic of most solid tumors. Clinically, TIFP is described as a physical parameter that hampers the uptake of anti-tumor drugs into tumor tissue and thus lack of therapeutic effects. Furthermore, it is assumed that the rare incidence of lymphatics in solid tumors is one of the main reasons for enhanced TIFP. In the present study it was investigated if tumors with reduced TIFP values might have a more dense lymphatic network in contrast to tumors with higher TIFP values. A431 squamous epidermoid vulva carcinoma cells or A549 epidermoid lung carcinoma cells were s.c. inoculated into the back of anaesthetised NMRI nude mice. A431 cells show a high TIFP (over 10 mmHg) while tumors derived from A549 cells exhibit a lower TIFP (ca. 5 mmHg). Tumors are excised after 3 weeks. Tumor tissue sections are analysed using immunohistochemical techniques on paraffin-embedded or cryo-conserved tissue sections for the expression of lymph vessel markers (e.g. podoplanin). Furthermore, RT-PCR experiments are performed to investigate the expression of VEGF-A, VEGF-C and HIF-1α in both tumor entities.In conclusion, our experiments show that the lymphatic distribution between two epithelial tumor entities (A431 and A549) differ strongly. Intratumoral lymphatic vessels are solely found in A431 tumors. Immunohistochemical findings were corroborated on the molecular level. A431 tumors showed a strong HIF-1α and VEGF-A expression suggesting a strong lymphangiogenesis, whereas A549 tumors showed no expression of these factors.

P199

Anti-vascular endothelial growth factor antibody bevacizumab in conjunction with chemotherapy in metastasising melanoma

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Background: The combination of the antiangiogenic antibody bevacizumab with standard chemotherapy has improved the prognosis in patients with metastastic colorectal cancer and other advanced cancers. The role of combined anti-VEGF and chemotherapy in metastastic melanoma is just starting to be elucidated. Patients/methods:We tested this notion in three stage IV melanoma patients with advanced and therapy-refractory disease. Bevacizumab (5 mg/kg) was given 1 week before start of chemotherapy and subsequently the application was repeated every 2 weeks. For chemotherapy, the combination of cisplatin and carboplatin or fotemustine was given every 4 weeks.

Results: Two patients achieved objective regressions after three courses of combined therapy. One of them had multiple skin and lung metastases, the other patient was treated for a large inoperable metastastic mass in his left axilla and multiple cervical lymph node metastases. Both responders received the combination of cisplatin/carboplatin and bevacizumab. The third patient, albeit progressing, demonstrated a pronounced liquid necrosis in bulky lymphnode metastasis. No bevacizumab related toxicity was observed. However, severe haematotoxicity occurred in one patient receiving cisplatin/carboplatin.

Conclusion: Herein, we report our rather encouraging, albeit preliminary experience with the combination of bevacizumab and chemotherapy in three patients. The two remissions observed clearly demand the need for larger studies.

P200

Dimethylfumarate impairs melanoma growth and metastasis

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Dimethylfumarate (DMF) inhibits signals transmitted by Rel proteins and is used for the treatment of inflammatory skin diseases such as psoriasis, but potential effects of DMF on tumor progression have yet not been analysed. We show that DMF reduced melanoma growth and metastasis in SCID mouse models. To identify targets of DMF action, we analysed mRNA expression in DMF-treated melanomas by gene chip arrays. Using BiblioSphere® software for data analysis, significantly regulated genes were mapped to Gene Ontology terms cell death, cell growth and cell cycle. Indeed, we found that DMF inhibited proliferation of human melanoma cells A375 and M24met in vitro. The cell cycle was arrested at the G2/M boundary. Moreover, DMF was pro-apoptotic, as shown by cell cycle analysis, annexin V and Apo2.7 staining. These results were confirmed in vivo. DMF reduced proliferation rates of tumor cells as assessed by Ki-67 immunostaining and increased apoptosis as assessed by TUNEL staining. In conclusion, DMF is anti-proliferative and pro-apoptotic and reduces melanoma growth and metastasis in animal models.

P201

Regulatory T cells within the inflammatory infiltrate of squamous cell carcinoma of the skin in immunocompetent patients versus solid organ transplant recipients

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Regulatory T cells play an important role in the control of the immune system. Next to autoimmune disorders, cancer and the physiological anti-tumor immune response may involve regulatory T cells. Squamous cell carcinoma of the skin is a skin tumor frequently occurring in the general population with a more than 60-fold increased incidence in solid organ transplant recipients, a group of patients with profound drug-induced immunosuppression. Published data shows evidence for altered levels of regulatory T cells in peripheral blood of solid organ transplant recipients.We intended to study regulatory T cells within the local inflammatory infiltrate surrounding squamous cell carcinoma of the skin. RNA was extracted from fresh biopsy material and analysed by reverse transcription-real time PCR for expression of transcription factors TBET, FOXP3 and GATA3 as well as cytokine mRNA expression for IFN gamma, IL-10, TGF beta, IL-4 in nine squamous cell carcinomas of immunocompetent patients and nine of organ transplant recipients. CD4 and CD8 mRNA expression was compared to GAPDH housekeeping mRNA expression, while cytokine and transcription factor mRNA was compared to CD4 mRNA expression. We found CD4 mRNA expression upregulated and CD8 mRNA downregulated in organ transplant recipients' inflammatory infiltrate. TBET mRNA expression was decreased, while GATA3 mRNA was increased with FOXP3 mRNA decreased in organ transplant recipients. TGFbeta and IL-10 were downregulated in organ transplant recipients' inflammatory infiltrate compared to immunocompetent patients.In summary, the inflammatory infiltrate of squamous cell carcinoma of the skin in organ transplant recipients suggests a shift away from Th1 to Th2 profile with decreased activation of regulatory T cells as compared to immunocompetent patients. These findings contribute to the observed phenomenon of increased carcinogenesis and metastasis of squamous cell carcinoma of the skin in the high-risk population of solid organ transplant recipients.

P202 (V06)

RAGE drives tumor development by sustaining inflammation

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Activation of the receptor for advanced glycation end products (RAGE), a multiligand receptor of the immunoglobulin superfamily, has been shown to be a key event in inflammatory conditions and studies with RAGE-deficient mice have confirmed its role in

functional innate immune responses and chronic inflammation.Recently, we found differential expression of the putative RAGE ligands S100A8 and S100A9 in a chemically induced tumor model of mouse back skin. We could demonstrate that S100A8 and S100A9 co-expression induces AP-1 and NF-κB-dependent gene transcription in epithelial cells accompanied by increased phosphorylation of JNK and Jun proteins. Moreover, S100A8/A9 induced the expression levels of MMP-2 and MMP-9, two well-known AP-1 target genes. Given the close association between chronic inflammation and epithelial cancer, we propose that RAGE activation by S100A8/A9 initiates a time-controlled feed-forward loop leading to sustained NF-κB and AP-1 activation and could thereby convert a transient pro-inflammatory response into sustained cellular activation, which over time promotes tumor development. Indeed, analysis of skin carcinogenesis using the classical two-stage DMBA/TPA protocol revealed impaired tumor development in mice deficient for RAGE. In the early stages of TPA promotion RAGE-/- mice were characterized by diminished keratinocyte hyperproliferation and dermal inflammation. Additionally, expression of RAGE ligands was significantly reduced in repeatedly TPA-treated skin indicating that this type of chronic skin inflammation is controlled by RAGE-mediated tissue activation.These data provide the first in vivo evidence that RAGE signaling represents a novel tumor-promoting axis in multistage carcinogenesis. Strategies that specifically block RAGE activation by targeting the S100 activity may be useful in cancer treatment and prevention in order to target both the inflammatory and the tumor component.

P203

Human papillomavirus type 26 (HPV26) is a high-risk type causing invasive digital squamous cell carcinoma (SCC)

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Persistent infection with oncogenic HPV is necessary for the development of cervical and a subset of other ano-genital cancers, whereas a role for HPV in keratinocyte skin cancer is unclear. As an exception, SCC of the nail unit has been causally related to infection with high-risk mucosal types, predominantly HPV16 and HPV31. In addition, there is limited evidence for a possible role of the rare type HPV26 in causing periungual SCC. Here we identified by broad-spectrum PCR followed by molecular DNA typing HPV26 in multiple pre-invasive and invasive digital SCC obtained from an HIV-positive patient. In addition, HPV 26 was identified from high-grade anal intraepithelial neoplasias. To obtain further evidence for a causal role in digital tumor progression, viral DNA was analysed quantitatively. A very high copy number of viral genomes per cell was detected by semi-quantitative PCR, strongly supporting a causal role of HPV26 in tumor development. Expression of the viral oncogenes was not detectable due to RNA degradation in the available tumor tissues. To determine if E6 oncogene nucleotide changes account for the oncogenic potential of genomes isolated from invasive SCC, the E6 ORF was sequenced and compared to E6 from preinvasive proliferations. No missense or synonymous nucleotide exchanges were observed for E6, when comparing DNA isolated from premalignant or malignant tumors, suggesting that co-factors in addition to persistent HPV26 infection are required for tumor progression. We propose to classify HPV26 as a high-risk type that may cause malignant keratinocyte progression to invasive SCC in immunosuppressed patients. Identification of HPV 26, besides known high-risk types HPV 16, 31, 33 or 35, may identify patients at risk for developing SCC of the nailbed, and possibly at other locations.

P204

Quantitative imaging of tumor angiogenesis by 125I-Gluco RGD in endogenous tumors

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Tumor growth beyond a certain seize depends on the formation of new blood vessels. Quantification of tumor angiogenesis by immunohistochemistry of structurally abnormal tumor vessels ex vivo is difficult. Thus, most studies measure changes in tumor size but not the alteration of the tumor vasculature. We used the well characterized RIP1-Tag2 mouse model to investigate the role of angiogenesis in tumor progression. In RIP1-Tag2 mice, the oncogene T antigene (Tag) is expressed in all insulin-producing beta cells. At around 4 weeks hyperplastic islets appear, leading to the development of adenomas and finally carcinomas. To monitor tumor growth we measured blood glucose level twice weekly. 7-weeks old RIP1-Tag2 mice show decreased blood glucose levels compared to C3H wildtype mice. As tumor growth progresses, blood glucose levels fall rapidly, leading to the death of RIP1-Tag2 mice at an age of 14–15 weeks. Although blood glucose measurement is a convenient tool to follow tumor progression non-invasively it is only an indirect method that does not reflect the exact tumor size. The integrin $\alpha v\beta 3$ is exclusively expressed on neovasculature but not on quiescent endothelial cells. Several studies showed, that Arg-Glyc-Asp (RGD) binds selectively $\alpha v\beta 3$. Here we studied the correlation of 125I-Gluco-RGD uptake and integrin expression in RIP1-Tag2 tumors. 3 h after intravenous injection of 125I-Gluco-RGD mice were sacrificed, pancreata isolated, snap frozen and 20 μm sections were taken. Digital storage film autoradiography of 7-weeks old RIP-Tag mice showed small areas of 125I-Gluco-RGD uptake corresponding to small islet cell tumors. 5 weeks later tumors developed multiple lesions with focal accumulation. Quantification of the uptake in 9–12-weeks old RIP1-Tag2 mice showed significantly higher ($P < 0.01$ Student's T test) accumulation (ratio tumor/normal pancreas 6.41) than in C3H wildtype mice (1.61). At an age of 14–15 weeks the ratio of pancreas to normal pancreas tissue was 8.82. In contrast, C3H wildtype mice showed a ratio of 2.37 ($P < 0.01$ Student's T test). Thus, assessing $\alpha v\beta 3$ integrin expression in tumors allows quantitative analysis of tumor angiogenesis. Our results may present a reliable marker for monitoring angiogenesis.

P205

Expression of cyclooxygenase-2 (COX-2) and peroxisome proliferator-activated receptor gamma (PPARγ) in skin tumors

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Background: To investigate whether protein expression of cyclooxygenase-2 (COX-2) and peroxisome proliferator-activated receptor gamma (PPARγ) is associated with clinico-pathologic characteristics in benign and malignant skin tumors.
Methods: Two tissue microarrays (TMAs) were used to analyse COX-2, PPARγ, TP53, and the Ki67 labeling index immunohistochemically. TMA-1 ($n = 323$) consisted of malignant melanomas, nevi, squamous cell carcinomas, basal cell carcinomas, Kaposi sarcomas, histiocytomas, capillary hemangiomas, and sebaceous adenomas. TMA-2 consisted of 88 malignant melanomas with follow-up data, 101 melanoma metastases and 161 benign nevi. Cytoplasmic COX-2 and nuclear PPARγ expression was scored semiquantitatively (0–3+).
Results: Using TMA-1, COX-2 and PPARγ expression of any intensity was detected in 82% (152/186) and 33% (69/212) of any analysable skin neoplasm, respectively. As regards TMA-2, COX-2 immunoreactivity significantly increased from benign nevi (51%) to primary melanomas (86%) to melanoma metastases (91%; $P < 0.001$). In case of primary melanomas, positive COX-2 staining was associated with advanced Clark levels ($P = 0.004$) and shorter recurrence free survival ($P = 0.0271$). COX-2 expression was not associated with overall survival. Accordingly, PPARγ immunoreactivity was significantly increasing from benign nevi (0%) to malignant melanomas (22%) and melanoma metastases (33%; $P < 0.001$). However, PPARγ expression in melanomas was not associated with any of the clinico-pathologic characteristics, including overall survival and tumor recurrence.
Conclusions: Expression of the enzyme COX-2 and the transcription factor PPARγ is a common phenomenon during the metastatic process of malignant melanoma, representing possible targets for an anti-inflammatory and anti-proliferative therapy.

P206

Mechanisms of T Helper cell mediated tumor arrest

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Various data suggest that tumor-reactive T helper cells control tumor growth more efficiently than cytotoxic T cells. Yet, the underlying mechanisms still remain enigmatic. Here, we investigated the effects of tumor-reactive Th1 cells on syngeneic murine colon carcinoma cells(CT26),that stably expressed the human epithelial adhesion molecule EpCAM. We generated EpCAM-reactive Th1 cells and started adoptive Th1 cell therapy 4 days after injection of tumor cells. Tumors were grown subcutaneously and Th1 cells were given i.p.; the control group received PBS. To determine the sequential effects of the therapy, we harvested tumors on day 6, 7, 9 and 14 posttreatment (pt), and analysed tumor weight, histology, ultrastructure of tumor cells and vessels, and intra-tumoral gene-expression. Inhibition of tumor growth was only observed in Th1 cell treated mice; and growth difference between Th1- and PBS-treated mice started on day 9 pt. Inhibition was most prominent on day 14 pt, with a 60–84% reduction of tumor weight as compared to control tumors. Quantitative real-time PCR of Th1- and angiogenesis-associated candidate genes was performed at the different days. The therapy did not influence the expression of VEGF, but adhesion molecule (ICAM-1) on day 9 and cytokines. The therapy induced especially IFN-gamma and cytokines of the IL-12 family, IL12/p35, IL23/p19 IL27/Ebi3. All four cytokines peaked on day 9 and remained elevated until day 14. As these cytokines may exhibit anti-angiogenic properties, we explored the influence of Th1-therapy on tumor neovascularization. Histology showed no major differences in tumor vascularity or in tumor cell necrosis. In sharp contrast, electron microscopy revealed disrupted endothelia and defects of the basal lamina, suggesting that the tumor vessels were non-functional capillaries in Th1-treated mice. As, until now, we did not observe any direct effect of Th1 cells on the tumors, the data strongly suggest that tumor-reactive Th1 cells inhibit tumor growth primarily by damaging the endothelia of tumor vessels. It is unlikely that T helper cells recognize endothelia directly. Further investigation have to characterize the target cells of the Th1 cells and the source of the anti-angiogenic cytokines. These data underline a crucial role of tumor-reactive Th1 cells in the control of tumor growth.

P207

Role of let-7 family microRNAs in melanoma development and progression

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MicroRNAs (miRNAs) have recently been described as a class of non-coding RNAs negatively regulating gene expression by RNA degradation or inhibition of protein translation. Based on current knowledge, at least one third of all genes are targeted by a few hundred miRNAs, making miRNAs central gene regulators. miRNAs have been reported to interfere with many cellular processes including cell proliferation, differentiation, and apoptosis.In the presented report, a large-scale miRNA expression screen was performed analysing more than 150 different miRNAs in laser-microdissected benign melanocytic nevi ($n = 10$), primary malignant melanomas ($n = 10$), and melanoma metastases ($n = 10$), using quantitative real-time PCR. Among a series of differentially regulated miRNAs members of the let-7 family were analysed in more detail, since these target central molecules involved in oncogenic transformation and growth regulation such as N-Ras and c-Myc. In accordance with its known function as a tumor suppressor, let-7a was found to be down-regulated in primary melanomas compared to benign nevi. Further functional in vitro studies showed that melanoma cell transfection with let-7a reduced Ras protein expression as determined by Western blotting and immunofluorescence. Let-7a targeting of Ras in melanoma cells was further tested by in vitro luciferase assays using luciferase reporter gene constructs containing different versions of the 3' untranslated region of Ras linked to the luciferase gene. Co-transfection of let-7a reduced in vitro luciferase

activity of these constructs, supporting its role for Ras downregulation via binding to its 3' untranslated region. Transfection of melanoma cells with antagonistic let-7a antagonil had an opposite effect on protein expression and luciferase activity. Moreover, let-7a exerted inhibitory effects on melanoma cell growth in clonogenic assays. Taken together, miRNAs linked to melanoma development and progression were identified and were shown to be functional in melanoma cells, targeting important oncogenes such as Ras. miRNAs might not only help to delineate central pathways of melanoma tumorigenesis, but may also serve as future innovative treatment options.

P208

Tumor immune escape by the loss of homeostatic chemokine expression

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The novel keratinocyte-specific chemokine CCL27 plays a critical role in the organization of skin-associated immune responses by regulating T cell-homing under homeostatic and inflammatory conditions. Here we demonstrate that human keratinocyte-derived skin tumors may evade T cell-mediated anti-tumor immune responses by down-regulating the expression of CCL27 through the activation of EGFR-Ras-MAPK-signaling pathways. Imiquimod, used effectively in the treatment of several cutaneous malignancies, bypasses the loss of this skin-homing signal for T cells by up-regulating the expression of inflammatory chemokines, including CXCL9, CXCL10 and CXCL11. In vivo, neutralization of CCL27 decreases leukocyte recruitment to cutaneous tumor sites and significantly enhances primary tumor growth. Collectively, our data identify a novel mechanism of skin tumors to evade host anti-tumor immune responses.

P209

Endothelial adhesion receptor expression and T cell distribution in metastatic melanoma

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Metastatic malignant melanoma is a devastating disease with poor survival rates. The prognosis for patients with Stage IV metastatic melanoma remains dismal with an average 5-year survival rate of <5%. Although it is known that immunotherapy can augment tumor specific immune responses in melanoma patients, to date these strategies have not proven effective at improving patient survival and require revision. To kill tumor, antigen-specific cytotoxic lymphocytes (CTL) must effectively target and enter tumor tissue. Tissue specific trafficking of lymphocytes, in turn, depends on expression and interaction between specialized lymphocyte and endothelial adhesion receptors and ligands. The purpose of this study was to examine the pathway of leukocyte migration to melanoma metastases and to develop a melanoma model to study modulation of adhesion molecule expression on human tumor vasculature.Our results show that the majority of melanoma metastases examined do not express the vascular adhesion receptors E-selectin, P-selectin and ICAM-1 on vessels within the tumor boundaries. In contrast, strong adhesion receptor expression was observed on vessels at the tumor margins and within adjacent tissue. Adhesion receptor ligands expressed by circulating CTL in the peripheral blood of melanoma patients did not show enhanced expression of tissue-specific homing receptors, nor was there preferential recruitment of tumor-specific CTL to the tumor. These results suggest that the expression of leukocyte homing receptors on the vasculature of metastatic melanoma is dysregulated. This results in a block to recruitment of activated CTL to melanoma metastases and is a likely factor limiting the effectiveness of current immunotherapy protocols. To investigate whether induction of adhesion receptors on tumor vessels would enable T cell recruitment to melanoma and enhance response to immunotherapy we have established a xenograft model in which human melanoma is implanted into human skin transplanted on immunocompromised mice which are also populated with human CTL. This model will allow modulation of the vasculature of human melanoma to be studied directly in a human skin setting which will simplify the transfer of results into clinical applications.

P210

RelB expression in mycosis fungoides (MF) and Sézary syndrome (SS); a potential marker for disease progression

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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. Such stage transitions are accompanied with changes in transcription factor activities, which cause changes in oncogene and tumor suppressor gene expression. It has been recently reported that MF and SS cells contain constitutive NFkB transcription factor molecules, consist of the classical p50 and p65 heterodimers. We tested MF and SS cell lines and skin lesions for the presence of another NFkB protein, RelB, to see whether this molecule may also be involved in the pathogenesis of these diseases.The presence and DNA binding of the RelB protein in MF and SS cell lines was tested by transcription factor (TF) ELISA. Biopsies from MF and SS skin lesions were tested for RelB using a RelB specific antibody for immune histochemistry. Several stages of MF and SS were compared.The TF-ELISAs showed that DNA-binding RelB was present in the nuclear extracts of the analysed MF and SS cell lines. In the skin lesions we found that RelB was present in all tumor stage patients of MF (6/6) and SS (6/6). In non-tumor skin lesions of the MF stage IB we found RelB staining in 2/6 patients. Interestingly we found the transition from RelB- to RelB+ in two

different biopsies from one patient, which were taken in the IB stage. Thus RelB activation may occur before the transition to the tumor stage. This may also apply for SS where we found RelB in one of two patients in the non-tumor stage III. Therefore RelB may be a good marker for disease progression of MF and SS.

P211

The BH3-only member Noxa induces apoptosis in melanoma cells: involvement of ROS and ASK1-JNK/p38 signalling pathways

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The reasons for resistance of melanoma to apoptotic stimuli are currently unknown despite intact p53. This has prompted us to explore the BH3-only protein Noxa to overcome the obstacle of melanoma resistance in response to genotoxic agents. The ectopic expression of the Noxa gene promoted melanoma cell death as demonstrated by the generation of reactive oxygen species (ROS), cytochrome c release and PARP cleavage in Western blot analysis of several melanoma cell lines. (BLM, A375, MV3). In addition, Noxa induced apoptosis via signal-regulating kinase 1 (ASK1) and its downstream signalling pathways c-Jun N-terminal kinase (JNK) and p38. The inhibition of Noxa-induced ASK1, JNK and p38 activation by the treatment with the ROS scavenger, N-acetylcystine (NAC), confirmed the importance of Noxa-induced ROS generation for the promotion of apoptosis. Treatment of Noxa-transfected cells with the inhibitor of JNK (SP600125) or those of p38 (SB203580) inhibited the DNA-binding activity of both AP-1 and ATF-2, and reduced apoptosis, suggesting an additional role for ROS-induced activation of ASK-1-JNK/p38 pathways in Noxa-induced apoptosis.These data provide evidence for the efficiency of Noxa gene transfer as an alternative approach for melanoma therapy and delineate its mechanism.

P212

Influence of cyclosporin A on DNA repair

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As can be demonstrated in organ transplant patients the immunosuppressive drug cyclosporine A (CsA) leads to an increased risk of UV-induced skin cancer. Other immunosuppressive drugs like sirolimus seem not to enhance UV-induced carcinogenesis suggesting that this might be a CsA specific effect. So far, the mechanism remains unclear. An insufficient elimination of tumor cells by the impaired immune system is discussed as well as a direct effect on tumor progression.In the rare disease xeroderma pigmentosum defects in nucleotide excision repair (NER) lead to an >1000-fold increased skin cancer risk. Interestingly, there is evidence from the literature that CsA may influence DNA repair. CsA may contribute to UV-induced carcinogenesis by reducing the NER capacity.We investigated this hypothesis using a modified host-cell-reactivation (HCR) assay. Briefly, the UVC-irradiated firefly luciferase reporter gene plasmid pcmvLUC is transfected into host cells for 48 h. Luciferase activity expressed by these cells indicates the amount of completely repaired UV-damaged pcmvLUC, which reflects their NER capacity. For exclusion of unspecific e.g. toxic effects and as an internal control for normalisation, the renilla luciferase reporter gene plasmid pcmvRL is cotransfected. Using this modified HRC assay we observed a relative firefly luciferase expression of ~14% after transfection of 1000 J/m^2 UVC-irradiated pcmvLUC compared to unirradiated plasmid in peripheral blood lymphocytes (PBL), the lymphoblastoid cell lines AG10107 and GM03715, and the fibroblast cell line GM00637 ($13.5 \pm 0.7\%$, $14.2 \pm 0.1\%$, $15.5 \pm 1.6\%$, and $13.5 \pm 1.1\%$ respectively). These results agree with data recently published for NER-competent cell systems. Incubation of these cells with increasing concentrations of CsA for 48 h resulted in a decrease of firefly luciferase expression for both, irradiated and unirradiated pcmvLUC, of 50–70% for 10 μM CsA and >90% for 20 μM CsA, but also in a similar decrease of renilla luciferase expression indicating a toxic effect of CsA. However, in recent experiments using CsA concentrations below 5 μM we observed a decrease of firefly luciferase expression without a corresponding decrease of renilla luciferase expression.These preliminary findings are suggestive for a potential inhibition of NER by CsA, contributing to the molecular pathogenesis of enhanced UV-induced carcinogenesis in CsA-treated patients.

P213

Small fragments of hyaluronic acid (HA) induce metalloprotease- and cytokine upregulation in human melanoma cells- role of TLR4

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Hyaluronic Acid (HA) is a major component of the extracellular matrix (ECM). HA turnover occurs constantly but is enhanced in areas of inflammation. During inflammation, high molecular weight HA is degraded into small molecular fragments (sHA) by activated hyaluronidases or reactive oxygen species. These fragments have been shown to induce metalloproteases and cytokines in dendritic and endothelial cells. Additionally, sHA is probably associated with tumor invasiveness and metastasis. Therefore, we investigated whether sHA also induces metalloprotease and cytokine upregulation in human melanoma cells. We report here that exposure of the human melanoma cell line Bro to sHA leads to enhanced expression of matrix metalloproteinase (MMP)2 and IL-8, factors that can contribute to melanoma progression e.g. by inducing angiogenesis or spreading of tumor cells. We suggest Toll like receptor (TLR)4 to be involved in this signalling pathway, similar to effects shown in dendritic and endothelial cells. We detected TLR4 on the surface of melanoma cells in vivo and in vitro. Small HA fragments induce IL-8 in melanoma cells independently from the HA receptor CD44 since these effects were found also in the CD44 deficient cell line RPM-MC. To further confirm the role of TLR4 in sHA-signalling we used specific siRNA to selectively silence TLR4. Inhibition of TLR4 resulted in suppression of IL8 transcription and translation after sHA stimulation. In addition, sHA stimulation leads to an increase of detectable bioactive NFk-B, a key component of the TLR pathway in the melanoma cells. This further confirms our hypothesis of TLR4 to be involved in the sHA signalling pathway in human melanoma cells. Our results suggest that small fragments of HA in melanoma might promote tumor invasiveness by inducing metalloprotease- and cytokine expression and thereby act as an endogenous danger signal by signalling through TLR4.

P214

Spatiotemporally regulated pericellular proteolysis controls extracellular matrix patterning for transition from individual to collective cancer cell invasion

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Invasive cell migration through tissue barriers requires pericellular remodeling of extracellular matrix (ECM) executed by cell surface proteases, particularly membrane-type 1-matrix metalloproteinase (MT1-MMP, MMP-14). Using time-resolved multimodal microscopy, we here show how invasive cells coordinate mechanotransduction and fibrillar collagen remodelling by segregating the anterior force-generating leading edge containing ß1 integrin, MT1-MMP, and F-actin from a posterior proteolytic zone executing fibre breakdown. Sterically impeding fibres are selectively realigned into microtracks of single-cell calibre, which become expanded by subsequent moving cell strands via large-scale degradation of lateral ECM interfaces, ultimately prompting transition towards in vivo-like collective invasion. The findings establish invasive migration and proteolytic ECM remodeling as interdependent processes essential for tissue micro- and macropatterning that underlie collective cell migration and invasive growth.

P215 (V26)

Mutant CDK4 increases the penetrance and decreases the latency of UV-induced, pigmented, metastasizing melanoma in the skin of C57BL/6 HGF mice

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Currently, novel mouse models of melanoma are being generated which recapitulate the histopathology and molecular pathogenesis observed in the human disease. Deregulated receptor tyrosine kinase signaling and impaired cell cycle control are hallmarks of both familial and sporadic melanoma. We previously observed that C57BL/6 mice which carry a mutant cyclin-dependent kinase 4 (CDK4R24C mice) and overexpress hepatocyte growth factor (HGF) rapidly develop multiple pigmented primary melanomas in the skin following carcinogen treatment. These carcinogen-induced autochthonous melanomas spontaneously metastasize in lymph nodes and lungs. Here we investigated the impact of neonatal UVB exposure on melanomagenesis in CDK4R24C x HGF C57BL/6 mice. New born mice were irradiated once on day 2–4 of life with a total dose of 6 kJ/m^2 UVB. In a cohort of 12 UV-treated CDK4R24C x HGF mice cutaneous melanocytic nodules appeared at an age of 4–6 months in all animals. Every mouse developed a few progressively growing melanocytic skin tumors at an age of 6–9 months leading to flattened, at times exulcerated melanomas. When sacrificed because of systemic signs of illness, each mouse displayed enlarged and heavily pigmented draining lymph nodes, 3–10 mm in diameter, and numerous small black nodules on the lung surfaces. Histopathologic examination confirmed primary melanoma of the skin with lymph node and lung metastases. one of the 12 mice also showed prominent melanoma metastases in liver and kidney. Importantly, tumors of other histology were not observed. Untreated CDK4R24C x HGF mice did not show any melanocytic tumors at an age of 6 months and only rarely developed melanomas. UV-treated HGF C57BL/6 mice developed melanocytic tumors several months later and showed progressively growing melanomas only in part of the mice. We conclude that mutant CDK4 in addition to HGF increases the penetrance and decreases the latency of UV-induced melanoma. This new experimental mouse model can now be exploited to further study the biology of melanoma and evaluate new treatment modalities.

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Casein kinase 1 alpha expression determines beta-catenin protein level and survival of melanoma cells

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Casein kinase 1 alpha (CK1 alpha) is a multifunctional Ser/Thr kinase which phosphorylates several substrates among those also beta-catenin, an important player in cell adhesion and Wnt signaling. Phosphorylation of beta-catenin by CK1 alpha is the priming reaction for the proteasomal degradation of beta-catenin. In most melanoma cells beta-catenin protein level is increased compared to the level found in benign melanocytes. One reason for the higher beta-catenin protein level in melanoma cells could be a reduced degradation of beta-catenin due to downregulation of CK1 alpha which leads to an accumulation of beta-catenin in the cells which in turn ends up in the transcriptional activation of oncogenic genes. Therefore, we examined in this study whether CK1alpha expression is downregulated during melanoma progression and whether this has a biological effect on beta-catenin signalling. We examined the expression of CK1alpha and beta-catenin during melanoma development and progression in vitro and in vivo using western blot analysis of lysates from melanoma cell lines and confocal laser microscopy on tissues of melanocytic nevi, primary melanoma and melanoma metastases. The expression analysis of CK1alpha in melanoma cell lines resembling different progression stages and in melanoma tissues revealed a downregulation of CK1alpha expression during melanoma progression in vitro and in vivo. This correlated with an increased beta-catenin expression and nuclear translocation of beta-catenin. Furthermore, we analysed the influence of CK1alpha in melanoma cells lines on beta-catenin protein level and on proliferation, survival, migration and gene expression. Our results indicate that CK1alpha has a key role in the determination of the beta-catenin level and beta-catenin-mediated signalling in melanoma cells which in turn influences melanoma progression.

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A signaling mute hCMV chemokine receptor prevents melanoma growth

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Chemokine secretion by tumor cells enhances tumor growth and leads to progression. Previously, we have shown that tumor survival and

growth is dependent on a chemokine gradient. In an effort to preserve chemokines from the tumor microenvironment, thus potentially abrogating tumor growth, we embarked on US28, a constitutively active human cytomegalovirus-encoded chemokine receptor homologue. US28 promotes different functions in vitro, like scavenging multiple chemokines from the cell microenvironment, enhancing migration of endothelial cells or inducing apoptosis.A primary low-tumorigenic (SBcl2) and a high-tumorigenic melanoma cell line (451Lu) were transduced with a lentiviral vector, encoding either green fluorescent protein (GFP), wild-type US28 (wt) or a previously described signaling mute version of US28 (R129A), which lacks the constitutive signaling activity of the wild type receptor while maintaining its endocytic properties. In vitro, CCL2 levels, as expected, were reduced in wt- and R129A-SBcl2 melanoma cells. When compared to GFP- and wt SBcl2 cells, the proliferation of R129A-SBcl2 cells was hampered and cell migration was reduced, as exemplified in a wound healing assay. In vivo, growth of 2x106 melanoma cells injected subcutaneously into the back of SCID mice was measured. We observed that wild type US28 exerts anti-proliferative properties in the low-tumorigenic melanoma cell line SBcl2, whereas the signaling mute R129A impressively prevents melanoma growth in both cell lines.The tumor-inhibitory properties of US28R129A are the first report, that a tailored mutation in this viral receptor prevents tumorgenesis and may represent a novel approach to interfere with tumor promoting effects of chemokines in solid tumors.

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Mechanisms of sensitivity and resistance of melanoma cells to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)

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Therapy resistance is crucial for the mortality of malignant melanoma. The death ligand TRAIL bears high potential as a new anticancer agent. However, its efficiency may be diminished by occurring resistance in cancer cells. We studied sensitivity to TRAIL-induced apoptosis and the functionality of its two agonistic receptors (DR4 and DR5) in melanoma cells. DR5 was found consistently expressed in all investigated melanoma cell lines, whereas DR4 was found in only 2/7 cell lines. High sensitivity to TRAIL-induced apoptosis was characteristic for DR4-positive melanoma cells, whereas DR4-negative cells showed a delayed response or were resistant. In parallel to apoptosis we found activation of the caspase cascade (caspase-8,-10, -3,-7) as well as cleavage of Bid, X-IAP and DFF45. Importantly, pronounced expression of DR4 as well as of DR5 was proven by immunohistochemistry in the majority of primary melanomas, and DR4 expression was often associated with a more favourable prognosis. In parallel to apoptosis, TRAIL was found to induce activation of NF-κB in DR4-positive melanoma cells, whereas DR4-negative cells were not responsive. Employment of selective DR4/DR5 blocking antibodies unequivocally proved the prevalent role of DR4 for TRAIL-induced apoptosis as well as for NF-κB activation. TRAIL-induced NF-κB activation was not associated with enhanced expression of antiapoptotic factors (c-FLIP, Bcl-xL, Bcl-2, XIAP, Survivin, Livin). Also in an established TRAIL resistance cell culture model for melanoma, resistance was neither associated with increased NF-κB activity nor with increased expression of antiapoptotic proteins. However, significant downregulation of caspase-8, caspase-10 and of DR4 was characteristic for selected, TRAIL-resistant melanoma cells, and sensitivity could largely be restored by their exogenous overexpression. Thus, DR4 expression in vivo and high efficiency of DR4-mediated apoptosis strongly suggests that TRAIL and especially DR4-based strategies could be suitable also for melanoma therapy. Initiator caspases and DR4 rather than NF-κB may control melanoma cell sensitivity to TRAIL, and approaches, which result in their upregulation may be useful for enhancement of TRAIL sensitivity.

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Increased resistance to death receptor-mediated apoptosis in cutaneous T-cell lymphoma cells

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Control of apoptosis via death ligands plays a basic role for lymphocyte homeostasis. In this study, we could demonstrate that they are also critical for cutaneous lymphoma development. Cutaneous T-cell lymphoma (CTCL) cell lines revealed pronounced resistance to death ligands as compared to cell lines of T-cell acute lymphoblastic leukemia (T-ALL). The proapoptotic activity of TNF-α was blocked, sensitivity to TRAIL was significantly reduced, and 1/4 CTCL cell lines was resistant to CD95 activation. In parallel, there was no activation of effector caspase-3 and initiator caspase-8 in non-responsive CTCL cells. Caspase-10 was cleaved selectively in sensitive CTCL cells but not in T-ALL cells. No indication for a responsibility of typical downstream regulators of apoptosis (Bcl-2, Bax, Survivin, X-IAP) or expression of TRAIL decoy receptors (DcR1, DcR2) was obtained, but loss of CD95 was found in 1/4, loss of TNF-R1 in 3/4, loss of caspase-10 in 2/4, loss of the proapoptotic Bcl-2 protein Bid in 1/4 and overexpression of cFLIP was found in 4/4 CTCL cell lines. This clearly indicates an inhibition of apoptosis early in the extrinsic cascade, namely at the formation of the death-inducing signaling complex (DISC). Parallels with regard to expression of apoptosis regulators were seen in PBMCs and biopsies of CTCL patients. Thus, the study underlines the significance of apoptosis control for cutaneous lymphoma and furthermore reveals several relationships to present CTCL therapies based on apoptosis regulation.

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The new iron-containing nucleoside analogue N69 triggers apoptosis in human melanoma cells by a caspase-independent way

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Malignancy of melanoma depends on high chemotherapy resistance related to defects in apoptotic signaling cascades. As all chemotherapeutics are virtually ineffective for melanoma therapy, new cytostatic drugs are urgently required to overcome apoptosis resistance. Nucleoside analogues in general have a high pharmacological potential for the treatment of cancer. Metal carbonyl complexes, such as the recently described nucleoside analogue N69, offer new

opportunities for drug development, since they are highly stable in solution and also reactive in the cell. These properties are basic prerequisites for an efficient drug.Our studies focused on the effects of N69 as well as on its mode of action in melanoma cells. A-375, Mel-HO, Mel-2a and SK-Mel-13 melanoma cell lines were investigated. After incubation with N69 high induction of apoptosis, as monitored by a DNA fragmentation ELISA, was found in A-375 and Mel-HO cells, whereas in Mel-2a and SK-Mel-13 cells apoptosis induction was only moderate. Cytotoxicity as determined by LDH release was low in all cell lines. Apoptosis induction increased with reaction time and concentration of N69. By investigating the mechanism of N69-induced cell death, typical hallmarks of apoptosis such as DNA fragmentation, TUNEL positivity and a shift of the sub-G1 peak in cell cycle analysis were seen. Interestingly we did not detect any activation of caspases, which is a common feature of apoptosis induced by other stimuli. After treatment with N69 neither caspase splice products were seen in Western blot analysis, nor did selective caspase inhibition achieve any reduction of the apoptosis rate. Apart from that we found release of mitochondrial factors as cytochrome c and AIF (apoptosis inducing factor). Interestingly, apoptosis induction by N69 was only partially blocked in stable transfected melanoma cell lines overexpressing the antiapoptotic Bcl-2 protein.These results demonstrate the high proapoptotic potential of the new iron-containing nucleoside analogue N69 in melanoma cells. New efficient drugs as well as new pathways leading to apoptosis may help for development of future strategies against malignant melanoma.

P221

The Resveratrol Analogue M8 (3,3′,4,4′,5,5′-hexahydroxystilbene) is a new and potent approach for the treatment of melanoma

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Resveratrol (3,5,4′ trihydroxystilbene) , a polyphenolic compound derived from various plants, displays beneficial effects on a variety of tumor cell lines. In an attempt to increase the anti-tumor effect of resveratrol, we generated the higher hydroxylated stilbene analogue 3,3′,4,4′,5,5′-hexahydroxystilbene termed M8. In vitro, M8 dramatically inhibits the proliferation of melanoma cells including the highly metastatic human melanoma cell line M24met. Furthermore M8 promotes G2/M arrest in the melanoma cell line M24met. The anti-tumor effects of M8 in the metastatic cell line M24met is significantly more pronounced in M8 treated cells compared to resveratrol. Interestingly, in the non-metastatic melanoma cell line A375 the anti-proliferative effect of M8 is only moderate. To evaluate the underlying mechanisms we performed proteomic analyses on different melanoma cell lines in vitro. We demonstrated that M8 exerts cell line specific modulations of proteins, which are involved in the regulation of proliferation, apoptosis and tumor cell migration. Crucial oncogenic stimuli such as Ras and Akt were analysed by Western blotting to evaluate the therapeutic impact of this new agent.In our recently established human melanoma SCID mouse model we demonstrated that M8significantly impairs tumor progression and decreases metastasis . In conclusion, M8 exerts its antiproliferative effects via blockade of oncogenic stimuli such as Ras and Akt, via involvement in cellular regulations such as apoptosis and tumor cell migration and via impairment of tumor growth and metastasis in vivo. M8 represent an innovative and potent approach for the treatment of melanoma.

P222

E-cadherin serum levels as a tumor marker in malignant melanoma

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The morbidity rate of malignant melanoma, the most malignant skin tumor, has increased continuously over the last decades. This tumor is known to metastasize early via the lymphogenic and hematogenic way and this step is associated with poor prognosis. Nevertheless, early detection of metastasis followed by surgical removal was shown to improve overall survival of these patients, which highlights the necessity of sensitive serum tumor markers. The most commonly used melanoma marker, S-100B, has low sensitivity in the early stages of melanoma. In most cases S-100B is not increased even though patients have lymph node metastases. Identification of additional, sensitive markers is therefore essential for earlier diagnosis and better survival rate. E- to N-cadherin switching is thought to contribute to melanoma progression. Proteolytic cleavage of the cadherin extracellular domain may serve as the first step in E-cadherin downregulation. To examine if melanoma progression correlates with increased E-cadherin ectodomain shedding we analysed serum collected from over 70 melanoma patients for soluble E-cadherin. Our experiments revealed that there is a significant increase in serum E-cadherin levels of melanoma patients with advanced disease compared to the control group correlating with increased S-100B protein values. More importantly, our data indicate that the levels of soluble E-cadherin are increased in a subset of melanoma patients with normal S-100B values. In summary, our results demonstrate that serum E-cadherin may be used as an early tumor marker for malignant melanoma.

P223

ERK phosphorylation in human melanoma in situ is not correlated with the presence of activating BRaf and NRas mutations

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Activating mutations either in the NRas or the BRaf gene are found with high frequencies in human melanoma. Exclusivity of both events suggests that activated Raf and Ras contribute to oncogenesis through the same signalling pathway, most likely by activation of the MAP kinases Erk1/2. We therefore analysed by means of immunohistochemistry phosphorylation of Erk1/2 in 98 primary and 72 metastatic melanomas and obtained several notable observations. (i) In contrast to an almost general and homogenous ERK protein

expression the presence of phospho-ERK was very heterogenous in melanoma tissues with as well as in melanoma tissues without activating NRas/BRaf mutation. (ii) In most cases ERK was phosphorylated only in a minority of the tumor cells and the percentage of phospho-ERK positive cells was not dependent to the presence of activating mutations in NRas or BRaf. (iii) In metastatic melanoma the amount of phospho-ERK positive cells was significantly decreased compared to the primary lesions. As a possible candidate responsible for silencing signal transduction from activated BRaf to ERK we analysed the expression of the Raf kinase inhibitor protein (RKIP). In virtually all melanoma samples RKIP was broadly expressed by the tumor cells. Staining was homogenous and did not reflect the inhomogeneity of phospho-ERK suggesting that RKIP is not critical for MAPK pathway inactivity in melanoma. Moreover, high intensity RKIP staining correlated rather with high percentage of phospho-ERK positive cells than the other way round. Neither the portion of phospho-ERK positive tumor cells nor the intensity of RKIP staining showed any correlation to the clinical course. In this study, we elucidated that phosphorylated ERK is present in only a minority of tumor cells, suggesting that MAPK pathway signalling may be important only in distinct subsets of tumor cells or in distinct phases of tumor development but not in manifest metastasis.

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Synergistic effects of Solaraze and of death ligands for induction of apoptosis in cutaneous SCC cell lines

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Actinic keratosis is a cancerous lesion which occurs primarily on sun-exposed skin areas and which has the potential to develop into squamous cell carcinoma (SCC). Solaraze gel (3% diclofenac, 2.5% hyaluronic acid) has been approved for topical treatment of actinic keratosis. Diclophenac is a non-steroidal anti-inflammatory drug with inhibitory activity on cyclooxygenase-2 (COX-2). COX-2 is upregulated in epithelial tumors and it catalyses the synthesis of prostaglandins which promote tumor growth and angiogenesis. However, the exact mechanisms how Solaraze inhibits growth of neoplastic keratinocytes in actinic keratosis are not known. In the present study, the effects of Solaraze and of the death ligands CD95L/FasL, TRAIL and TNF-α on the regulation of apoptosis were investigated in four cutaneous SCC cell lines. Apoptosis was monitored by DNA fragmentation analysis, by detection of caspase cleavage products, by caspase inhibition experiments and by cell-cycle analysis. Significant induction of apoptosis by an agonistic CD95 antibody (CH-11), by TRAIL and TNF-α was demonstrated in all cell lines. Induction of apoptosis was also found in three SCC cell lines grown for 24 h in the presence of 0.3% of Solaraze, whereas one of the cell lines remained resistant. Diclophenac was identified as the apoptosis-inducing compound. Furthermore, combined treatment of SCC cell lines with Solaraze and CH-11, TRAIL or TNF-α resulted in a synergistic enhancement of apoptosis. These results for the first time describe a direct proapoptotic function of Solarace on SCC cells. The mechanism seems to be related to death receptor function. Thus, the therapeutic effect of topical Solaraze treatment for actinic keratosis may be based on the sensitization of neoplastic keratinocytes to death ligand-induced apoptosis.

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Melanoma cells adhere to human endothelial cells via luminal secreted ultra large von Willebrand fibres (ULVWF) under conditions of laminar flow

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To investigate adhesion of melanoma cells to the vessel wall under defined shear flow we established a microfluidic system (volume: ~50 μl). The system enables to generate a laminar flow ranging from 0 s-1 to 10 000 s-1. Human umbilical vein endothelial cells (HUVEC) were grown in this channel system to confluence before human melanoma cells were added. Superfusion of HUVEC by melanoma cells at a shear rate of 3000 s-1 was recorded over 10 min by video microscopy to detect early steps of cancer cell adherence. Afterwards cells were fixed and stained by anti-vWF antibodies. In agreement to recent results, we observed that melanoma cells stimulate HUVEC to release von Willebrand Factor (vWF). Immunofluorescence staining revealed ultra large vWF fibres (ULVWF) attached to the luminal membrane of HUVEC. Moreover, melanoma cells were found attached to these vWF fibres (12.33 ± 2.02 cells, $n = 3$, $t = 3$ min). Above this, an increased number of melanoma cells were observed to bind to ULVWF after stimulation of HUVEC by Histamine (50 μM) (19.75 ± 2.78 cells, $n = 3$, $t = 3$ min). Addition of anti-vWF antibodies attenuated melanoma cells adhesion by ~90% within the observed time frame (1.66 ± 1.66 cells, $n = 3$, $t = 3$min). Interestingly, blockage of P-Selectin resulted also in diminished melanoma cell adhesion (8.33 ± 1.45 cells, $n = 3$, $t = 3$min), although less effective compared to blockage of vWF. We conclude that circulating melanoma cells stimulate HUVECs to secrete luminal ULVWF. We show that melanoma cells subsequently adhere to HUVEC via ULVWF and P-Selectin. Melanoma induced ULVWF release and melanoma adhesion to ULVWF can be detected instantly after start of superfusion. We therefore, assume that this mechanism displays a pivotal role during early processes of tumor cell extravasation.

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Increased lactate levels of melanoma cells after uva exposure: possible correlation between senescence and the Warburg effect

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It has been recently reported that tumors are able to ferment glucose to lactate even in the presence of oxygen. Aerobic glycolysis, also called Warburg effect, is a metabolic pathway in carcinomas characterized by the anaerobic degradation of glucose even in the presence of oxygen that leads to the production of large amounts of lactate. During tumorigenesis, an increase in glucose uptake and lactate production have been detected. The pentose phosphate pathway (PPP) allows glucose conversion to ribose for nucleic synthesis and glucose degradation to lactate. The non-oxidative part of the

PPP is controlled by thiamine-dependent transketolase enzyme reactions. Transketolase enzyme activities have been related to neurodegenerative diseases, diabetes, and cancer.Senescence-associated growth control represents an important protection from tumors including malignant melanoma. We could show that uva irradiation has an influence on the senescence mediated growth control of melanoma cells in vitro. In the present study we could show an increase of lactate levels after uva exposure of melanoma cells. For a time period of three weeks, melanoma cells with different stages of tumorigenesis (RGP, VGP, MM) were exposed to 4 J/cm^2 and 6 J/cm^2, respectively. After each week lactate levels, senescence and transketolase enzyme activity were measured. We could detect an altered transketolase enzyme activity after uva exposure which correlates with the senescence levels measured in the melanoma cells, representing different stages of tumorigenesis. Dependent on the phenotype, the melanoma cells discriminate regarding there susceptibility towards uva radiation showing distinguishable senescence levels and transketolase enzyme activities.We postulate a novel concept for the understanding of the physiological changes in malignant conversion in melanoma cells after uva exposure.

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Melanoma-derived MMP-1 induces an acute prothromotic, proinflammatory and cell adhesive endothelial surface

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As a hallmark of malignant tumors, cancer cells leave their original site and migrate via the vascular system to settle up metastases. A critical step in this process is extravasation, the ability of circulating tumor cells to adhere to and pass the endothelium as the physiological barrier between blood and tissue. This multistep process requires efficient communication between tumor cells and endothelium. In the present study, we analysed melanoma-derived soluble factors interacting with endothelial thrombin receptor (PAR¬1) the principal mediator of endothelial cell activation. Melanoma cell derived soluble MMP-1 canonically cleaves endothelial PAR1 receptors displayed by calcium fluxes and acute release of IL-8 and von Willebrand factor. Von Willebrand factor (vWF), stored in endothelial cells (EC) and platelets, is the major player of the first response of primary haemostasis and therefore a core protein for the initiation of thrombus formation. Our data indicate that this haemostatic active vWF is also released by EC and immobilized at the vessel wall upon melanoma cell exposure. Surprisingly, high molecular weight multimers of vWF were found to form elongated fiber-like structures (>200 μm in length) on the EC cell surface. After adding platelet-poor plasma during a 5 min stimulation time cell surface attached platelets were found to be almost exclusively sticking to vWF fibres. What is more, these VWF fibers are capable to anchor melanoma cells under flow conditions. 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 cross-talk via an intravascular MMP1/PAR1 axis and might serve as a future target for therapeutical prevention of tumor-derived thrombosis.

P228

Physical limits of cancer cell migration by shape change and deformation

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Physico-chemical processes underlying cancer cell movements depend on cellular strategies to overcome the biophysical resistance of tissue scaffolds, including basement membranes or interstitial extracellular matrix (ECM). Two principle mechanisms how cells overcome narrow tissue regions have been established, (i) the mesenchymal mode, executing pericellular proteolysis and structural widening of ECM gaps, and (ii) the non-proteolytic, amoeboid mode, maintained by cell shape change and squeezing in the absence of ECM remodelling. Albeit shape change and squeezing are of fundamental importance for cell translocation, the physical limits of cell deformation relative to the very narrow tissue structures are unknown. Using highly invasive HT-1080 fibrosarcoma cells migrating through polycarbonate filters of different pore diameter (0.45–8 μm) or high-density 3D collagen lattices, we here show the physical limits of cell movement. Compared to baseline migration rates, the cellular efficiency to cross filter pores was diminished by 80 % for 3 μm pore diameter and near-completely abrogated by 1 μm pore diameter, suggesting a gradual process with a decreasing capability to minimize the cell diameter between 5 μm and 1 μm. Live-cell 4D confocal microscopy of cell shape and matrix scaffold was used to determine the limits of cell deformation in 3D high-density fibrillar collagen supplemented with 5-component protease inhibitors to prevent proteolytic fiber degradation. Migration was abrogated in the range of 1.5–2.5 μm pore diameter, whereas gaps of 3–4 μm were readily transmigrated, confirming the findings of transwell filters for an interstitial tissue. Thus, both experimental systems represent physically different, yet equally valid models to measure deformability of cells during the migration process. Using HT-1080 cells expressing cytoplasmic RFP and nuclear histone H2B/GFP, the nucleus was identified as the critical, most rigid cell region limiting squeezing through pores below 3 μm, whereas cytoplasmic protusions were frequently detected in gaps of 1 μm diameter. In conclusion nuclear deformability limits cell movement through regions smaller than 3 μm in a gradual manner, suggesting that preexisting tissue structures determine whether cells overcome barriers by non-proteolytic mechanisms, such as during protease inhibition based therapy of cancer disease.

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Tumor growth of established RMA-Tag tumors is delayed after treatment with anti-DEC205-Tag protein conjugates

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Tumor antigens, coupled to the anti-DEC205 antibody, can target dendritic cells in vivo and were successfully applied in murine preventive tumor vaccination studies using transplantation tumor models. In this study our aim is to analyse whether anti-DEC205-protein conjugates have the potential to reduce the tumor growth of established tumors in the murine autochthonous RIP1-Tag5 tumor model. Here the Tag protein served as tumor antigen to

activate the immune system against the Tag expressing tumors. In initial experiments the Tag protein and the anti-DEC205-Tag conjugates were used to immunize mice in an in vivo kill experiment, to test whether they have the potential to activate the immune system antigen specifically. Both, protein alone and protein conjugate induced a comparable kill of injected Tag peptide loaded cells. To further analyse their potential to reduce established tumors we first tested the anti-DEC205-Tag conjugates in a subcutanous transplantation tumor model for better observation of the tumor growth using RMA-Tag tumor cells. When the tumors had reached an average diameter of 4 mm the mice were treated with anti-DEC205-Tag conjugate or uncoupled Tag protein. Groups of mice were injected with the respective proteins plus poly I:C and CpG at day 5, 8 and 11 after tumor challenge or were left untreated. The tumor growth was reduced after injection of the anti-DEC205-Tag conjugate compared to the tumor growth of the untreated mice. Moreover, the growth of the tumors in the anti-DEC205-Tag treated mice resumed 1 week after the last immunization. In contrast, the mice injected with the uncoupled Tag protein did not show a significant reduction of the tumor growth as compared with untreated mice. Since both vaccines, i.e. Tag and anti-DEC-Tag, induced similar in vivo kill activity, but differ in their activity to protect from tumor growth we conclude that the anti-DEC205-Tag conjugates induce a rather comprehensive anti-tumor immune response as compared to the uncoupled Tag protein and that the in vivo kill activity does not necessarily correlate with the in vivo anti tumor activity. Furthermore, the anti-DEC205-Tag conjugates seem to be a suitable tool to be tested in the autochthonous RIP1-Tag5 tumor model.

P230

Interaction of mast cells and cutaneous tumors

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The expression of mast cells (MC) is increased in the vicinity of various tumors. However, little is known about MC actions on tumor homeostasis. There is evidence that the number of peri-tumorous MC is positively correlated with the probability of metastasis and with a poor prognosis. Since MC express a number of cytokines and growth factors, it can be assumed that they may have in impact on tumor growth.Studies dealing with MC tumor interactions in the human system are rare and frequently based on experiments using malignant leukaemic MC. Addressing this problem, we established an MC-tumor coculture system for the study of possible supportive or suppressive effects of MC on tumor growth. Advantage of this system is the usage of non-transformed primary human MC in combination with melanoma cells. Different melanoma cell lines were co-cultivated with increasing numbers of primary, dermal MC (1:250, 1:50, 1:10, 1:5, 1:1) for 48 h, and the metabolic activity of melanoma cells was estimated by WST-1-assay in order to look for a potential dependency of tumor metabolic activity on MC number. In addition to native MC this panel was expanded by the use of pre-activated MC. This activation was achieved by incubation of the MC with stimuli such as anti-IgE, Substance P, Stem Cell Factor, IL-4 and a combination of Stem Cell Factor with IL-4.

The results of the in vitro experiments revealed that MC stimulate tumor metabolic activity up to 40%. Activation of MC with SCF or anti-IgE led to a significantly increased metabolic activity of the melanoma cells. The increase in metabolic activity was correlated to mast cell number.

Our data suggest that MC may play a supportive role in the progression of cutaneous tumors.

P231

Mechanisms of hepatocyte growth factor induced regulation of cell adhesion in melanocytic cells

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Hepatocyte growth factor/Scatter Factor (HGF/SF) is a known mitogen, motogen, and morphogen for many epithelial cells. Previous work from our lab has shown that HGF down-regulates E-cadherin in melanoma cell lines. Mechanisms of HGF induced down regulation of cadherins in melanoma are unknown. Based on recent reports, which showed that the Snail superfamily of zinc-finger transcription factors represses E-cadherin and upregulates N-cadherin, we hypothesized that HGF downregulation of E-cadherin may be mediated through these transcriptional repressors or Src, a non-receptor tyrosine kinase. The status of Src, Snail, Slug and Twist has been determined in the cytoplasm and the nuclear fraction of melanoma cell lines, melanocytes, keratinocytes and fibroblasts by immunoblotting. Recombinant HGF has been probed for changes in expression levels of these proteins and transduction with an adenoviral vector will determine changes after continuous exposure to HGF. Additionally, we will perform electrophoretic mobility shift assays (EMSA), to study binding of Snail and Slug to E-boxes of the E-cadherin and N-cadherin promoter. Results show that the cell lines express Src, Snail, Slug and Twist in a cell type specific manner. Differences in the localisation and the concentration of all these proteins between the cytoplasm and the nuclear fraction were observed. For example, the monomer of Twist could be detected in the cytoplasm whereas the dimer of Twist was detected in the nuclear fraction. Interestingly, Src was detected in both fractions (cytoplasm and nucleus). Snail was abundantly found in the nuclear fraction of some cell lines. After exposure to recombinant human HGF for 8 h at 10 ng/ml or 50 ng/ml modulation of expression levels were observed. For example, some melanoma cell lines showed an increase in Src as well as the Twist dimer in the nuclear fraction. No significant difference in expression levels was observed for either of the two concentrations of HGF. Previous work has shown that HGF effects Snail in human hepatoma cells. Our results indicate that Snail is not the only target of HGF, but rather acts via different pathways and transcription factors.

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Relevance of PDGF receptors to the pathogenesis of desmoplastic malignant melanoma

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Platelet-derived growth factor (PDGF) is a potent mediator of connective tissue formation and confers to re-modelling of the skin architecture. An increase in expression of PDGFRs, especially PDGFR-beta was observed in solid tumors and may render tumorigenicity. Desmoplastic malignant melanomas (DMM) are rare clinicopathological entities. In these tumors, the neoplastic cells undergo fibroblastic differentiation and they have a strong tendency for local infiltrative growth and local recurrence. The intent of this project is to decipher the importance of PDGFRs for the overall biology in DMM.To this purpose we investigated

PDGFR expression in tissues of DMM compared to primary cutaneous malignant melanoma by immunohistochemistry. To test for different effects on functional and phenotypical changes we transduced a melanoma cell line (SBcl2) with a lentiviral vector, encoding either green fluorescent protein (GFP), PDGFR-α or -β. The constructs encoding PDGFR-α or -β were confirmed by sequencing. The expression of PDGFR was determined by RT-PCR, MACS and immunoblotting. Changes in random migration, proliferation, anchorage independent growth and invasion are studied. Immunohistochemical analyses of tissue samples from DMM and primary cutaneous melanoma revealed a positive staining of PDGFR-α for both entities. PDGFR-β, however, was predominantly expressed in DMM only. In scratch assays (wound healing assays), SBcl2 cells transduced with PDGFR-β and showed a significantly slower wound closure than non-transduced cells or melanoma cells expressing GFP. Differences in migration were observed after 24, 48 and 72 h. These preliminary data indicate that PDGFR-β might govern a specific etiopathogenic role in DMM.

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UVB-induced expression of Ah-receptor and CYP1A1 in a epithelial squamous cell carcinoma cell line in vitro

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Normal human keratinocytes express the aryl hydrocarbon receptor (AhR) in vivo. Expression decreases from the stratum corneum to the stratum basal. Squamous cell carcinoma of the lung and mucosa also express AhR. It was shown that AhR by inducing CYP1A1 to metabolize dioxins from cigarette smoke causes squamous cell carcinoma of the mucosa and lung. As of now it was not known, whether AhR is expressed in epithelial squamous cell carcinoma cells in vitro or in vivo. We examined the hypothesis that AhR is capable of inducing AhR and CYP1A1 after irradiation with UVB light. The human epithelial squamous carcinoma cell line SCC12 was irradiated with 100 mJ UVB-Light. AhR and CYP1A1 expression was determined by PCR, western blot and immunhistochemistry. PCR revealed a 6-fold increase in AhR and a 13-fold increase in CYP1A1 expression after irradiation with UVB-light. This upregulation could also be verified on protein level with western blot and immunhistochemistry. After addition of the AhR specific inhibitor MNF, no upregulation of AhR nor CYP1A1 could be detected on RNA or protein level. Our results demonstrate that indeed AhR and CYP1A1 can specifically be induced by UVB-light in the SCC12 cell line in vivo. Although these are preliminary results, they might be of importance in the development of new therapeutic approaches for treatment of epithelial tumors.

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Reconstitution of the gene defect in Netherton syndrome by rAAV2-LEKTI: effect on trypsin-activity in human keratinocytes

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Background: Netherton syndrome (NS, MIM 256500) is a severe autosomal-recessive disorder characterized by the clinical triad of variable erythroderma, hairs haft anomalies, and atopic diathesis. NS is caused by different mutations in SPINK5 (serine protease inhibitor Kazal-type 5) on human chromosome 5q32, which encodes the 1064aa serine-protease inhibitor LEKTI (lympho-epithelial-Kazal-type related inhibitor). Low levels or non-functional truncation of LEKTI seem responsible for the high desquamation observed in NS. Due to the lack of inhibitory activity by LEKTI the two serine proteases human kallikrein 5/stratum corneum chymotryptic enzyme (hk5, SCCE) and human kallikrein 7/stratum corneum tryptic enzyme (hk7, SCTE) are unregulated and remain constantly active resulting in a higher degradation of desmosomal proteins.The aim of this study was to reconstitute the SPINK5-gene defect in keratinocytes of NS patients by gene transfer of functional human SPINK5-cDNA.
Methods: A recombinant adeno-associated virus vector type 2 was constructed containing the human cDNA for LEKTI under control of a CMV-promoter (rAAV2-LEKTI). Transfected NS cells were compared with mock-transfected cells (rAAV2-GFP) and normal human keratinocytes (NHK) for changes in LEKTI-expression by RT-PCR and FACS analysis. A fluorimetric trypsin assay was used for homogenates of transfected NS cells and NHK for detection of changes in hydrolytic activity.
Results: LEKTI expression was 1.5–3.5 times higher in rAAV2-LEKTI than in rAAV2-GFP transfected NS cells. Trypsin activity of rAAV2-GFP transfected NS cells was 4.8 times higher than that of NHK. In 75% of rAAV2-LEKTI transfections performed, this elevated hydrolytic activity could be reduced for more than 60%.
Conclusion: In view of these results rAAV2-LEKTI gene transfer could be a promising future tool for reconstituting the defect in NS.

P235

Accelerated collagen lattice contraction of human dermal fibroblasts overexpressing manganese superoxide dismutase is due to an accumulation of H2O2 and subsequent release of TGF-β1

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Manganese superoxide dismutase (MnSOD), a mitochondrially localized antioxidative enzyme dismutates superoxide anions to H2O2. These reactive oxygen species (ROS) play an important role in wound healing. However, prolonged generation of toxic concentrations of ROS most likely contributes to impaired wound healing. Here we addressed the role and mechanism of MnSOD expressed at different levels in the organization and contraction of the dermal equivalent as a model for wound contraction. For this purpose, we generated human dermal MnSOD overexpressing fibroblasts and murine MnSOD deficient fibroblasts which were seeded into free floating collagen lattices. By means of the scopoletin assay the concentration of H2O2 was found to be increased in MnSOD overexpressing fibroblasts due to an imbalanced overexpression of MnSOD with unchanged activities of the H2O2 detoxifying enzymes. Seeding MnSOD overexpressing fibroblasts into collagen lattices led to a substantially increased contraction, whereas collagen lattices containing MnSOD deficient fibroblasts showed a significantly delayed contraction. Immunofluorescent staining against F-actin revealed cytoskeletal reorganization with enhanced spreading and formation of protrusions of MnSOD overexpressing fibroblasts together with more efficient network formation. High

concentrations of TGF-β1 were found in supernatants of MnSOD overexpressing fibroblasts seeded in collagen lattices compared to the control. Inhibition of the TGF-β I-receptor signaling by means of the inhibitor of the TGF-β receptor I ALK 5 kinase resulted in a significantly reduced contraction. Vice versa, incubation of control fibroblasts with TGF-β1 enhanced the contraction. Incubation of MnSOD overexpressing cells with the H2O2 scavenger Ebselen strongly reduced the enhanced contraction as well as the release of TGF-β1. Collectively, these data show that the imbalance of MnSOD expression with an increase in H2O2 and subsequent TGF-β1 release promote collagen lattice contraction dramatically. It remains to be seen, whether dysregulation of the antioxidant defense with enhanced TGF-β1 release contributes to pathologic scar contraction.

P236

Biphasic regulation of AP-1 subunits during human cutaneous wound healing

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Cutaneous wound healing is a well coordinated process that includes inflammation, proliferation and differentiation. Activator protein 1 (AP-1) subunits have been implicated in the regulation of genes important for these processes and have been shown to be involved in wound healing. However, investigation of human healing and non-healing wounds in vivo and ex vivo, and the comparative analysis of several members of the jun and fos families are still missing. Here, we show that spontaneous human wound healing is bi-phasic. In the first phase all AP-1 subunits investigated, i.e. c-Jun, Jun B, Jun D, c-Fos and Fos B are absent from the nuclei at the wound margins/leading edges. This downregulation coincides with that of the gap junction protein connexin 43. Later on, c-Jun, Jun B, Jun D and c-Fos re-appear in the nuclei of the leading edges in a time dependant manner. In non-healing wounds the downregulation of nuclear AP-1 subunits is mostly absent, a more intensive staining at the wound margins is often observed. Our findings suggest that coordinated down- and up-regulation of the various AP-1 subunits in the course of wound healing is important for its undisturbed progress, putatively by influencing inflammation and cell–cell communication.

P237

Ultraviolet-B radiation induces the expression of antimicrobial peptides in keratinocytes

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Ultraviolet-B radiation (UVB) suppresses the immune system. This effect certainly plays an important role in photocarcinogenesis. Exacerbation of skin infections is suggested as a further biological consequence of UV-induced immunosuppression. However, this may only apply to certain viral infections like herpes simplex but not to bacterial infections. In contrast, impetiginized dermatoses can improve upon UVB-exposure without antibiotic or antiseptic treatment. There is evidence that for the bacterial defense the innate immune response is much more relevant than the adaptive immune response. Keratinocytes exhibit the capacity to release various antimicrobial peptides (AMP), including human beta defensins (HBD), RNase 7 and psoriasin. Therefore, we asked whether UVB induces the release of AMP. Normal human keratinocytes and HaCaT cells were exposed to various UVB doses. FACS analysis and RTq-PCR revealed a dose-dependent increase of HBD-2, -3, RNase7 and psoriasin. Doses exceeding 150 J/m^2 caused a decrease which was associated with the initiation of apoptotic cell death as shown by annexin-V staining. These findings were supported by in vivo experiments. Healthy persons were irradiated with different doses of UVB on the buttock. At distinct timepoints after irradiation rinsing fluids and biopsies were obtained from the irradiated skin regions and subjected to ELISA and immunhistochemical staining to determine the amount of AMP. A UVB-dose dependent and also time-dependent expression of AMP could be shown. Patients suffering from atopic dermatitis show decreased levels of AMP which may be due to an over-expression of interleukin-10 (IL-10). Accordingly, incubation of cells with IL-10 caused a decrease of the constitutive expression of AMP. UVB was able to abolish the inhibitory effect of IL-10 on the expression of AMP. Together, these data demonstrate that UVB is able to induce the expression of AMP, thereby explaining the rare frequency of bacterial superinfections upon UVB exposure. This also indicates that UVB exerts diverse effects on the immune system, suppressing the adaptive but inducing the innate immune response.

P238 (V09)

Accelerated aging in mice with connective tissue specific deficiency of the manganese superoxide dismutase -in vivo evidence for the free radical theory of aging

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Until now the free radical theory of aging is heavily debated as it lacks final experimental evidence. This theory postulates that increased mitochondrial production of free radicals during oxidative phosphorylation is the major determinant of life span, and age-related diseases/malfunctions including skin aging, osteoporosis, atherosclerosis and neurodegeneration among others. The manganese superoxide dismutase (SOD2) is localized in the mitochondria and represents the first line of defense against the oxidative attack of superoxide anions. To circumvent the early lethality of SOD2 deficient mice, we have used a connective tissue-specific strategy with introduction of lox P sites flanking exon 3 of the SOD2 gene. Breeding the collagen type I (α2 I) promoter Cre transgenic mice to a SOD2 'floxed' mouse resulted in a connective tissue specific SOD2 deficient mouse line. Crossing the collagen type I promoter Cre transgenic mice to a lac Z indicator mouse, or staining with antibodies against the Cre protein, showed that the Cre recombinase is distinctly active in connective tissue cells of all organs including the skin, the brain, the bone, liver, kidney and heart to mention, the most important. Southern blot analysis of dermal fibroblasts generated from the connective tissue specific SOD2 deficient mice revealed a complete excision of exon 3 of the SOD2 gene resulting in a functional deficiency for SOD2. Mice deficient for the connective tissue specific SOD2 displayed a complex aging phenotype with a reduction of the life span, skin atrophy of the dermis with decrease in interstitial collagens, atrophy of the subcutaneous tissue, severe osteoporosis, as assessed by radiology and bone densitometry as well as neurodegeneration. These data provide strong in vivo evidence for the free radical theory of aging. This connective tissue specific SOD2 deficient mouse model will be particularly suited to close

persistent gaps and to answer fundamental questions regarding the nature of the relevant intracellular targets of reactive oxygen species and how oxidative modification of these target molecules drive the aging process.

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Integration of bioinformatics and RNA expression profiles: A systems biology approach to determine regulatory elements of immediate-early AP-1 response genes

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Cis-regulatory regions govern the intersection of cell signaling, transcriptional regulation and gene expression. Deciphering the localization of such regions, the transcriptional factors that bind to it, and ultimately the logic behind the combinatorial control is crucial to understand a specific expression pattern of a controlled gene. As a proof of concept we set out to obtain an overview of the AP-1 family gene expression program and to identify the responsible key regulatory elements by the combination of a systems biology approach and the analysis of RNA expression profiles. Human HaCaT keratinocytes were stimulated with proinflammatory IL-1β for 0, 0.25, 0.5, 0.75, 1, 2, 6, 12, and 24 h and RNA levels of the AP-1 family members, i.e. c-fos, fosB, fra-1, fra-2, c-jun, junB and junD were determined by real-time PCR. Due to their RNA expression profiles AP-1 family members could be classified into immediate early and early genes, subdivided by low and high transcription rates. Exemplarily, IL-1β resulted in a very similar expression profile for c-fos and c-Jun with maximal RNA-levels (50- and 80-fold induction, respectively) already after 45 min with a rapid subsequent decline to basal levels of RNA expression after 2–6 h. Based on the identified expression patterns, we hypothesized common transcription factor binding sites in the respective promoter region of these genes. Using the open source TOUCAN bioinformatics tool for regulatory sequence analysis (http:/www.esat.kuleuven.be/~saerts/software/toucan.php) we identified particularly CREB and SRF motifs in the promoter region over-represented in the majority of the AP-1 family members as being responsible for the prototypical gene induction profile of these transcription factors during proinflammatory cytokine responses. Together these results demonstrate the feasibility to integrate a systems biology-driven approach and RNA expression profiles to decipher significant regulatory elements. Moreover, our findings implicate that comparative promoter analysis may be useful as an approach to identify under physiological and/or pathophysiological conditions clusters of synchronous gene expression archetypes.

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Regulation of epidermal tight-junctions (TJ) during Staphylococcal skin infections

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Recently, tight junction (TJ) -proteins have been described in the epidermis. Together with other cell–cell junctions like adherens junction (AJ) and desmosomal proteins they constitute a barrier function within the skin. As we learnt from simple epithelia, TJ proteins are targets of bacteria and their toxins in the intestine. Therefore, we investigated the influence of various *staphylococcus* strains on TJ, AJ, desmosomal proteins and actin expression in a human keratinocyte infection culture of the cell line HaCaT as well as in an ex vivo porcine skin infection model. Confirmed by immunofluorescence staining, we found that the pathogen *S. aureus* induces down-regulation of TJ and zonula adherens proteins within porcine skin. The same applied to TJ and AJ proteins at the cell–cell borders of HaCaT cells, dependent on the amount of bacteria and period of incubation. In both models there were no visible changes in desmosomal protein and actin expression. Neither in HaCaT cells nor in porcine skin the commensal *S. epidermidis* did cause alterations. Interestingly, disintegration of TJ proteins proved to be regulated in a time-dependent manner after infection with *S. aureus*. First of all the TJ protein ZO-1 was lost, secondly, Claudin-1. With respect to occludin, first an up-regualtion was seen, followed by a loss until 24 h after infection with *S. aureus*. Taken together our results show that exfoliative toxin-negative strains of *S. aureus* may down regulate cell–cell junction proteins by as yet unknown mechanisms. Therefore, TJ are likely to be involved in the pathogenesis of different *Staphylococcus* strains. By showing a time-dependent order of TJ protein expression, a dynamic mechanism for bacterial TJ regulation may be unveiled.

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An ex-vivo oral mucosa infection – model for the evaluation of the topical activity of antifungal agents

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Although nystatin has been used since the 1950s as a non-absorbable antifungal agent, there is still no reliable in-vivo data available stating a dose-effect relationship of nystatin-suspension in the treatment of oropharyngeal infection with *Candida albicans*. Here, we studied the efficacy of a commercially available topical nystatin suspension and furthermore introduce a new ex-vivo model of candidiasis using porcine oral mucosa. After 48 and 96 h of *Candida albicans* infection, 230 IU nystatin (standard dosage), 100 and 20 IU proved to be equally efficacious. Multiple applications of nystatin were not superior compared to single application. In dosages of 10 and 0.1 IU the activity of nystatin suspension against *Candida albicans* was no longer confirmed. In an agar diffusion model the minimal biocidal concentration of nystatin proved to be 0.25 IU. Our results suggest that the proposed porcine ex-vivo model is much closer to the in-vivo situation of the treatment of muco-cutaneous candidiasis and may provide a substitute for animal models in the investigation of antifungal agents. Additionally, it seems to be a valuable tool for further investigations of the pathogenesis of *Candida albicans* infections.

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T-oligos modulate human hair growth and pigmentation

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Small DNA oligonucleotides homologous to the 3' overhang of human telomeres, called T-oligos, stimulate pigmentation in human epidermal melanocytes in vitro and in vivo and can also up-regulate p53 and induce apoptosis in human epidermal keratinocytes, down-regulate COX-2 expression and inhibit NFkappaB However, it is unknown how T-oligos affect human hair follicle keratinocyte and melanocyte functions. Female human scalp hair follicles in anagen were freshly isolated, then three intact pigmented anagen hairs were placed into each well and were stimulated either with 100 μM T-oligo, (sequence: 5'-phosphate-GTTAGGGTTAG-3') or incubated in culture medium only. Follicles sets were incubated for 48 h up to 9 days.T-oligo treated hair follicles showed a striking, 4-fold increase in pigmentation after 48 h, as discernable both on gross examination and after FM staining, which only slightly increased over the next 9 days, while hair matrix proliferation was reduced by 65%, as assessed by Ki67 staining. T-oligo-treated hair follicles showed a moderate, but significant inhibition of hair shaft elongation by 24% when compared to diluent treated controls within 72 h ($P < 0.05$). Notably, all hair follicles remained in anagen VI and revealed no significant increase in apoptosis as confirmed by TUNEL analysis. These findings demonstrate that human hair follicle hair growth and pigmentation can be potently modulated by telomere homologue oligonucleotides. Strikingly, T-oligos have opposite effects on hair pigmentation and hair follicle growth. This is of special interest, as – under both physiological and pathological conditions –melanogenesis in the hair-follicle pigmentary unit is stringently coupled to maximal hair matrix keratinocyte proliferation and active hair shaft formation. The potential biological insights that may be distilled from this new model system in follow-up studies (including the assessment of classic markers of skin aging, alterations in cell cycle control modulators, hormonal changes, telomere length alterations, inflammatory responses, etc. will far extend beyond hair biology, pathology, and gerontology, since they are likely to be relevant to aging and DNA repair strategies of peripheral neuroectodermal–mesodermal interaction systems in general.

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NF-κB inhibition reveals differential mechanisms of TNF versus TRAIL-induced apoptosis upstream or at the level of caspase-8 activation independent of cIAP2

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Tight regulation of apoptosis is crucial for tissue homeostasis in the skin. Death ligands activate not only a death program but also regulate inflammatory signalling pathways, e.g. through NF-κB induction. While TRAIL and TNF both activate NF-κB in human keratinocytes, only TRAIL potently induces apoptosis. However, when induction of NF-κB was inhibited with a kinase dead IKK2 mutant (IKK2-KD), TNFbut not TRAIL-induced apoptosis was dramatically enhanced. Acquired susceptibility to TNF-induced apoptosis was due to increased caspase-8 activation. To investigate the mechanism of resistance of keratinocytes to TNF-induced apoptosis, we analysed a panel of NF-κB-regulated effector molecules. Interestingly, the inhibitor of apoptosis protein (IAP) family member cIAP2, but not cIAP1, XIAP, TRAF1 or TRAF2 was downregulated in sensitive but not in resistant keratinocytes. Surprisingly however, stable inducible lentiviral expression of cIAP2 was not sufficient to render IKK2-KD-sensitized keratinocytes resistant to TNF and reduction of cIAP2 alone using retroviral stable siRNA expression did not increase the sensitivity of keratinocytes to TNF. In conclusion, we demonstrate that inhibition of NF-κB dramatically sensitizes human keratinocytes to TNF- but not to TRAIL-induced apoptosis and that this sensitization for TNF was largely independent of cIAP2. Our data thus clearly exclude the candidates proposed to date to confer TNF apoptosis resistance and suggest the function of an unanticipated effector of NF-κB critical for the survival of keratinocytes upstream or at the level of caspase-8 activation. The clinical use of IKK inhibitors might thus unexpectantly change the functional outcome of TNF receptor triggering in human skin.

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Chitosan-induced cell death in fibroblasts and keratinocytes is dependent on molecular weight

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Introduction: Chitosan, a biomaterial produced from the natural polymer chitin, is a linear polysaccharide composed of β-(1–4)-linked N-acetyl-D-glucosamine and D-glucosamine units. Apart from their antimicrobial effect, chitosans accelerate wound healing. Proliferative and antiproliferative effects on fibroblasts and keratinocytes have been described. Therefore, the aim of this study was to investigate the influence of molecular weight and concentration on the effect of chitosans with similar degree of deacetylation (≥85%) on primary human dermal

fibroblasts (NHDF) and primary human epidermal keratinocytes (NHEK).
Materials and methods: N-acetyl-D-glucosamine (Sigma, Taufkirchen, Germany), chitosan oligosaccharide lactate (Mn< 5 kDa, Sigma, Taufkirchen, Germany) and a chemically modified chitosan (chitosan 1130, 120 kDa, Chipro, Bremen, Germany) were studied. The toxicity was determined by measurement of intracellular ATP content (ATPLite™-M, PerkinElmer, Boston, USA) and induction of apoptosis was monitored by the activation of caspases 3/7; 8 and 9 (Promega, Madison, USA). In addition, we tested the ability of these materials to quench or promote radical formation by ABEL-superoxide/peroxynitrite assay (Knight Scientific Limited, Plymouth, UK) and a cell-based test with lucigenin (Fluka Chemie AG, Buchs, Switzerland), respectively.
Results: A molecular weight and concentration dependent toxicity and induction of apoptosis in NHDF was observed, when cells were incubated with 0.0001–0.001% (w/v) of the compounds for 48 h. Apoptosis was caspase 9- but not caspase 8-dependent. An inhibition of proliferation was not apparent for NHEK. Although a slight induction of caspase 3/7 could be demonstrated, the treated keratinocytes were negative for increased activation of caspase 8 or 9. Quenching of ROS/RNS was not found with the investigated materials. In contrast to N-acetyl-D-glucosamine and chitosan oligosaccharide lactate, chitosan 1130 showed a strong pro-oxidative effect in the cell-based assay with lucigenin.
Conclusions: The results demonstrate that the biological activity of chitosan depends on the molecular weight, concentration, and the cell type analysed. None of the materials quenched ROS/RNS, but chitosan 1130 promotes radical formation which could possibly be a cause for proliferation inhibition and apoptosis. The cytotoxic effect of chitosan 1130 on fibroblasts should be considered in applications of this material for modern wound management.

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Fibroblasts, keratinocytes and HaCaT-cells proliferation influenced by polihexanide

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In the wound management wound dressings combined with antimicrobial agents (like silver, povidone-iodine, polihexanide) are utilized increasingly for the treatment of critical colonized or infected chronic wounds. Povidone-iodine and octenidine are considered more or less equivalent for acute wounds whether infected or colonized, whereas polihexanide is regarded first choice for chronic wounds because of its low cytotoxicity and very good skin tolerance beside its antimicrobial effects. Furthermore, a positive influence of polihexanide on wound closure was observed in individual clinical cases. Therefore we investigated the influence of polihexanide on the proliferation of three different cell lines. The influence on proliferation of human fibroblasts, keratinocytes and HaCaT cells (changes of dsDNA and ATP content) were investigated by the PicoGreen® dsDNA quantitation kit and the ATPLite™-M ATP assay. The Quant-iT™ PicoGreen® DNA reagent (molecular probes, Eugene, Oregon, USA) is an ultrasensitive fluorescent nucleic acid stain for quantization of double-stranded DNA (dsDNA). The determination of proliferation was also carried out on the basis of a lu-minometric ATP measurement by means of ATPLite™-M (Packard Bioscience BV, The Netherlands). This assay is based on the detection of light generated by the ATP dependent enzymatic conversion of D-luciferin by luciferase. The amount of the emitted light corresponds directly to the ATP concentration. A significant increase of the proliferation of human fibroblasts, keratinocytes and HaCaT cells by polihexanide alone was found (significant versus control: 0.2–2 μg/ml). Lavasept® (polihexanide + macrogolum) also enhanced the progeny of all cell types (significant vs control: 1–2 μg/ml). Macrogolum alone showed no increase. In higher concentrations a decrease of proliferation was observed dose-dependently. These in vitro results on different cell lines demonstrate an impressive positive influence on the proliferation. The observations are in accordance with in vivo studies recently published (Kramer A et al., 2004). On experimental superficial aseptic wounds in piglets (assessed by computerised planimetry) polihexanide (0.4%) showed a promotion of wound closure in contrast to octenidine or Ringer solution alone. Therefore polihexanide seems to be an ideal antimicrobial substance in wound dressings for treating chronic wounds because of its low cytotoxicity, good skin tolerance and positive influence on proliferation.

P246

Impact of ultraviolet light and oxidative stress on Nrf1-3 in human skin cells

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Human skin is constantly exposed to ultraviolet (UV) light and other proinflammatory stressors which can lead to increased amounts of intracellular reactive oxidative species. Nrf1-3 are transcription factors of the Cap' n' collar family and have been demonstrated to play a crucial role in regulation of protective genes against oxidative stress. However, only little is known about Nrf1-3 in human skin cells. Conventional RT-PCR and real-time PCR analysis revealed constitutive expression of all Nrf members at the RNA level in the majority of epidermal and dermal human cell types with highest amounts in epidermal melanocytes. Western blotting confirmed expression of Nrf1 and 2 at the protein level in all tested cell types. Immunohistochemistry of healthy skin disclosed the presence of Nrf1 in nuclei of various epidermal cells, dermal fibroblasts and various epithelia of adnexal structures while Nrf2 was detected primarily in the latter cells within the cytoplasm. The subcellular localization of Nrf1 and Nrf2 in human skin was confirmed by immunofluorescence studies on melanocytes, keratinocytes and fibroblasts growing in vitro. Exposure of H202 (500 μM, 20 min) rapidly increased the relative RNA amounts of Nrf1-3 while UVB irradiation (10 mJ/cm^2), surprisingly, led to a dramatic downregulation of Nrf1-3 expression in both epidermal melanocytes and keratinocytes. In contrast to fibroblasts exposed to oxidative stressors, UVB irradiation of keratinocytes and melanocytes however did not alter the subcellular localization of Nrf1 and Nrf2. Interestingly, UVB-induced downregulation of Nrf1-3 was markedly counteracted by alpha-melanocyte-stimulating-hormone (alpha-MSH), a peptide hormone which recently was shown to have cytoprotective effects. Long-term treatment with alpha-MSH alone, furthermore, increased the steady state levels of Nrf1-3. Our data present a first insight into the expression and regulation of Nrf members in human skin cells in response to oxidative stressors and also point towards a novel functional role of alpha-MSH as a hormone modulating oxidative stress responses.

P247

Kindlin-1 is a phosphoprotein involved in regulation of polarity, proliferation and motility of epidermal keratinocytes

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A novel family of focal adhesion proteins, the kindlins, is involved in attachment of the actin cytoskeleton to the plasma membrane and in integrin mediated cellular processes. 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, progressive skin atrophy, photosensitivity and, occasionally, carcinogenesis. Here we characterized authentic and recombinantly expressed kindlin-1 and show that it is localized in basal epidermal keratinocytes in a polar fashion, close to the cell surface facing the basement membrane, in the areas between the hemidesmosomes. We identified two forms of kindlin-1 in keratinocytes, with apparent molecular masses of 78 and 74 kDa, corresponding to phosphorylated and desphosphorylated forms of the protein. In kindlin-1 deficient skin, basal keratinocytes show multiple abnormalities: cell polarity is lost, proliferation is strongly reduced, and several cells undergo apoptosis. In vitro, deficiency of kindlin-1 in keratinocytes leads to strongly reduced cell proliferation, decreased adhesion, undirected motility and intense protrusion activity of the plasma membrane. Taken together, these results show that kindlin-1 plays a role in keratinocyte adhesion, polarization, proliferation and migration. It is involved in organization and anchorage of the actin cytoskeleton to integrin-associated signaling platforms.

P248

The effect of L-glutamate and L-aspartate on the intracellular calcium concentration of keratinocytes

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Several studies indicate that glutamate receptors are expressed not only in neuronal cells but also in non-neuronal cells like keratinocytes. These investigations were concentrated on ionotropic glutamate receptors of the N-methyl-D-aspartate (NMDA) receptor type. While most studies used the in-vitro agonist N-methyl-D-aspartate for functional experiments, only little is known about the effect of the physiological transmitters at NMDA-receptors of keratinocytes. To prove this we studied the intracellular calcium concentration of normal human epidermal keratinocytes (NHEK) upon stimulation with L-glutamate (1–1000 μM) or L-aspartate (1–100 μM). Measurement of the intracellular calcium concentration was performed by laser scanning microscopy. Cytotoxicity was investigated using crystal-violet-method.The application of L-glutamate resulted in an elevated intracellular calcium concentration in up to 44% of all investigated cells. L-aspartate only had minor effects on the intracellular calcium concentration of NHEK. The selective NMDA-receptor antagonist DL-2-amino-5-phosphonovaleric acid (AP5) inhibited the L-glutamate-effect only partially, as about 20% of keratinocytes still showed elevated intracellular calcium levels after stimulation with L-glutamate. There was no evidence for a cytotoxic potential of L-glutamate. The physiological glutamate-receptor agonist L-glutamate increased the intracellular calcium concentration of NHEK. It is a non-toxic substance for keratinocytes. Interestingly the L-glutamate-induced increase of intracellular calcium seems to be not exclusively NMDA-receptor-dependant. Future studies must elucidate the possible presence of other glutamate receptors in keratinocytes.

P249

Somatostatin receptor 3 is regulating tight junctions via heterotrimeric G-proteins

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Tight junctions (TJ) are cell–cell junctions that are located in the uppermost living layers of the epidermis. It has been demonstrated that they play a role in barrier function of the skin. Heterotrimeric G-proteins have been shown to regulate the formation and permeability of TJ in simple epithelia, yet hormone/receptor systems which might control these junctional G-proteins have so far been elusive. Here we show that the human somatostatin receptor 3 (SSTR3) is present in the stratum granulosum of human epidermis in a staining pattern which is consistent with a localization at tight junctions. In addition, somatostatin is present in epidermis. By yeast two hybrid screening we identified the multiple PDZ protein MUPP1, a tight junction associated protein which is also present in the epidermis, as an interaction partner for the C-terminus of the receptor. Interaction between both proteins in vivo can be demonstrated in epithelial cell lines such as MDCK-II and MCF-7. As a result of the interaction with MUPP1, the hSSTR3 is targeted to a large protein complex at tight junctions. On a functional level, the activation of the receptor by agonists leads to an increase in transepithelial resistance (TER). Our data raise the possibility that hSSTR3, due to its interaction with MUPP1, can modulate the permeability characteristics of epidermal TJs in normal skin but also in pathological conditions, e.g. psoriasis.

P250

Polyunsaturated fatty acids (PUFAs) synergistically increase antiproliverative effects of the vitamin D analogue calcipotriol on human melanocytes in vitro

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The active form of vitamin D, 1,25-dihydroxyvitamin D3 (1,25(OH) 2D3, calcitriol) represents a seco-steroid hormone that is a key

regulator of calcium homeostasis in the human body. Additionally, 1,25(OH)2D3 is in a broad variety of tissues involved in many other cellular processes including regulation of cell growth. We have analysed whether polyunsaturated fatty acids (PUFA) modulate the antiproliferative effects of vitamin D analogues on human melanocytes. Combined treatment of human melanocytes with the vitamin D analogue calcipotriol and α-linolenic acid (ALA) or docosahexaenoic-acid (DHA) resulted in pronounced inhibition of cell proliferation as compared to treatment of melanocytes with calcipotriol alone. Treatment of melanocytes with newly synthesized calcipotriol-PUFAs (calcipotriol conjugated at the side chain with ALA or DHA) did not result in pronounced inhibition of cell proliferation, as compared to treatment of melanocytes with calcipotriol alone. In conclusion, we demonstrate that PUFAs synergistically increase the antiproliferative effects of calcipotriol, indicating that a combination of PUVAs and calcipotriol may be effective therapeutically, e.g. in the treatment of malignant melanoma or the skin disease psoriasis.

P251

Connexin 43 plays a central role during early wound healing

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Connexins (Cx) are transmembrane proteins that form Gap Junctions (GJ), communicating channels that allow the exchange of small molecules, e.g. metabolites and second messenger molecules, between neighbouring cells. GJ are important for migration, differentiation and proliferation of cells. Connexin 43 has been shown to be ubiquitously expressed in human epidermis and to be down regulated during early wound healing. To investigate whether this is a central event of wound healing or a secondary effect only, we treated ex vivo wound healing models with peptides that inhibit Cx43 and investigated wound healing progress as well as the staining patterns of connexins 43 and 26, Ki67, protein ZO-1 and occludin. Moreover we used Chitosan which is known to inhibit wound healing at high concentrations and promote wound healing at low concentrations and investigated its effect on connexin expression. We observed an acceleration of wound healing when using Cx43 inhibitory peptides. In models treated with high concentrations of Chitosan, the loss of Cx43 staining at the wound margins, which is found in normal wound healing, was absent. Therefore we conclude that the down regulation of Cx43 is a central event in the course of early wound healing. The staining patterns of Cx26, Ki67, ZO-1 and occludin are discussed.

P252

JunB silencing in human primary keratinocytes inhibits regular epidermal development in organotypic skin cultures

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The differential expression of the jun family members of the transcription factor AP1 during human epidermal keratinocyte differentiation caused us to examine the involvement of JunB in this process. Specific JunB knock down in a human in vitro skin equivalent model based on differentiating epidermal keratinocytes transfected with stealth siRNA for JunB was confirmed by Western blot analysis of cell lysates and immunohistochemistry. JunB knock out organotypic epidermal cultures displayed a disorganised phenotype comprising a thin stratum corneum, a reduced or absent granular layer, and the appearance of foamy cells and big vacuoles in the suprabasal layers. The differentiation associated proteins loricrin, filaggrin, cytokeratin1/10 and caspase-14 are downregulated, whereas involucrin and cytokeratin 5/14 expression remained unaffected. JunB silencing had no influence on the proliferation rate of keratinocytes. Human Genome U133 Plus 2.0 Affymetrix DNA chips that contained oligonucleotide arrays of approximately 38 500 well-characterized human genes were used to identify JunB regulated/dependent genes in epidermal skin equivalents. The majority of the regulated genes were involved in cell adhesion, cytoskeleton restructuring, signalling transduction, and cell cycle. In conclusion our results indicate that silencing of JunB in a human skin equivalent model leads to alteration of regular keratinocyte differentiation in vitro.

P253

Extracellular phosphorylation of collagen XVII by protein kinase ecto-CK2 inhibits ectodomain shedding

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Ecto-phosphorylation is emerging as an important mechanism to regulate cellular ligand interactions and signal transduction. Here we show that extracellular phosphorylation of the cell surface receptor collagen XVII by ecto-casein kinase 2 controls its ectodomain shedding in keratinocytes. Collagen XVII, a member of the novel protein family of collagenous transmembrane proteins and a component of the hemidesmosomes, mediates adhesion of the epidermis to the dermis in the skin. Its ectodomain is constitutively shed from the keratinocyte surface by metalloproteinases of the ADAM family, mainly by TACE, but the mechanisms controlling ectodomain cleavage remain unclear. In this study we used biochemical, gene transfer and structural modeling approaches and show that extracellular phosphorylation of collagen XVII interferes with TACE cleavage. Incubation with the cell-impermeable gamma-P32-ATP led to collagen XVII labeling, which was significantly diminished by a broad-spectrum extracellular kinase inhibitor or a specific CK2 inhibitor. Collagen XVII peptides containing a predicted CK2 recognition site were phosphorylated by CK2 in vitro, and Ser542 and Ser544 were identified as the respective phosphorylation sites. Inhibition of CK2 kinase activity increased ectodomain shedding in HaCaT keratinocytes, whereas overexpression of CK2-alpha inhibited cleavage of collagen XVII. Moreover, Ser-to-Ala mutation of Ser544 significantly increased shedding as compared to the wild-type protein. Structural modeling indicated that phosphorylation prevented TACE binding to its substrate. In conclusion, extracellular phosphorylation of collagen XVII by ecto-CK2 represents a novel mechanism of regulating ectodomain shedding and is part of the machinery controlling adhesion and motility of epithelial cells in the skin.

P254

The effect of progestogens on human melanocytes

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It is well known that the use of oral contraceptives may lead to melasma, an epidermal hyperpigmentation with an increase in melanocytes in sun-exposed areas of the face. Oral contraceptives contain a combination of ethinylestradiol and a progestogen and melanocytes express receptors for estrogen and progesterone. It is assumed that elevated estrogen levels in the serum of pregnant women or patients taking oral contraceptives are responsible for the development of melasma. To further study the role of progestogens, we analysed the effects of estrogen, progesterone and chlormadinon acetate on melanocytes in vitro. Human melanocytes were cultured in growth medium containing different concentrations of estrogen and progestogens. They were analysed at day 9 by a proliferation assay and a 3H-tyrosine assay, which measures the activity of tyrosinase, the key-enzyme of pigmentation. Estrogen (1 nM) led to an increase in proliferation and tyrosinase activity in melanocytes as shown previously. On the contrary, progesterone and chlormadinon acetate (100 nM) reduced the proliferation rate by 50–70% and 30–50%, respectively. A combination of estrogen and progesterone or estrogen and chlormadinon acetate led to an increased proliferation of melanocytes, but to a less extent than by estrogen alone. Since melasma only occurs in sun-exposed areas of the face, we were interested in the importance of ultraviolet light (UV) and exposed human melanocytes both to estrogen/progestogen and to UVA (10 or 20 J/cm^2) or UVB (10 or 20 mJ/cm^2). The tyrosinase activity in UVB-irradiated melanocytes was again stimulated by estrogen and inhibited by progesterone, but not by chlormadinon acetate. Taken together, our data suggest an inhibitory effect of progesterone and chlormadinon acetate on human melanocytes, which counteracts the stimulatory effects of estrogen. This could be important for the choice of which progestogen is used in oral contraceptives to prevent the development of melasma.

P255 (V37)

The collagen VII hypomorph, a viable mouse model for dystrophic epidermolysis bullosa, allows testing of molecular therapies

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Gene-, protein- or cell-based therapy approaches have been developed for different genodermatoses, but the evaluation of the efficacy and side effects has been impeded by the lack of suitable animal models. In case of dystrophic epidermolysis bullosa (DEB), a hereditary skin blistering disorder caused by mutations in the gene COL7A1 encoding collagen VII, all animal models used so far exhibit major draw-backs. Successful treatment depends considerably on the immunogenicity of introduced collagen VII, but most studies have relied on skin grafts on athymic mice to demonstrate therapeutic effects. Immunocompetent animals with COL7A1 mutations, e.g. sheep and dogs, have not proven practical due to the large size and excessive cost of care. Finally, the collagen VII null mouse dies early due to massive blistering and is therefore unsuitable for establishment of therapies. Therefore we developed a collagen VII hypomorph mouse model for evaluating therapies. Mice homozygous for intronic insertion of a pgk-neo-cassette in Col7a1 (Col7a1 flNeo/flNeo) display hemorrhagic blisters at birth which heal within the first week of life. Blistering of the tongue and oral mucosa begins in the third week of life, aggravates with age and causes severe growth retardation and death by malnutrition at the age of 8–9 weeks. Adult animals display dystrophic nails and frequently miss digits. RT-PCR analysis of murine keratinocytes revealed an abnormal splicing pattern of the collagen VII mRNA in Col7a1 flNeo/flNeo, with only a minor percentage of correctly spliced mCol7. Hence, quantitation of protein levels in skin shows a 90% reduction in type VII collagen. Electron microscopy showed normal hemidesmosomes in Col7a1 flNeo/flNeo skin but strongly reduced numbers of anchoring fibrils with sub-lamina dense blister formation. Taken together, Col7a1 flNeo/flNeo represents a viable mouse model for DEB which displays all clinical and biological characteristics of human non-Hallopeau-Siemens DEB. Beside analysis of disease pathogenesis and anchoring fibril biology, it will be used to analyse efficiency and adverse effects of novel therapies for DEB, i.e. fibroblast-, gene- or protein therapy, as well as stem cell therapy.

P256

Triple helix stability of collagen VII as an indicator for skin fragility in dystrophic epidermolysis bullosa

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Dystrophic epidermolysis bullosa (DEB) is a heterogeneous skin blistering disorder caused by mutations in the gene for collagen VII, the major component of anchoring fibrils. Glycine substitutions within the collagenous domain represent a subgroup of the more than COL7A1 300 mutations associated with DEB. They may exert dominant negative effects on fibril function, but very little is known about genotype-phenotype correlations underlying different degrees of skin fragility. Triple helix stability is likely to be a key factor defining the phenotype of a given glycine mutation, and systematic analysis of triple helix stability provides insights into possibilities for therapeutic correction of genetic aberrations. We used recombinant expression and site directed mutagenesis to generate wild type and mutant collagen VII and measured their helix-to-coil transition (or melting temperature, Tm) using resistance to limited proteolysis as a probe. Coexpression of wild type and mutant collagen VII was used to determine stability of hybrid triple helices in mimicry of a heterozygous expression. Recombinant wild type (wt) collagen VII had a Tm of 41°C, similar to endogenous collagen VII in keratinocytes. Recessively inherited glycine substitutions decreased the Tm to 38°C when expressed in combination with the wild type protein (G1845R/wt, G2316R/wt). Hybrid triple helices of mutant collagen VII with glycine substitutions found in patients with dominant DEB showed significantly lower Tm (e.g. G1776R/wt, 31°C; G2006R/wt, 32°C; G2015R/wt, 34°C; G2207R/wt, 34°C). Interestingly, mutations associated with a minimal clinical phenotype, such as nail dystrophy, reduced the Tm only to 36°C (G1815R/wt). These results demonstrate that there is a significant difference in triple helix stability of collagen VII mutants associated with recessive and dominant DEB and that the Tm reflects the severity of the clinical phenotype. We will use this experimental system to determine the amount of wild type collagen VII necessary to overcome the dominant negative effect of a given glycine substitution, thereby paving the road for gene or protein therapy for dominant DEB.

P257

The concentration of charged dyes has effects on the fluorescence response upon membrane potential alterations of human cells

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The fluorescence of cationic and anionic charged dyes is used to measure both qualitatively and quantitatively membrane potential alterations of human cells by flow cytometry. However, some dyes possess concentration-dependent characteristics that lead to a non-linear relationship between fluorescence and potential change. One of the underlying mechanisms is the quenching of fluorescence by formation of dye aggregates. This is especially known for cationic dyes which often accumulate in the mitochondria at very high concentrations, even if the dye concentration in the staining solution is very low. So, a higher dye uptake of cells does not result in higher but rather in unchanged or decreasing fluorescence. In addition, one dye may bind to several cellular sites having different quantum yields. Saturation effects and spectral shifts both for excitation and emission are further sources of unlinearity. Therefore, the knowledge of a special dye response is a prerequisite for using appropriate dye concentrations and for reliable interpretation of data. IGR-1 melanoma cells as a model were stained with a series of different concentrations of three anionic and three cationic dyes. The fluorescence response upon a definite depolarization as a function of the dye concentration in the staining buffer was determined using a FACScan flow cytometer. Two anionic dyes exhibited first a proportional signal change followed by saturation with increasing dye concentrations, while the third anionic dye showed a completely anomalous response. The change in fluorescence of all cationic dyes decreased to zero with increasing dye concentrations. For two dyes at intermediate concentrations, however, the course of the response was found to be a plateau.

P258 (V02)

Oligonucleotides cause IL-8 suppression in skin keratinocytes in vitro and show anti-inflammatory effects in vivo

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Recent findings suggest that DNA do not only codes for genetic information but offers also additional function, particularly in recognition of microorganisms. In the present study, two classes of oligodeoxynucleotides (ODN) were tested in skin keratinocytes. One ODN comprised two cytidine-phosphate-guanosine (CpG) motifs and one poly-cytidine served as control. Interestingly, both ODN suppressed the basal and the TNFalpha-induced IL-8 levels measured in keratinocyte cell culture supernatants. Experiments using deletion mutants revealed a critical length of approximately 16 nucleotides conveying IL-8 suppression. Also the ODN backbone was analysed: experiments offered that phosphorothioate-bondings are critical for significant IL-8 suppression. In order to substantiate the anti-inflammatory response, a contact hypersensitivity (CHS) mouse model was utilized. Topical application of poly-cytidine-containing ointments reduced ear thickness in sensitized mice corroborating our in vitro data. Both fluorescent-labeled ODN were taken up into mouse skin already after 2 h. Taken together, these findings suggest an anti-inflammatory effect of ODN in epithelial cells in vitro and in vivo, indicating that DNA molecules offer distinct biological activities restricted to the physiological compartment applied. This effect seems to be independent from Toll-like receptor 9 (TLR-9) as ODN methylation or transfection of HaCaT cells with dominant-negative constructs against TLR-9 and the adaptor protein MyD88 showed no influence in the IL-8 suppressive effect of ODN.

P259

Regulation of endothelial cell function by dimethylfumarate

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Increasing evidence indicates that angiogenesis is crucially involved in the pathogenesis of chronic inflammatory diseases. Psoriasis, a chronic inflammatory disease of skin and small joints, is characterized by a strong proliferation of endothelia and enhanced expression of Vascular endothelial growth factor (VEGF). Moreover, psoriasis can be induced in mice by transgenic overexpression of VEGF in keratinocytes. These data suggest a pivotal role of angiogenesis in the pathogenesis of psoriasis. Fumaric acid esters (FAE) such as Dimethylfumarate (DMF) are immunomodulatory molecules, which improve inflammatory diseases accompanied by strong angiogenesis, such as psoriasis. However, the underlying mechanisms of the anti-psoriatic effect are not fully understood. To determine whether FAE might improve psoriasis due to an anti-angiogenic effect, we investigated the influence of DMF on endothelial cell functions. Therefore, we first performed in vitro sprout formation assays using human microvascular endothelial cells (ECs) and mouse aortic rings. In both assays, sprout formation was significantly reduced by DMF in a dose-dependent manner. Migration and proliferation of ECs were also strongly decreased by DMF treatment. In contrast, apoptotic cell death as a potential cause for the effects mediated by DMF was excluded. Interestingly, the analysis of candidate signal transduction pathways mediating the impairment of EC function by DMF revealed no significant involvement of ERK-1/ERK-2 or the p38 kinase, respectively. Since we observed that DMF increased reactive oxygen species (ROS) in ECs, we next asked whether the observed effects of DMF on endothelial cell function are directly mediated by DMF or might be related to increased ROS formation. The application of antioxidants completely restored sprout formation of both, microvascular ECs and aortic rings as well as EC proliferation after DMF treatment. These data suggest, that the increase in ROS was responsible for the observed inhibitory effects of DMF on ECs. Using the murine cornea micropocket assay we could demonstrate a significant reduction of neovessel formation due to DMF treatment also in vivo. Taken together, these results suggest, that the therapeutically established anti-psoriatic molecule DMF might improve, at least in part, the inflammatory disease through ROS-mediated anti-angiogenic effects. The strong anti-angiogenic potential of DMF may provide new therapeutic options.

P260

T-cell induced keratinocyte apoptosis is blocked by cFLIP in eczematous dermatitis

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In healthy skin epidermal keratinocytes display low levels of Fas receptor (CD95) and are highly resistant to CD95-mediated killing. However, in the elicitation phase of eczematous dermatitis dermis-infiltrating, IFN-γ secreting activated T-cells pave the way to apoptosis, in part by promotion of CD95 expression on keratinocytes. CD95-mediated death of keratinocytes is mediated by T-cells through direct CD95L/CD95 interactions as well as paracrine secretion of soluble CD95L. This mechanism was previously recapitulated in vitro using an eczematous dermatitis model, in which keratinocytes that were cocultured with stimulated CD45RO+ T-cells upregulate CD95 expression and undergo CD95-induced apoptosis. In this study, we have investigated the impact of intracellular inhibition of death ligand-mediated apoptosis in human keratinocytes. Caspase-8 and/or caspase-10 are critically required for CD95-mediated apoptosis, and these initiator caspases are inhibited by the catalytically inactive caspase-8/-10 homologue cFLIP long (cFLIPL). In order to test the impact of inhibition of upstream caspase activation in eczematous dermatitis, we used the keratinocyte cell line HaCaT that were retrovirally transduced with cFLIPL. These cells were then cocultured for 48 h with anti-CD2/-CD3/-CD28 mAb activated, purified human CD4+ T-cells from peripheral venous blood. While vector carrying keratinocytes efficiently died by apoptosis in HaCaT/CD4+ T-cell cocultures, cFLIPL-expressing keratinocytes were partially protected against T-cell mediated apoptosis. Our findings further support the concept that CD95-mediated apoptosis of keratinocytes plays a central role in the pathogenesis of T-cell mediated eczematous dermatitis.

P261

Low concentrations of gycyrrhizin stimulate cell growth and activate growth-related kinases

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Glycyrrhizin is an active component of the root of *Glycyrrhiza glabra* (licorice) which is one of the most commonly used herbs in Traditional Chinese medicine. Glycyrrhizin offers a variety of pharmacological effects including a positive effect in wound healing. In the present study we investigated the effect of glycyrrhizin in human keratinocytes (HaCaT). Besides basal parameters such as cell growth, membrane integrity, and apoptosis we determined the activity of kinases involved in cell growth. We found in the presence of 0.05–4 mM glycyrrhizin a concentration dependent phosphorylation of EGF-R, ERK 1/2 and Akt/PKB. At higher concentrations the phosphorylation declined. Likewise, to this pattern the BrdU incorporation rate showed also a biphasic effect. At low concentrations we detected a concentration dependent increase in BrdU incorporation peaking at 1 mM followed by a decrease at higher concentrations (>1 mM) down to 70% compared to untreated controls. Glycyrrhizin offered no toxic or apoptotic effects at concentrations between 0.5 and 4 mM. Our in vitro results suggest a positive effect of glycyrrhizin on tissue repair in vivo at low concentrations. Higher concentrations may offer a contrary effect.

P262

Differentiation signals for human Langerhans cell precursors identified by sequential migration of monocytes

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Precursors of dendritic cells (DC) are myeloid cells transported via the blood stream, which migrate into tissues, where they differentiate to DC, most probably following locally released chemotactic signals. Using human peripheral blood monocytes, we investigated the impact of trans-endothelial migration on phenotype and function of Langerhans cell (LC) precursors, which are paradigmatic DC from the epithelial layer in the skin. On their way from the blood vessels to the epidermis, LC precursors need to cross the endothelial layers. They are guided by chemoattractants from dermal fibroblasts and from epidermal keratinocytes. Several chemokines are secreted by both, fibroblasts and keratinocytes; in addition, both cell types produce a small number of fibroblast-specific or keratinocyte-specific chemokines. In an in vitro transwell system, we could show that monocytes migrated towards medium conditioned by fibroblasts. Only CD14low cells, which had migrated towards fibroblast conditioned medium, began to express CD1a, additionally a population of the CD14low cells became CCR6 positive after migration and incubation. 14 h later, in a second migration assay, only monocytes having migrated towards fibroblast-conditioned medium could migrate towards keratinocyte-conditioned medium. But neither monocytes having migrated spontaneously to control medium nor non-migrated monocytes, which were incubated for the same time period responded to keratinocyte-conditioned medium. Thus, trans-endothelial migration of human peripheral blood monocytes towards fibroblast-conditioned medium enabled them to subsequently migrate towards keratinocyte-conditioned medium. This would be a feature expected from LC precursors. The investigation of chemokine receptors of freshly isolated monocytes and after their various migration steps revealed that their trans-endothelial migration towards fibroblast-conditioned medium rescued the expression of chemokine receptor CCR2, which was otherwise rapidly down regulated. We therefore hypothesize that trans-endothelial migration of monocytes is a critical step for their differentiation to Langerhans cells.

P263 (V29)

Tryptophan deprivation contributes to the generation of regulatory dendritic cells

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Indoleamine 2,3-dioxygenase (IDO) is produced by dendritic cells (DCs) and plays a critical role in peripheral T cell tolerance. Given the great importance of DCs in the initiation of a T cell response, surprisingly little is known about the impact of a high IDO activity, i.e. a tryptophan (trp)-deprived milieu, on DCs themselves. We investigated

the biology of human monocyte-derived DCs differentiated under trp-deficient conditions in vitro (DCs-trp). We show here that low concentrations of the essential amino acid tryptophan during differentiation causes, both, immature and mature DCs to acquire a tolerogenic phenotype [CD80low, CD40low/ Immunoglobulin-like transcript 2 (ILT2)high, Immunoglobulin-like transcript 3 (ILT3)high]. This is intriguing, as it demonstrates that the deficiency of trp not only acts through mere starvation, but, actively increases the expression of regulatory signals such as inhibitory receptors ILT2 and ILT3 on these DCs. Interestingly, the kinetics done with ILT3 showed that (i) on day 3, there is a peak of ILT3 expression on DCs-trp and DCs + trp; (ii) ILT3 is significantly and constantly higher expressed on DCs-trp than on DCs + trp (up to two-fold higher on day 3); (iii) the commitment to tolerogenic DCs following trp-starvation seems to act early in the differentiation pathway since external addition of trp on day 3 did not fully downregulate ILT3. In fact, trp-deprived DCs-trp show a reduced capacity to take up antigens and to stimulate TT-specific autologous T cells in vitro. In conclusion, a tryptophan-deprived environment due to ongoing IDO activity such as in allergic inflammation contributes to the generation of regulatory DCs with immune suppressive properties.

P264

Accelerated wound closure in mice deficient for interleukin-10

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The impact of the local inflammatory response on the process of wound healing has been debated for decades. In particular the question whether infiltrating macrophages and granulocytes promote or impede tissue repair has received much attention. Interleukin-10 (IL-10), one of the most potent immunoregulatory cytokines, limits innate as well as adaptive immune responses and is thought to protect the host from immune-mediated tissue damage. The role of Il-10 during tissue repair is not well understood. In the present study, we show that wound healing is accelerated in mice deficient for the anti-inflammatory cytokine IL-10. IL-10-/- mice closed excisional wounds significantly earlier as compared with IL-10 competent control littermates. This effect was due to accelerated epithelialization as well as enhanced contraction of the wound tissue in the mutant animals. Increased smooth muscle actin expression in IL-10 deficient mice suggests that augmented myofibroblast differentiation is responsible for the enhanced contraction of wounds in mutant mice. The number of macrophages infiltrating the wound tissue was significantly increased in IL-10-/- mice compared with control littermates suggesting that this cell type mediates the accelerated tissue repair. Functional analysis of tissue breaking strength revealed a decreased biomechanical strength of wound tissue in IL-10 deficient mice during the early phase of repair, reflecting the increased proportion of cells and provisional wound matrix versus collagen at the wound site. However, during later stages of repair, microscopic analysis of collagen fibrils and quantification of collagen content indicated significantly increased deposition of extracellular matrix in mutant mice. These results show for the first time that IL-10 is a negative regulator of wound repair and support the view that activation of the innate immune response is a central event during wound healing.

P265

Immunohistological phenotyping of the human hair follicle bulge region as a stem cell niche

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Since the discovery of epithelial hair follicle stem cells (eHFSC) in the bulge region ('Wulst') of mouse hair follicles and the identification of equivalent cells in human hair follicles, a biologically and clinically important debate has unfolded: How many eHFSC populations are there, where are they localized, which are the best immunohistological markers for their identification in situ, and what characterizes the bulge stem cell niche? In the current study, we attempt to contribute to this ongoing, controversial debate by summarizing published markers of murine eHFSC and comparing the immunoreactivity pattern of selected markers in normal human scalp skin. We show that, in addition to keratin 15 and beta1-integrin, extra cellular matrix proteins such as tenascin-C, fibronectin and fibrillin-2 and the immunoregulatory surface antigen CD200, which is found e.g. in immunoprivileged tissues, are upregulated in the bulge region of human scalp hair follicles. Similar to what has previously been reported in murine hair follicles these may contribute to define the – as yet obscure – stem cell niche conditions of human hair follicles. In contrast, alpha6-integrin, nidogen and LTBP-1, in our hands, show no up regulation in the human bulge region. Contrary to what has been reported in mice, prominent nestin immunoreactivity was not usually seen in the human bulge region. Connexin43 and CD34 did not show any expression in this region and may thus be useful negative markers for the bulge region (note that murine bulge eHFSC are CD34+ !). Thus, the key challenge to identify selective immunohistological markers that allow to reliably distinguish human bulge stem cells from their progeny (i.e. transient amplifying cells) and that selectively demarcate the stem cell niche(s) of human hair follicle is still unmet. However, using the panels of positive and negative markers indicated above is a pragmatic first approach to the immunohistological characterization of the human bulge region.

P266

Exploring the functional role of beta1-integrin-mediated signalling in the human hair follicle

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Integrins are transmembrane adhesion proteins that exert crucial signalling properties ranging from the control of cell proliferation, death, and differentiation during developmental and in stem cell biology to immunomodulatory functions. In human skin and hair follicle (HF) biology, beta1-integrin (beta1) and its ligands (e.g. fibronectin, collagen, laminin) are of particular interest since epithelial stem cells presumably up-regulate beta1 expression and since its functional deletion in mutant mice induces severe defects in HF development and growth. However, it is as yet entirely unknown whether beta1 plays any role in the regulation of human hair growth, and the expression pattern of beta1 in the human pilosebaceous unit is still ill-defined. Here, we show that immunoreactivity for beta1 can be found in distinct compartments of human scalp HFs in situ and in vitro [e.g. connective tissue sheath and

dermal papilla fibroblasts, outer root sheath (ORS) and hair matrix keratinocytes, myoepithelium of the sebaceous gland]. Beta1 was most prominent in the ORS and the surrounding connective tissue sheath, and was slightly up-regulated in the bulge region of the ORS ('Wulst'), which represents the major seat of epithelial HF stem cells. When microdissected, organ-cultured human scalp HF in the growth stage of the hair cycle were exposed to beta1-antibodies in vitro, two different activating anti-beta1-antibodies (12G10 and TS2/16) promoted hair growth in vitro: they caused a significant increase in hair shaft elongation, stimulated hair matrix keratinocyte proliferation and highly significantly inhibited spontaneous hair follicle regression (catagen development) and HF keratinocyte apoptosis. A beta1-inhibitory antibody (mAb13) did not exert significant hair growth-modulatory effects. The current results from a physiologically highly relevant organ culture assay support the concept the beta1-mediated signalling is also important in the control of human HF growth and cycling, and invite one to further explore the targeted, local stimulation of HF beta1-mediated signalling as an innovative new strategy for hair loss management.

P267

In situ targeting of epithelial stem cell populations in the human hair follicle by use of the human keratin15-promotor

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Previous work has shown the utility of the keratin15-promotor for targeting epithelial hair follicle stem cells (eHFSC) in mice. The murine keratin15-(K15)-promotor preferentially identifies hair follicle bulge cells in adult mice, and K15-mRNA is overrepresented in bulge outer-root-sheath cells, consistent with the increase in K15-protein detected in the BrdU or 3[H]-thymidine label retaining region that could be defined as the counterpart of the human anagen hair follicle bulge, a discrete microenvironment, located at the base of the part of the hair follicle that is established during morphogenesis but does not degenerate during hair cycle. Since K15-promoter-driven GFP-expression in transgenic mice has successfully been employed for cell-sorting and gene profiling of murine eHFSCs, it would be highly desirable to utilize this promoter also for demarcating eHFSCs in the human system. However, it remains to be demonstrated that this is feasible. To attempt this, we have cloned the human K15-promotor into a GFP-expression system and have transiently transfected microdissected whole, organ-cultured human anagen-VI hair follicles from normal scalp skin in vitro, using modified pEGFP-N1.Preliminary results with this technique indicate that K15-promoter-driven expression of GFP can indeed be seen in the bulge region of human hair follicle. This has not been achieved before in an intact human organ. However, since GFP expression can also be seen in unexpected human hair follicle regions not previously recognized to show K15-promoter activity, this method requires further controls, better characterization, and stringent controls. Nevertheless, this in vitro method already holds substantial promise as an experimental tool for demarcating human eHFSC and for distinguishing these from other hair follicle stem cells populations as well as from epidermal keratinocyte stem cells. In addition, targeting the human K15-promoter may become exploitable for gene and stem cell therapeutic purposes in human skin, and may be recruited as a powerful new therapeutic tool for the management of selected hair growth disorders.

P268 (V11)

Human scalp hair follicles are an extrathyroidal target and an extrapituitary source of thyroid-stimulating hormone (TSH)

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Pituitary thyroid-stimulating hormone (TSH) regulates thyroid hormone synthesis via receptors (TSH-R) expressed on thyroid epithelial cells. However, cultured human skin cells also transcribe TSH/TSH-R in vitro. Since the hair follicle (HF) is uniquely hormone-sensitive and generates other 'pituitary' hormones, we therefore asked whether human HFs are both an extrapituitary source of and an extrathyroidal target for TSH. TSH and TSH-R expression were examined by RT-PCR and immunohistology. TSH-R-mediated signaling was assessed by cAMP-measurement, DNA microarray and RT-PCR analysis of organ-cultured human scalp HFs. TSH and TSH-R were expressed in intact human scalp skin and by microdissected HFs on the gene and protein level. Thyroglobulin transcripts were also detected. TSH immunoreactivity was limited to epithelial skin compartments, while TSH-R was expressed by HF fibroblasts. Organ-cultured HFs secreted TSH into the medium. Their stimulation with TSH induced differential gene expression changes (e.g. of filamin Aα, connective tissue growth factor, acidic hair keratin-1), upregulated thyroglobulin and thyroid transcription factor-1, and induced cAMP secretion – thus documenting TSH-R functionality. These data introduce human HFs as extrapituitary sources of TSH and as extrathyroidal targets for TSH-R-mediated signaling, and suggest intrafollicular signaling between epithelial TSH and mesenchymal TSH-R as a hitherto unknown parameter in HF biology. Intrafollicular TSH-R and thyroglubulin may be targeted by 'anti-thyroid' autoimmunity, and thyroglobulin may exert peripheral tissue functions beyond thyroid hormone synthesis and storage. The human HF invites exploitation as a clinically relevant model system for dissecting non-classical, peripheral activities of major neuroendocrine messengers in a prototypic neuroectodermal–mesodermal interaction system.

P269

Elucidation of the modulatory effect of alpha-melanocyte-stimulating hormone on collagen turnover in human dermal fibroblasts

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Alpha-melanocyte-stimulating hormone (α-MSH) elicits pleiotropic biological effects on many cutaneous cell types including fibroblastic cells. We recently demonstrated that fibroblastic cells of the skin express functional melanocortin-1 receptors. A-MSH suppressed the inductive effect of transforming growth factor-beta1 (TGF-β1) on

collagen synthesis in vitro and in vivo as demonstrated in a cutaneous fibrosis model of newborn mice. In order to extend our knowledge on the anti-fibrogenic potential of α-MSH we investigate its effects on bleomycin-induced collagen synthesis. Bleomycin has previously reported to increase the RNA amounts of collagen type I in human dermal fibroblasts in vitro and is widely used as a fibrogenic agent in animal models. Moreover, bleomycin has been shown to induce fibrotic disorders in man. However, the mechanism by which bleomycin directly induces collagen synthesis is unknown. We demonstrate that α-MSH significantly reduces the RNA amounts of collagen type I and III in human dermal fibroblasts treated with non-cytotoxic doses of bleomycin in vitro. The upregulating effect of bleomycin on collagen synthesis was not due to direct induction of the smad signaling pathway since the drug did not affect nuclear translocation of smad2/3. Bleomycin also failed to induce nuclear translocation of NFκβ/p65 suggesting that the NFκβ pathway is also not involved in the mechanism of bleomycin-induced upregulation of collagen synthesis. Coincubation with actinomycin D, a blocker of mRNA transcription, largely abrogated the effect of bleomycin on collagen type I and III suggesting that bleomycin regulates collagen synthesis primarily by transcriptional upregulation and not by increased RNA stability. Most interestingly, various anti-oxidants including ascorbic acid, melatonin, and dithiothreitol, profoundly inhibited the inductive effect of bleomycin on collagen transcription suggesting that production of reactive oxidative species are crucially involved in the molecular mechanism by which bleomycin regulates collagen synthesis. Our data furthermore suggest that α-MSH uses similar effector pathways for blocking bleomycin-induced collagen transcription in fibroblasts.

P270 (V13)

Type XXIII collagen: functional analysis of a novel transmembrane collagen in skin

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Collagen XXIII, a transmembrane collagen, belongs to the growing subclass of type II orientated membrane bound collagens. A common feature of all these proteins is the presence of two versions of the protein: a membrane bound and a shed form. Spatial distribution of collagen XXIII in mouse was analysed by in situ hybridization, Western blot and immunofluorescence analysis, revealing restricted expression in skin (epidermis and hair follicles), lung, gut, brain and kidney. In brain collagen XXIII is mostly shedded whereas in the lung and the skin the full-length protein is the major form, indicating that the shedding is tissue specific regulated. To further analyse the shedding process of collagen XXIII, mutation were introduced into the two putative cleavage sites. This study clearly supported the notion that furin, a major physiological protease, is necessary for the shedding. To study the influence of the microenvironment in the plasma membrane, localization of collagen XXIII in respect to lipid raft and non-lipid raft marker proteins was analysed. Most of the protein is present in lipid rafts and the disruption of the lipid rafts through the partial removal of cholesterol clearly enhances the shedding. Biochemical screen for interaction partners using solid phase assays revealed the binding of a2b1 integrin to collagen XXIII whereas binding of the integrins a1b1, a10b1 and a11b1 was ruled out. This data was confirmed by cell attachment assay using immortalized cell lines as well as primary keratinocytes of wt and delta-a2 mice. Furthermore the interaction could be visualized by rotary shadowing of the a2-I-domain – collagen XXIII complexes.

P271

Unique and redundant functions of the collagen receptor α2β1 integrin in skin

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The α2β1 integrin functions as the major receptor for collagen type I on a large number of different cell types and has been suggested to be important for many biological processes. Surprisingly, mice deficient for α2β1 develop normally and show no obvious deficits. Therefore, we hypothesized that interactions of cells with surrounding collagens may be maintained by compensatory action of other collagen integrin receptors, such as α1β1 or α11β1, and that functional deficits would be uncovered in situations which challenge the cell-collagen interactions. In vitro functional assays showed that keratinocytes critically depend on α2β1 for adhesion to collagen type I, IV and XXIII, the latter being identified as a novel ligand for α2β1. Although skin fibroblasts can partially compensate for loss of α2β1, they fail to generate mechanical tension in a 3D collagen environment. These deficits could be attributed to alterations in focal adhesions and activation of Rho GTPases. To address the question whether challenges to the skin may uncover functional deficits in vivo in α2-deficient mice, we studied the healing of full-thickness wounds. Unexpectedly, re-epithelialization of excisional wounds of α2β1 integrin-null mice was not impaired, indicating that keratinocytes do not require contact with collagen for wound closure. Whereas wound contraction and myofibroblast differentiation was normal, scar properties and inflammatory infiltration were changed. Interestingly, formation of new blood vessels in wounds of α2β1 null mice was strongly increased. In conclusion, loss of α2β1 results in severe cellular deficits that can only partly be compensated for during wound healing.

P272

Influence of human intrinsic skin aging on the cytoskeleton – the cellular response

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As we age, the body undergoes many natural changes associated with aging skin. Genes determine the natural aging process (intrinsic aging).

On cellular level, the molecular structure and machinery shows significant alterations. The contribution of extracellular matrix components in intrinsic aging has been investigated thoroughly, however, less data exist on cytoskeletal proteins. We investigated the differential expression in human foreskins of aged probands in comparison to skin samples of young probands on transcriptional and protein level. We demonstrated reduced mRNA expression of cytoskeletal components a-plakoglobin, a-tubulin, keratin 19 and b-actin in intrinsically aged human skin by real-time PCR as well as Northern blot analyses. These cytoskeletal proteins were also less abundant in total skin protein extracts analysed by Western blot analysis. Furthermore, reduced in vivo expression of F-actin is demonstrated in cryosections of aged human skin. These molecular features of intrinsic aging highlight the importance of the cellular compartment in this process and imply a still functional relationship of the cytoskeleton and the extracellular matrix in aging.

P273 (V04)

IGF-1 receptor signaling, but not insulin receptor signaling is essential for normal skin morphogenesis

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Diabetes mellitus type II is increasingly affecting the western population and is often associated with skin complications. It is presently unclear if this is a direct consequence of impaired insulin signaling in the different skin cell types or due to secondary complications, such as hyperglycemia. To examine if insulin, or its close relative IGF-1, mediated signaling in keratinocytes directly contribute to skin complications, we have inactivated the insulin receptor (IR) or the IGF-1 receptor (IGF-1R) specifically in the epidermal compartment. Whereas the IR knockouts are viable, the epidermis-specific IGF-1R knockouts die within 1 day after birth. The epidermis of IGF-1R deficient mice is abnormally thin and translucent, similar to that found in complete IGF-1R knockouts. This shows that the number of epidermal layers is determined by cell autonomous IGF-1 signaling. However, differentiation seems to be normal in the absence of IGF-1R, as judged by H&E staining and the expression of Keratin 14, Keratin 10 and Loricrin. Nevertheless, the expression of Keratin 6 in the IGF-1R knockout skin is strongly upregulated, suggesting a pathological skin condition. Surprisingly, no obvious changes were found in either proliferation or apoptosis in newborn skin, suggesting that a change in the balance between proliferation, differentiation and apoptosis is not the reason for the thin epidermis. However, primary cultures of IGF-1R negative keratinocytes were growth impaired compared to controls when cultured in the absence of fibroblasts. Molecular characterisation of the downstream pathways showed no obvious changes in MAPK activity, whereas total AKT levels were considerably increased. At present we are further analysing the underlying mechanism by which epidermal IGF-1R signaling regulates the size and thickness of the epidermal compartment.

P274

Fibrin promotes matrix remodeling and active vessel formation in a 3D 3-cellular in vitro skin reconstruct, suitable to study wound healing in vitro

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The development of 3D multicellular tissue reconstructs is mandatory to study mechanisms in wound healing and tumor biology in an in vivo-like context. Such models should mimic key features of the skin, including 3D multicellular architecture with orthotropic cell positioning, a stable basal membrane, as well as extracellular matrix enabling vessel formation. We developed an organotypic 3D model based on a composite three-cellular skin equivalent, engineered into a dermal matrix consisting of fibrin or collagen. Fibroblasts isolated from human neonatal foreskin were combined with human microvascular endothelial cells (HMEC) within the respective matrix, overlaid after one week of culture with human primary keratinocytes, and lifted to the air-liquid-interface to form a multilayered dermo-epidermal equivalent. Epidermal differentiation, basal membrane formation, matrix remodeling and 3D vessel formation in fibrin- versus collagen-based reconstructs were investigated by immunohistology, electron-, confocal- and multiphoton microscopy. In fibrin-based reconstructs not however in collagen scaffolds, branched capillary-like networks formed spontaneously until day 7 consisting of CD31-positive cells surrounded by a type IV collagen positive layer. Electron microscopic characterization revealed active capillary vessels with pericytes and signs of transendothelial exchange. The multilayered epidermal compartment consisted of an intact basal lamina (positive for laminin-5 and collagen IV) with anchoring filaments and fibrils, physiological keratinocyte differentiation (positive for cytokeratin 10 and involucrin), and an orthokeratotic stratum corneum. The multicomponent-skin model was validated for spontaneous in vitro reepithelialization of induced punch defects in the absence or presence of wound fluids collected from acute and chronic human wounds. Regeneration of punch biopsies was fastest by using normal culture media as well as wound fluid collected from acute wounds and, conversely, delayed in chronic wound fluid. These findings establish fibrin as a major proangiogenic matrix in organotypic skin equivalents and 3D three-cellular dermo-epidermal reconstructs as a useful tool for future in vitro long-term studies of wound healing and tumor biology.

P275 (V22)

Major translocation of calcium upon barrier insult: calcium dynamics visualized by -fluorescence lifetime imaging

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Calcium controls an array of key events in keratinocytes and epidermis: localized changes in Ca2+ concentrations and their regulation are therefore especially important to assess in epidermal barrier homeostasis and repair, neonatal barrier establishment, in differentiation, signaling, cell adhesion, and in various pathologic states. Yet, tissue- and cellular Ca2+ concentrations in physiologic and diseased states are

only partially known, and difficult to measure. Here we report a method using Calcium Green as the calcium sensor and the phasor-plot approach to separate raw lifetime components. This enables us to quantitatively assess and visualize dynamic changes of Ca2+ in ex-vivo biopsies of unfixed epidermis, exploiting fluorescence lifetime imaging. Our first results comparing undisturbed epidermis with epidermis following a barrier insult revealed major shifts from intra- to extracellular, and, more importantly, a mobilization of high amounts of Ca2+ shortly following barrier disruption, presumably from intracellular stores. These results partially contradict the conventional view, where barrier insults abrogate a Ca2+ -gradient towards the SG. Methodologically, the latter is based on Ca2+-precipitation followed by electron microscopy, or proton-induced x-ray emission. Both techniques require fixed tissue, for electron microscopy also a chemical precipitation and are limited in that they can determine Ca2+ in only very small sample volumes, at or below light microscopic resolution levels, or, in the case of PIXE, determine only total calcium, irrespective of ionization or binding. So far, neither cellular and/or subcellular localization can be determined through these approaches. We believe that our approach will overcome these limitations in the observation of epidermal Ca2+ dynamics, and contribute to elucidating basic physiology as well as various pathologic situations in epidermis.

P276

Impact of sunscreen advertisements on sun protective behaviour of adolescents

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Objective: Media and fashion trends have great impact on sun protective behaviour of adolescents. The purpose of this study was to investigate the effect of sunscreen advertisements in magazines on sun protective attitude of adolescents.

Methods: Adolescents aged 15–21 years and attending different types of secondary schools (offering general/vocational education) participated in the survey. We selected five sunscreen advertisements with different settings from recent fashion magazines. In a field test, we showed each student a randomly assigned advertisement. To assess adolescents# perceptions of the advertisement the participants had to fill in a detailed and pilot-tested questionnaire. Answers were made on a 5-point Likert scale ranging from 'I strongly disagree' (rating 1) to 'I strongly agree' (rating 5).

Results: 750 students (m 46.8%, f 53.2%) from 16 schools in Vienna were included in the study. The most representative answers out of a total of 21 questions are given here: 65.2% answered that none of the sunscreen advertisements showed that sun exposure might be harmful. Four out of five advertisements gave them a strong feeling of the sun being pleasant (rating 5: 44.5%, 33.1%, 34.5%, and 38.5%) and strongly raised the desire of spending holidays in the sun. Advertisement 1 and 4 strongly convinced that tanned skin was desirable (rating 5: 34.9% and 26.6% respectively). Although all of the sunscreen advertisements conveyed to the majority of the students that sun protection is important, all of them – except advertisement 5 – also gave them the feeling that one could stay longer in the sun when using the promoted product.

Conclusion: Our study clearly shows that sunscreen advertisements have a dual message: although they convince adolescents that sun protection is important, they promote sunbathing and tanned skin as desirable. They give a false sense of security and therefore encourage extended UV exposure.

P277

Dual role of Cockayne syndrome protein B in RNA polymerase I transcription

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Mutation in the Cockayne syndrome protein B (CSB) gene leads to Cockayne's syndrome, a severe genetic disorder with autosomal recessive inheritance. The patient's phenotype is reminiscent of premature aging presenting physical and mental retardation, along with the progressive degeneration. Here, we propose the first model describing at which steps of rRNA transcription CSB is operational in vitro. Nuclear extracts from CSB-mutated cells fail to support full length 375 nt rRNA transcription. Although nuclear extracts from CSB-mutated cells successfully initiate transcription producing abortive 4nt RNA in vitro, RNA polymerase I stalls at position 12. Moreover, RNA polymerase I slides along the transcribed region to synthesize 4nt transcripts remaining bound to its transcription initiation factors. The preincubation of recombinant CSB with the rDNA promoter overcomes stalling of RNA polymerase I and is necessary to recover normal rRNA synthesis. Addition of CSB to the reaction mixture when 12nt is already formed does not restore full length transcription. Additionally, CSB stimulates RNA polymerase I enzymatic activity. Chromatin immunoprecipitation experiments revealed that CSB does not only bind to the promoter, but also to the gene internal 28S transcribed region. This imply that CSB travels with the polymerase. Taken together, these experiments revealed that CSB functions in both transcription initiation and elongation by RNA polymerase I.

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Growth factor mediated stimulation of RNA polymerase I transcription is impaired in Werner syndrome cells

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Werner syndrome is a segmental progeroid disease characterized by increased cancer and acceleration of specific age-related phenotypes, due to loss of a protein known as WRN. The early onset of symptoms related to normal aging include osteoporosis, ocular cataracts, graying and loss of hair, diabetes mellitus and arteriosclerosis. WRN possesses multiple DNA-dependent enzymatic activities (ATPase, helicase and exonuclease). The exact functions of WRN remain still unclear, but if WRN function is lost problems with replication, DNA damage processing, recombination and transcription arise.In yeast it is known that polymerase I transcription is inhibited if SGS1 function (yeast homolog of WRN) is abolished. WRN shows a serum-stimulated trafficking in human cells, leading to a migration of WRN from nucleoplasm to nucleolus, which is the side of RNA polymerase I transcription. Therefore we were interested which growth factor(s) induces the WRN accumulation in the nucleolus.Human fibroblasts transfected with pEGFP-WRN and stimulated with different single growth factors,

namely PDGF-AB, FGF-b, IGF-I and -II, EGF and VEGF, lead to an accumulation of EGFP-WRN in the nucleoli, place of Pol I transcription. Concomitant Pol I transcription is stimulated by growth factors, as shown in Northern blots. This growth factor dependent stimulation of RNA Pol I transcription is impaired in WS cells. This highly interesting finding suggests that growth factor depending signal transduction is impeded in WS cells. In addition nuclear extracts of Werner syndrome lymphoblastoid cells are inactive in 'in vitro' transcription. So the situation of diminished polymerase I transcription in vivo can be mirrored in vitro. Furthermore inactive nuclear extracts of WS cells can be reactivated in adding recombinant WRN protein to the reaction. This novel and interesting finding suggest a role of WRN in the signal transduction of growth factor signalling.

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The huPBL-SCID/skin allograft model in drug discovery

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Inflammatory diseases have a high prevalence in Western countries, and pharmaceutical companies spend increasing amounts of money to develop drugs for these disorders. However, their complex pathophysiology is only partially represented in classical rodent models, and new compounds frequently fail in clinical trials. Moreover, efficacy of agents such as antibodies against human antigens cannot be tested. Therefore, we characterized the huPBL-SCID/skin allograft model (1) and adopted it for use in drug discovery. In this model immunodeficient SCID/Beige mice are transplanted with human skin and injected with human peripheral blood mononuclear cells from a different donor, resulting in a humanized alloreactive response. We found that the model reflects several key aspects of human T cell-dependent immune diseases, such as host versus graft and graft versus host disease as well as Th1-dependent dermatitis. Thus, T cell numbers, epidermal thickness, and papillomatosis were significantly enhanced compared to control skin, as determined by quantitative analysis of immuno-histology of skin grafts. In addition, skin and plasma levels of cytokines such as IL-6, IFNg and TNFa were increased. Furthermore, the effect of immunosuppressive therapies, such as calcineurin inhibitors, could be quantified. Therapy led to significant reduction of (immuno-)histological markers and cytokine levels. In this way, the huPBL-SCID/skin allograft model is valuable for target validation and compound characterization in drug discovery.

1 Pober JS *et al.* PNAS. 1994: 91: 9146–50.

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Acanthosis nigricans als kutanes Leitsymptom des HAIR-AN syndroms

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Acanthosis nigricans ist eine relativ häufige, bei Kinder jedoch eher seltenere kutane Veränderung. Sie kann idiopathisch auftreten, häufig steht jedoch eine systemische, meist endokrinologische Erkrankung dahinter. Wir berichten über einen knapp 14-jähriges adipöses Mädchen, welches an unserer Klinik mit einer Acanthosis nigricans in den Axillen sowie am Stamm vorstellig wurde. In der anschließenden endokrinologischen Untersuchung zeigte sich eine Hyperandrogenämie sowie deutliche Hinweise einer Insulinresistenz. Im Ultraschall stellten sich in den Ovarien mehrere Zysten dar. In Anbetracht der Befunde wurde die Diagnose HAIR-AN, gestellt. Als HAIR-AN Syndrom wird das Zusammentreffen von Hyperandrogenämie, Insulin-Resistenz und Acanthosis nigricans bezeichnet. Es gilt als fünfte polyendokrinopathische Form des SAHA Syndroms (Seborrhoe, Akne, Hirsutismus und Androgenämie) neben den adrenal, ovariell, hyperprolaktinämischen und idiopathischen Formen. Die primäre Störung scheint hierbei eine Insulinresistenz mit sekundärer Erhöhung der Insulinspiegel und konsekutiver Überproduktion ovarieller Androgene zu sein. Als Ursache der kutanen Manifestation der Acanthosis nigricans werden die persistierende Hyperinsulinämie in Zusammenhang mit der Hyperandrogenämie diskutiert. Vielfach werden diese Veränderungen zusammen mit klassischen Viriliserungserscheinungen von den Patienten als störend empfunden. Der behandelnde Dermatologe sollte aber hierbei auch an die schwerwiegenderen systemischen Endokrinopathien bei Diagnose und Therapie denken.Anhand der Kasuistik werden die Charakteristika des HAIR-AN Syndroms beim adoleszenten Mädchen dargestellt.

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Quantitative extraction of tattoo pigments from skin to determine the concentration of pigments in tattooed skin

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In the western world millions of people have a tattoo to decorate the skin. Frequently tattoo colorants are mixtures of organic pigments containing also precursors and byproducts of pigment synthesis. Exposure to UV light or laser removal is proven to result in potentially toxic or carcinogenic decomposition products. To assess the health risk of tattooing and the light interaction with the respective pigments, it is important to determine the concentration of pigments in tissue. Surprisingly, this concentration is unknown. Thus, we developed an extraction method and quantified the amount of pigment that is punctured in the skin. Pigskin is tattooed using a standard tattooing machine and the original tattoo pigment Pigment Red 22 (purity of 80%), as well as the highly pure synthesised pigment with the concentration of 10% and 25% (w/vol). After tattooing, the skin specimen is punched and gently dissolved using a tissue lysis buffer. The pigment is extracted via liquid extraction with isopropanol/dichloromethane and concentrated in a keeper by heating and nitrogen stream. The samples are dissolved in methylene chloride and analysed by HPLC. Preliminary studies have yielded recovery rates of the extraction method with >95%. The amount of pure pigment punctured into skin is ranging from 140 to 500 mg per 100 cm^2 of tattooed skin. Our results show that the concentration of pigments in the skin depends critically on the size of the pigments and the concentration of the pigment applied to the skin surface. In light of these high concentration punctured into the skin we should remind the fact that the application of such pigments have no established history for safe use. Moreover, after puncturing into the skin a part of the pigments is transported away into the human body and can be found in other

organs like the lymph nodes.The work is supported by a DFG grant(BA1741/3-1).

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The effects of intense pulsed light (IPL) on blood vessels investigated by mathematical modeling

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Intense pulsed light (IPL) sources have been successfully used for coagulation of blood vessels in clinical practice. However, the broadband emission of IPL hampers the clinical evaluation of optimal light parameters. We describe a mathematical model in order to visualize the thermal effects of IPL on skin vessels, which was not available, so far.-One IPL spectrum was shifted towards the near infrared range (NIRSS, near IR shifted spectrum) and the other was heavily shifted toward the visible range (VSS, visible shifted spectrum). The broadband emission was separated in distinct wavelengths with the respective relative light intensity. For each wavelength, the light and heat diffusion equations were simultaneously solved with the finite element method. The thermal effects of all wavelengths at the given radiant exposure (15 or 30 J/cm^2) were added and the temperature in the vessels of varying diameters (60, 150, 300, 500 μm) was calculated for the entire pulse duration of 30 ms. VSS and NIRSS both provided homogeneous heating in the entire vessel. With the exception of the small vessels (60 μm), which showed only a moderate temperature increase, all vessels exhibited a temperature raise within the vessel sufficient for coagulation with each IPL parameter. The time interval for effective temperature raise in larger vessels (diameter >60 μm) was clearly shorter than the pulse duration. In most instances, the vessel temperature was higher for VSS when compared to NIRSS. We presented a mathematical model capable of calculating the photon distribution and the thermal effects of the broadband IPL emission within cutaneous blood vessels.

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Synergistic effects between podoplanin and epidermal growth factor receptor

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The 37 kDa mucin-type glycoprotein podoplanin promotes migration and adhesion of endothelial cells by reorganization of the cytoskeleton. Recently, we demonstrated strongly induced podoplanin expression on the tumor progressing side of squamous cell carcinomas (SCC). Because activation of the epidermal growth factor receptor (EGFR) is predicted to enhance the metastatic potential of SCC, and podoplanin expression is known to be increased by EGF in keratinocytes in vitro, an active role of podoplanin in epithelial tumor progression has been proposed. In the present study we investigated the putative connection between podoplanin and EGF stimulation/EGFR expression in tumor cell lines and primary cutaneous cells in vitro and in SCC, basal cell carcinomas (BCC) and psoriatic skin lesions in vivo using quantitative real time PCR, Western blot analysis and immunohistochemistry. SCC positive for podoplanin also expressed EGFR. However, while podoplanin expression was mainly restricted to the edge of tumor progression, EGFR expression was also seen in the middle of proliferating tumor strands. In psoriatic skin lesions, EGFR expression was demonstrated in all nucleated cell layers of the epidermis, whereas podoplanin was found only at the suprapapillary plates. Downregulation of podoplanin by siRNA in an SCC cell line leads to concomitant downregulation of EGFR. Therefore, based on the present findings of podoplanin and EGFR expression, we conclude that synergistic effects between podoplanin and EGFR result in specific regulation of tumor cell behaviour.

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Cross species expression studies on 5alpha reductase type I and type II in mouse, rat, hamster, and man, and evaluation of the hamster flank organ as a model for 5aRI targeting acne therapy

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Drug discovery for acne therapy is hampered by the lack of animal models. Nevertheless, hamster flank organs (FO) have been used to profile topical anti-androgens for acne. The FO model responds to oral anti-androgens and inhibition of 5alpha reductase (5aR). Yet, two isozymes of 5aR (5aRI and 5aRII) with little sequence homology and differential tissue expression exist in man, mouse, and rat. To evaluate the FO model regarding comparability and predictivity we asked whether homologues of the human 5aRI and 5aRII exist in the hamster, and if so, whether tissue distribution is comparable to man, mouse and rat.Hamster tissues were analysed by RT-PCR using primers for human, mouse, or rat 5aRI or 5aRII. Amplicons were sequenced and hamster-specific primers were then used to quantitatively analyse 5aRI and 5aRII regarding a variety of tissues including skin, FO, and sex glands. Human equivalents were analysed using a RNA array technology.The expression profiles of 5aRI were similar in mouse, rat, and hamster, while differences existed in man. Thus, 5aRI predominated in rodent liver, followed by preputial glands (mouse, rat), FO (in hamster), and brain. 5aRI mRNA in normal rodent skin was not or only moderately stronger than in lung, testes, or kidneys. In contrast, 5aRI predominated in normal human skin followed by liver and different parts of the brain. In rat, 5aRII mRNA predominated in epididymis over testes and prostate while virtually all other tissues were free of 5aRII. Expression patterns in hamster were similar, yet with smaller differences and greater interindividual variation. In humans, 5aRII predominated in epididymis over liver, thalamus, trachea, and bladder, while all other organs were devoid of 5aRII. In conclusion, 5aRI and II homologues were demonstrated in the hamster for the first time. Rodents including the hamster show a similar 5aRI and II distribution pattern. However, 5aRI expression is more prominent in human than in rodent skin. Preputial glands of rats and mice and hamster FO may in contrast be suited for 5aRI inhibition studies. Caution should be applied though since inhibitors specific human 5aRI may lose isozyme specificity in rodents due to limited 5aR sequence homology between the species.

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The 'follicular trochanter': an uncharacterized compartment of the human hair follicle epithelium in the bulge region

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The 'bulge' region of the outer root sheath (ORS) of both mouse and human hair follicles, i.e. the area where the arrector pili muscle (APM) inserts into the upper ORS, is now firmly established as at least one major site of epithelial hair follicle stem cells. During the embryonal development of human scalp hair follicles, two separated ORS protrusions begin to grow at the posterior side of the follicle. At first, the lower one, the 'bulge', is the larger one of the two, but later on it becomes relatively smaller. The upper one develops into the sebaceous gland and its duct. The lower one becomes the future attachment site of the developing APM. Normally, the APM attaches to this central ORS compartment without any major ORS protrusion detectable. Even though this special ORS region had already been described in human hair follicles by 19th century authorities and is prominent during fetal human hair follicle development, it is difficult to identify in adult human hair follicles. Therefore, the insertion of the APM is generally employed as a lead structure to point one to the bulge region, whose identity is then further supported by immunohistology, using e.g. cytokeratin 15 and CD200 immunostains. However, among 144 examined normal scalp hair follicles from 12 different individuals of both sexes, we noted a stringently localized ORS protuberance at the site of APM insertion in 8% of the hair follicles. This protuberance occurred in 42% of all the different human scalp skin samples we examined. This suggests that the occurrence of this structure is not that rare and that it is more than a biologically irrelevant structural peculiarity displayed only by the odd individual. This beak-, hook- or papilloma-like protrusion of the ORS may reflect 'snapshots' of distinct developmental stages in a dynamic process. It will be interesting to clarify in future studies, whether these different developmental stages are linked to distinct stages of hair follicle cycling.

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The German Network for Systemic Scleroderma: new data on organ involvement and immunosuppressive therapy based on a registry encompassing 1556 patients.

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Systemic scleroderma is a rare heterogeneous disease, characterized by different degrees of organ involvement and disease progression. To improve clinical care and provide well-characterized biologic material a German Network for Systemic Scleroderma (DNSS) was established, presently including 37 centers. The Network gathers principal subspecialties involved, i.a. dermatology, rheumatology, pulmonology and nephrology. To date, the Network's patient registry contains data on diagnosis, organ involvement and therapy of 1556 patients. An overview of disease subgroups shows that 47% suffer from lim. disease, 31% from the diffuse form, 9% from an overlap syndrome, 7% from an undifferentiated form and 1% from scleroderma sine scleroderma. Pulmonary hypertension was present in both groups (19% dSSc, 13% lSSc), while pulmonary fibrosis was more often in dSSc patients (58% vs 22%). Centers also used a novel urine-microelectrophoresis and discovered early grades of renal damage in up to 50% of patients. Renal dysfunction was reported in 13% of diff. and lim. patients; renal crisis was rare (1%). GI involvement appeared in 69% of dSSc- and 55% of lSSc-patients. Distribution of ANAs showed that patients with lSSc were mostly affected by ACA (43.5%), while patients with dSSc were most frequently Scl-70 positive (45.9%). Use of corticosteroids has previously been shown to damage kidney function. However, a detailed analysis of DNSS-patients' treatment demonstrated that it still plays an important role in SSc general therapy. Immunosuppressants used included methotrexate (7.8%), azathioprine (6.4%), cyclophosphamide (5.8%), chloroquine (2.3%), cyclosporine A (1%) and others. 35% of dSSc-patients and 15% of lSSc-patients received immunosuppressants. The multidisciplinary approach also allowed to differentiate between therapeutic preferences. Data demonstrate that dermatologists used significantly less systemic therapies in their patients.

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Skin surface ammonium levels: differences between smoking and non-smoking women when regarding ammonium levels with respect to localization and age

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Both mainstream and sidestream cigarette smoke contain high amounts of ammonia (NH3) that may be converted into ammonium ions (NH4+) when reaching the acid environment of the upper epidermis. In consequence the amount of NH4+ on the skin surface might indicate cutaneous effects of cigarette smoking. Therefore this study aims at investigating if analysis and comparison of NH4+-levels reveal differences between smokers and non-smokers. Overall 20 female non-smokers (aged 18–60) and 20 female smokers (aged 18–60) were enrolled. NH4+-levels were determined on the forearm, forehead and palms using a spectrophotometric assay (Spectroquant® Ammonium-Test, Merck). The statistical analysis consisted of comparing the average NH4+-values of the non-smokers and smokers at corresponding sites of investigation, of comparing the values assessed at different sites as well as comparing age-dependent changes of the NH4+-levels in the two groups. The comparison of the NH4+-levels at corresponding sites of investigation showed no significant difference between smokers and non-smokers (all $P > 0.05$). Analysis of the NH4+-levels of the different sites of investigation revealed significant differences between the localizations in the non-smokers (all $P < 0{,}01$). In smokers there were significant differences between the NH4+-levels of the palms and the two other sites ($P < 0.01$), but not between forearm and forehead ($P = 0.158$). Regarding the NH4+-values with respect to age an increase of the NH4+-amount with age could be

measured in the forearm and the forehead region of smokers and non-smokers. On the palms there was an increase with age in the non-smokers and a decrease with age in smokers. Correlation of the NH4+-levels with age revealed significant results on the forearm and forehead of the non-smokers ($r = 0.472$; $P = 0.036$), ($r = 0.494$; $P = 0.027$). No significant correlation could be found on the palms or in the smokers (all $P > 0.05$). The results obtained show no major differences between smokers and non-smokers when comparing absolute NH4+-values. On the other hand when relating the NH4+-values to localization and age several differences could be assessed. This indicates rather complex effects of smoking on the NH4+-levels. Further studies are needed to elucidate possible mechanisms leading to these changes.

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A widely applicable optimized protocol for lentivirus production and virus concentration

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Lentiviral gene delivery has received widespread interest due to its enormous potential both for in vitro as well as gene therapeutical applications. The achievement of adequate virus titers is very sensitive to numerous factors including producing cell line, DNA purity, packing system etc. and represents the greatest limitation in lentiviral technology. Reported protocols for virus production in general are optimized using a single transfer vector, thus commonly limiting their applicability for transfer vectors designed of modified by other groups. Here we show that the efficiency of a frequently employed protocol for lentivirus production, though robustly allowing titer generation of 10exp7 units/ml with small transfer vectors, greatly decreases when applied to vectors exceeding a size of 9 kb. By contrast, a modified protocol allows efficient titer generation in five different vectors tested up to a size of 13 kb. Addition of the histone deacetylase inhibitor sodium butyrate further increases virus titer specifically in large vectors. Across various virus production protocols we find that virus titer correlates most closely with the size of the packaged insert ($r = 0.92$), rather than the size of the complete transfer vector. Finally, we demonstrate that the efficiency of lentivirus concentration by ultracentrifugation also depends on the size of the packaged insert. We describe a modified concentration protocol allowing virus recovery of more than 50% even in virus particles packaged with inserts as large as 7.4 kb. The protocols described herein should greatly facilitate the production and concentration of a wide range of lentiviral vectors.

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Both immunoadsorption (IA) and rituximab (Rtx) induce prolonged clinical responses in *Pemphigus vulgaris* (PV)

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PV is a severe and at times a life-threatening autoimmune bullous disorder associated with IgG against desmogleins (Dsg) 1 and 3, components of the desmosomal adhesion complex of epidermal keratinocytes. Despite advances in immunosuppressive treatment, therapy-resistant PV still remains a major clinical challenge. Recently, IA has been shown great promise in reducing pathogenic serum IgG autoantibodies. Rituximab (Rtx) a monoclonal antibody against CD20 on B-cells has been highly effective in the treatment of refractory *Pemphigus*. The aim of this study was to follow up on patients with recalcitrant PV who had received either IA or Rtx, respectively, utilizing a recently established autoimmune bullous skin disorder intensity score (ABSIS), and ELISA with recombinant Dsg 3 and 1. Five PV patients with extensive skin involvement (55 ± 13.9 years; treatment duration: 27 ± 6.8 days) were subjected to IA while five patients with predominantly severe mucosal PV (age 55 ± 19.1 years; treatment duration: 21 ± 3.7 days) were subjected to Rtx treatment. Both, IA and Rtx groups showed a significant decrease of anti-Dsg3/1-specific IgG within the first 2 months (IA.: 80% reduction; Rtx: 60% reduction) and a further decrease within 6 months (IA: 90%; Rtx: 60–70%). In the IA group, cutaneous involvement was determined by the ABSIS skin score and was reduced by 80% (after 2 months) and 95% (after 6 months), respectively. In the Rtx group, the ABSIS mucosal score documented a decrease of oral lesions by 60% (after 2 months) and 80–90% (after 6 months). Systemic prednisolone was given orally in the IA group at 0.34 ± 0.1 mg/kg (after 2 months) and at 0.15 ± 0.1 mg/kg (after 6 months), while patients on Rtx received prednisolone at 0.24 ± 0.3 mg/kg (after 2 months) and at 0.05 ± 0.1 mg/kg (after 6 months). Thus, both IA and rituximab had a comparable effect on the clinical activity and anti-Dsg autoantibody titers in cutaneous dominant and mucosal dominant PV, respectively.

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