V01

Dependence of functional lymphoid tissue on lymphotoxin-a

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Antibody-cytokine fusion proteins (FP) can efficiently target cytokines to the appropriate antigen-source. In this regard, we previously demonstrated that targeting of lymphotoxin-a (LTa) to the microenvironment of melanoma metastases elicited an efficient immune response associated with the induction of a peripheral lymphoid-like tissue in C57BL6J mice. To scrutinize the relevance of the induced tissue we performed a series of experiments in LTa $^{-/-}$ mice which lack peripheral lymph nodes. To this end, treatment of tumor-bearing LTa $^{-/-}$ mice with an antibody-LTa FP led to the eradication of established tumors. Macroscopic examination of tumor residues revealed the presence of oval-shaped, opalescent structures resembling lymph nodes. Indeed, immunhistochemistry analyses of tumor sites of treated animals demonstrated a strong inflammatory infiltrate with defined T- and B- cell areas, similar to the architecture of lymph nodes. Furthermore, *in situ* TRP-2/K^b tetramer staining revealed the presence of expanded numbers of tumor specific T cells among the infiltrate. Since immune responses in LTa $^{-/-}$mice are not totally devoid, we tested if the antibody-LTa FP only enhanced a preexisting immune response. Thus, we performed experiments with an antibody-IL2 FP known to boost preexisting immune responses. In melanoma-bearing wild type mice the IL2 FP treatment led to the destruction of established tumors, whereas this treatment in LTa $^{-/-}$mice had no therapeutic effect. Additional transfer of tumor conditioned T cells, however, restored the therapeutic effect of IL2 FP administration. Thus, our experiments demonstrated that LTa FP treatment induced a tertiary lymphoid tissue at the tumor site capable of priming tumor-specific T cells.

V02

Generation of regulatory T cells from naive human and murine T cells by retroviral gene transfer of Foxp3

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Immunological unresponsiveness to self is mediated in part by regulatory CD4+CD25+ T cells (Treg), which can actively suppress self-reactive T cells that have escaped thymic selection. The isolation of large numbers of Treg is difficult, since CD4+CD25+ cells constitute only a small subset of CD4+ T cells. Recently, the transcription factor Foxp3 has been identified to be a key regulatory gene for the development of Treg in mice and man. The Foxp3 cDNA was cloned into a MCSV-based retroviral vector encoding eGFP under control of an IRES site and used to transfect the GPE86 packaging cell line. For controls a retrovirus containing only eGFP was employed. Retrovirus-containing culture supernatants were utilized for Foxp3 gene transfer into murine naive CD4+CD25- T cells to investigate if these cells would develop a regulatory phenotype. After transfection sorted eGFP+ T cells were stimulated. Control virus-transfected T cells proliferated, whereas Foxp3 transfected T cells were anergic. Flow cytometry analysis of Foxp3 transfected T cells revealed expression of CD25, CTLA-4, PD-1, CD103, and neuropilin-1, all molecular markers associated with Treg. Subsequently, GFP+ T cells were added to CD4+CD25- T cells to study their suppressor function. Naive CD4+CD25- cells that were control virus-treated failed to inhibit T cell proliferation. In contrast, naive T cells that had been transfected with the Foxp3 containing retrovirus showed strong suppressor activity. A mechanism by which Treg suppress other T cells is the production of IL-10. Only Foxp3 gene-transfected T cells but no control virus-treated T cells expressed IL-10. We are currently using Foxp3 transfected T cells for the treatment of systemic autoimmunity in K14-CD40L transgenic mice in order to ameliorate disease. In other experiments gene transfer of Foxp3 into naive human CD4+ T cell lines induced suppressor activity and also the expression of Treg-associated surface molecules. Together, gene transfer of Foxp3 converts naive murine and human T cells towards a regulatory phenotype similar to that of natural Treg. Furthermore, Foxp3 gene transfer may be useful to generate large numbers of Treg for immunotherapy of autoimmune disorders

V03

Ephrin-B2 dependent cell-cell contact signaling induces migration competence and apoptosis resistance in B16 melanoma cells

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Ephrins are well characterized cell-surface bound cell-cell signaling molecules that represent key regulators of cell migration in organogenesis. Since they are frequently overexpressed in various cancers, a functional role in cancer progression has been postulated, but the evidence is still scarce. Based on our findings that Ephrin-B2 is upregulated in the invasive front of advanced human malignant melanomas (MM) and metastases, we addressed the question, how this pathway may contribute to MM progression. Therefore, we studied the effects of both constitutive overexpression by transfection of B16 cells with the complete or a dominant-negative, signaling-deficient construct, and the transient activation by treatment with recombinant Ephrin-B2 activating fusion-proteins: Upon activation of this pathway the cells adopt a highly migration-competent phenotype characterized by induction of multiple lamellipodia, a high rate of actin polymerisation, activation of focal adhesion kinase (FAK), and integrin-receptors. Functionally, this confers a significant increase of attachment of the cells to fibronectin coated surfaces. Furthermore, the migratory net effect is a significant increase of migration/invasion in both scratch wound healing assays and in Boyden chambers. Apart from that, we show that due to activation of MAP kinases (JNK), there are also multiple changes occurring on the transcriptional level. Affymetrix® chip analyses revealed that Ephrin-B signaling is linked to upregulation of 14-3-3 zeta, a co-activator of the B-Raf pathway, and several other factors (SOX-4, ATF-2), which have been linked to the vertical growth phase and the metastatic phenotype. Consequently, in C57Bl6-mice, subcutaneous MM expansion was significantly reduced, if dominant-negative transfected cells were injected compared to mock-transfected ones. Accordingly, the rate of apoptotic, TUNEL-positive cells was grossly enhanced. We conclude, that cell-cell contact signals via the Ephrin-B pathway can modulate migration and invasion of MM cells. Further links to other cancer-pathways (B-Raf, apoptosis) make it a candidate for molecular targeted future therapies.

V04

$cFLIP_L$ inhibits death receptor-mediated NF- ?B activation and apoptosis induction at the death-inducing signalling complex (DISC) in human keratinocytes

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Human keratinocytes express death receptors like CD95, TRAIL-R1/-R2 and undergo apoptosis following treatment with TNF-related apoptosis-inducing ligand (TRAIL) or CD95 ligand (CD95L). The intracellular inhibitor $cFLIP_L$ inhibits death receptor-mediated apoptosis and is highly expressed in primary human keratinocytes but absent in transformed HaCaT keratinocytes. In order to study the role of $cFLIP_L$ in more detail, we established a panel of stable monoclonal $cFLIP_L$-overexpressing HaCaT keratinocytes by viral transduction using a retroviral bicistronic vector system. Functional analysis revealed that relative $cFLIP_L$ levels correlated with resistance to TRAIL in these lines, while CD95, TRAIL-R1-R4 and initiator caspase expression were unchanged compared to control lines. Biochemical characterization of caspase activation following TRAIL treatment showed that complete caspase 8 activation was inhibited at the death inducing signalling complex (DISC) in $cFLIP_L$-overexpressing lines, while DISC recruitment and partial cleavage of caspase 8 to p43/41 was unaffected. We next asked if DISC-mediated nonapoptotic signals like activation of the transcription factor NF-?B are modulated by $cFLIP_L$. Surprisingly $cFLIP_L$ specifically blocked TRAIL-induced NF-?B activation and TRAIL-dependent induction of the NF-?B target gene IL-8 on RNA and protein level.

Our data demonstrate that $cFLIP_L$ is not only a central anti-apoptotic modulator of death receptor signals, but also an inhibitor of TRAIL-induced NF-?B activation and proinflammatory target gene expression. $cFLIP_L$ may thus represent a central "silencer" of proapoptotic and proinflammatory responses to death receptor ligation in keratinocytes and its modulation may not only influence apoptosis sensitivity but may also lead to altered death receptor-dependent inflammation of the skin.

V05

CCL18 is upregulated in atopic dermatitis and mediates *in vivo* recruitment of T cell into human skin

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The chemokine CCL18, also known as Parc/DC-CK1, is produced by monocytes, myeloid dendritic cells (DC) and germinal center DC. The receptor of CCL18 is still not identified but CCL18 characteristically attracts na? T cells and has therefore been implicated in the induction of immune responses. However, CCL18 expression is not restricted to lymph nodes. Particularly, CCL18 has been detected in inflamed lesions of allergic lung disease. The secretion of CCL18 is increased by Th2 cytokines such as IL-4/IL-10 and decreased by IFN-?. Since atopic dermatitis (AD) is Th2 cytokine mediated, we aimed to investigate the role of CCL18 in this disease. Interestingly, the expression of CCL18 was strongly upregulated in peripheral blood derived antigen presenting cells (APC's) from patients with atopic dermatitis compared to healthy individuals, as assessed by intracellular cytokine staining and ELISA after 48h culture with or without IL-4 stimulation. Most importantly, CCL18 expression was detected in the upper dermis of lesional atopic dermatitis skin in a series of samples, whereas CCL18 could not be detected in normal skin. Moreover, co-staining revealed that CCL18 expression was restricted to HLA-DR$^+$ APC's confirming our *in vitro* results. Using FITC-labeled CCL18 as a tool to detect receptor expression, we demonstrated CCL18 binding predominately on naïve CD45RA$^+$, CCR7$^+$, CD62L$^+$ peripheral blood CD4$^+$ and CD8$^+$ T cells. However, a fraction of memory effector CD4+ Th cells isolated from the skin of AD patients also bound CCL18-FITC. Similar results were obtained using a long term cultured AD skin derived Th cell clone, which in addition migrated in response to CCL18. To further investigate the relevance of CCL18 for the pathophysiology of AD, the chemokine was evaluated in an *in vivo* skin homing assay using SCID mice transplanted with human skin grafts (SCID-hu skin mice). Indeed, in contrast to PBS, injection of CCL18 mediated recruitment of human CFSE labeled T cells to human skin grafts *in vivo*. Thus our results implicate that CCL18, in addition to recruiting na? T cells to APC's, contributes to the recruitment and/or maintenance of T memory effector cells to inflamed AD skin.

V06

Keratin5-Cre/LoxP mediated deletion of Vascular Endothelial Growth Factor severely compromises Mammary Gland Function

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In the adult, the process of blood vessel formation, or angiogenesis, is restricted to settings of increased vascular requirements, such as wound healing, tumor growth and the female reproductive tract during pregnancy and lactation. Expression of the blood vessel endothelial cell mitogen, Vascular Endothelial Growth Factor (VEGF A), is upregulated in keratinocytes during wound healing and epidermal tumors, and has been shown to be regulated in concert with mammary gland development during pregnancy and lactation, suggesting that this factor is important for angiogenesis in these settings. Using the Cre/LoxP system under the control of the keratin5 promoter, we have generated mice in which VEGF has been inactivated in all keratin5 expressing tissues, including the female reproductive tract. We have shown previously that in these mice (K5/Cre VEGF F/F) wound healing is delayed and tumor formation is virtually abrogated, and now report that mammary gland development and function in mutant female mice is severely compromised: pups suckled by mutant female mice have less milk in their stomachs compared to those suckled by controls, and gain weight much more slowly. Mammary glands of pregnant and lactating mutant females show a reduced development of ducts, with fewer lobules, and a reduced number of proliferating ductal cells. In accordance with the reduced amount of milk, the milk proteins casein and WAP are also decreased in the mutant mice. In addition, mammary gland morphogenesis in mutant virgin mice during puberty is retarded compared to controls. We conclude that VEGF contributes to virgin mammary gland development, but may be able to be replaced by other angiogenic factors, such as PlGF. In contrast, VEGF appears to be more important for mammary gland differentiation and function during pregnancy and lactation, since inactivation of VEGF in mammary gland epithelia results in a severe reduction of milk production.

V07

CXCR6 and its ligand CXCL16 mediate T cell recruitment into psoriatic skin

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In recent years, much effort in research has focussed on the identification of tissue and disease specific chemokine receptors. Targeting major receptors of an inflammatory process will offer for the first time the possibility for disease specific therapeutic interventions. To this end, chemokine receptor expression was analyzed in psoriatic skin derived T cell lines and compared to PBL derived lines from the same donors. Remarkably, CXCR6 was expressed more prominently in the skin derived T cell lines, both in the CD4$^+$ and CD8$^+$ subset. Moreover, CXCR6 expression was exclusively found in the CCR7$^-$ population, marking them as effector memory T cells ready to respond vigorously to activation. Indeed, these CXCR6$^+$ T cell lines produced the Th1 cytokines IL-2 and IFN-? but not Th2 cytokines like IL-4. In the contrary, Th cells from atopic dermatitis with a Th2 cytokine pattern were all CXCR6 negative. The CXCR6$^+$ CD4$^+$ and CD8$^+$ T cells from psoriatic skin responded to the CXCR6 ligand CXCL16 as determined by intracellular Calcium mobilization assays. Moreover, psoriatic skin derived T cells migrated to CXCL16 *in vitro* and, *in vivo*, CXCL16 recruited human T cells to human skin grafts previously transplanted onto SCID mice. Analysis of expression profiles by immunohistochemistry demonstrated the presence CXCR6$^+$ T cells in psoriatic skin. Comparing different leukocyte subsets as well as resident skin cells for the production of CXCL16, monocytes were identified as the major source of CXCL16 by intracellular FACS staining and protein analyses by ELISA. Consequently, CXCR6+ T cells migrated in response to monocyte supernatants. As monocytes may produce several T cell attracting chemokines, the relevance of this finding was further investigated by neutralizing CXCL16. Indeed, addition of blocking anti-CXCL16 mAbs to the supernatants or CXCR6 desensitization by adding an excess of CXCL16 to the T cells strongly inhibited this migration. These investigations indicate that CXCL16 and CXCR6 are new and apparently important players in the process of psoriatic inflammation. Thus, targeting CXCR6 in psoriasis may be a new therapeutic approach which not only reduces skin inflammation but is also devoid of systemic side effects as seen with immunosuppressants.

V08

\`UV-induced tolerance is thymus-dependent\`

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\`Application of haptens onto UV-exposed skin does not result in induction of contact hypersensitivity (CHS) but induces hapten-specific tolerance which can be adoptively transferred. This is mediated by the generation of suppressor T cells which belong to the CD4+CD25+ subtype of regulatory T cells (Treg). In general, Treg have been recognized to play an important role in preventing the development of various autoimmune disorders, including diabetes and thyroiditis. Thus, the primary function of Treg appears to be the inhibition of autoreactive T cells which have not been eliminated by negative selection in the thymus (central tolerance). Therefore, Treg are important components of peripheral tolerance. Although the involvement of Treg in UV-induced tolerance is undisputed, it is still unclear how and where UV-induced Treg are generated. To study whether the thymus is involved in this process, C3H/HeN mice were thymectomized at day 3 after birth. At the age of 8 to 10 weeks, CHS against dinitrofluorobencene (DNFB) was induced by epicutaneous application. In comparison to sensitized control mice, thymectomized mice showed a more pronounced CHS response, implying that the generation of negative regulatory T cells but not of T effector cells may be impaired by thymectomy. Application of haptens onto UV-exposed skin did not result in sensitization in control mice, while the CHS response was not suppressed in UV-exposed thymectomized mice. Resensitization of the mice after 2 weeks with the same hapten did not cause sensitization in control mice, indicating that tolerance had developed. In contrast, thymectomized mice showed an unimpaired ear swelling response upon resensitization, suggesting that UV-induced tolerance does not develop in thymectomized mice. Finally mice which were thymectomized at day 3 received a retransplantation of age-matched thymuses into the kidney capsules at week 8. Upon thymus retransplantation originally thymectomized mice regained the ability to develop UV-induced tolerance. Taken together, the data demonstrate that development of UV-induced tolerance and consequently the generation of UV-induced regulatory T cells is thymus dependent. \`

V09

Deficiency of the Retinoblastoma Binding Protein 2-Homolog 1 (RBP2H1) in human malignant melanoma: A new mechanism contributing to uncontrolled growth.

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Using RNA arbitrarily primed PCR, we identified transcripts with cell cycle-related differential expression in human melanocytes. Among the partial cDNAs cloned, a novel cDNA was identified encoding a 174 kDa protein with 54% identity to the recently characterized retinoblastoma binding protein 2, a cell cycle controller that directly stabilizes active, hypophosphorylated pRB (retinoblastoma protein). Therefore, the new gene was termed retinoblastoma binding protein 2-homolog 1 (RBP2H1). A computerized sequence analysis of the cloned RBP2H1 revealed highly conserved motifs with possible subcellular regulatory potential: Two DNA-binding zinc finger/LAP protein motifs indicate a possible role as transcription factor. More interestingly, a further domain (non-T/E1A-pRB binding domain) possibly mediates direct binding and interaction with pRB. Subsequent analyses showed an ubiquitous expression of RBP2H1 in normal tissues, e.g. adult thymus, uterus, adult and fetal brain, colon, lymphocytes and, most intensely, in the testis. In contrast, real time TaqManTM-RT-PCR showed a significant downregulation of RBP2H1 in malignant melanomas (MM) and melanoma metastases (MMM) compared to common melanocytic nevi (MN) [MN: n = 6; MM: n = 5 and MMM: n = 7], which was further substantiated by in-situ-hybridization. Based on these findings, we suppose a potential role of RBP2H1 in melanoma progression due to a loss of modulation of the cell cycle controller pRB. In order to confirm this concept, a melanoma cell line (A375-SM) was stably transfected either with RBP2H1 full length cDNA or with a truncated construct (missing the non-T/E1A pRB-binding domain). The results show reduced cell growth in cells with re-established full-length RBP2H1 expression. Furthermore, Western blot analysis with antibodies raised against three different phosphate residues of pRB (Ser795, Ser780, Ser807/811) could detect hypophosphorylation in full-length transfected SM cells compared to non-transfected cells and cells transfected with the truncated version. We conclude that RBP2H1 exerts G1/S transition control by stabilizing the active, hypophosphorylated pRB. Our study adds a novel mechanism to the current concepts of how melanomas can escape from growth control, which may ultimately lead to new therapeutic options.

V10

Design of a novel proteolysis resistant $VEGF_{165}$ variant

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During cutaneous wound repair the development of granulation tissue requires the formation of new capillaries and the absence of this tissue is a typical feature of chronic non-healing wounds. Recently we demonstrated that in chronic venous ulcers plasmin cleaves and inactivates VEGF165 protein, indicating that VEGF proteolysis may constrain VEGF mediated actions in non-healing wounds. These data inspired us to investigate whether VEGF165 can be proteolytic stabilized by mutating the plasmin cleavage site. This type of modification would be expected to increase the period that topically applied VEGF protein is active in the chronic wound environment. By a site directed mutagenesis approach we generated novel, active plasmin resistant VEGF165 variants. As indicated by western blotting and endothelial cell proliferation assays substitution of Arg110 with Ala110 or Gln110, and Ala111 with Pro111 yielded plasmin-resistant and biologically active VEGF165 mutants. In addition, substitution of Ala111 with Pro111 resulted in a substantial degree of stabilization when incubated in wound fluid obtained from non-healing wounds. To analyze whether the regressive endothelial response in impaired healing wounds can be rescued by topical application of VEGF165 we evaluated the overexpression of VEGF165 wild type and VEGF mutant plasmid DNA in a wound healing impaired mouse model. As revealed by immunohistochemical staining for PECAM-1 and the VEGF receptor flk-1 a single application of the plasmid cDNA encoding VEGF165 wild type in excisional cutaneous wounds could significantly promote angiogenesis and wound closure. Preliminary data indicate that in comparison to VEGF165 wild type protein proteolysis resistant VEGF prolongs the angiogenic response in this mouse model. Our data provide a molecular strategy to stabilize the VEGF165 molecule in the proteolytic environment of chronic wounds and provide preclinical data indicating that the topical application of a proteolysis resistant VEGF165 molecule may be regarded as a novel therapeutic tool for the treatment of chronic wounds.

V11

Cancer Regression induced by Immunostimulatory CpG Oligodeoxynucleotides in Patients with Metastatic Melanoma ? the Results of a Clinical Phase II Trial

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Stimulation of Toll-like receptor (TLR) 9 by pathogen-derived compounds leads to direct activation of human antigen-presenting plasmacytoid dendritic cells (pDC) and B cells and indirectly dramatically increases cytotoxic T and natural killer cell responses. The synthetic oligodeoxynucleotide CPG 7909 contains CpG motifs optimized to specifically interact with TLR9 and is a strong activator of both innate and specific immunity. CPG 7909 crossreacts with mouse TLR9 and has shown impressive antitumor activity in preclinical tumor models when used as monotherapy.
In a clinical phase II trial in stage IV melanoma patients (n=20), we applied a fixed dose of 6 mg CPG 7909 s.c. at weekly intervals. To particularly trigger tumor-specific adoptive immunity, we preferentially injected CPG 7909 in the surrounding of peripheral lymph nodes draining tumor-bearing skin areas. This treatment induced objective tumor responses -as assessed by EORTC-RECIST guidelines- in 2/20 patients and significant antitumor activity in further 3 patients. Adverse events included transient injection site reactions, fever, and arthralgias. Hematological and non-hematological toxicities were limited. So far (7/20 pts.), FACS-based phenotyping of PBMC revealed consistent activation of BDCA-2+ pDC with increased CD86 and HLA-DR expression, activation of CD4+ T cells with increased numbers of CD4+CD40L+ cells, and an increased frequency of CD19+CD38++ plasma cells (IgM-producing) during CPG 7909 therapy. The one responding patient analyzed thus far, was found to have additional changes with increased numbers of activated CD8+CD40L+ T cells as well as CD8+CCR7-CD45RA- effector memory lymphocytes and decreased frequencies of IL-10-producing CD4+ and CD8+ T cells. Consistent changes in NK cell activity were not detected, assays to determine frequencies of antigen-specific T cells are ongoing. We conclude that CPG 7909 monotherapy exerts objective anti-tumor activity in melanoma patients, is well-tolerated, can be applied safely and induces a phenotypic signature in PBMC associated with exposure and, possibly, response to therapy.

V12

Mutations in ALOX12B and ALOXE3 cause decreased synthesis and impaired activity of epidermal lipoxygenases in autosomal recessive congenital ichthyosis (ARCI).

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Autosomal recessive congenital ichthyosis (ARCI) forms a heterogeneous group of severe hereditary keratinization disorders showing clinical and genetic heterogenity. Up to present five loci of ACRI have been mapped to chromosome 2q33-35, 14q11, 17p13, 19p13 and 19p12-q12. Mutations in TGM1 on chromosme 14q11 account for approx. one third of ARCI cases. We and others have identified several different mutations in ALOX12B and ALOXE3 on chromosme 17q13, which code for two different epidermal lipoxygenases, in patients with ARCI from Turkey, France, North Africa, Germany, Czechia and India.
Epidermis-type lipoxygenases comprise newly identified members of the protein family of lipoxygenases (LOX), synthesized preferntially in the skin. Corresponding to other LOX 12R-LOX metabolizes unsaturated fatty acids, like arachidonic acid, to hydroxyperoxyderivates (HPETEs). ELOX-3, however, does not oxydize those typical LOX substrates, but converts HPETEs to distinct epoxyalcohols. In order to determine the enzyme activity using the genuine substrates, we have expressed recombinant ALOXE3 and ALOX12B in HEK cells. We could demonstrate that the product of 12R-LOX is the educt of eLOX-3, thus both enzymes are subsequent members of the same pathway. Until now we have found a total of eight different point mutations in these genes. Recombinant, mutant ALOXE3 and ALOX12B was generated by site-directed mutagenesis. We could show slightly reduced expression as compared to the wildtype. Furthermore, enzymatic activity was completely abolished in mutant LOX proteins. So we could demonstrate that mutations in ALOXE3 and ALOX12B result in deficieny of unsaturated fatty epoxyalcohols, as 12R-LOX and eLOX-3 are involved in the same disease mechanism. These findings shed new light on the question of genotype/phenotype correlation in ARCI and explain the similar ARCI phenotype caused by mutations in different genes.

V13

The dynamics of T-APC encounters visualized in living mice by intravital microscopy

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Interaction between antigen-presenting cells and lymphocytes in lymphatic tissues is the basis for the antigen specific activation of T cells. There is currently considerable debate as to the kinetics of this process in vivo. To clarify these issues we intended to visualize T cell APC interactions in the lymphatic tissue of living mice.
Bone marrow dendritic cells (bmDC) or naïve B cells were loaded with a peptide of chicken ovalbumin which is recognized by TCR transgenic CD4 T cells from the DO11.10 mouse model. After staining with different fluorescent dyes, bmDC were injected subcutaneously close to the inguinal node of a Balb/c mouse. B cells and T cells were given i.v. At different time points after the injection mice were anaesthetized, ventilated and the inguinal lymph node was prepared without disrupting its vascularization. The blood and lymph flow was visualized via injection of a dye labelled protein, while surrounding cells in the node and matrix fibres were stained with a variety of dyes. Imaging of migrating cells and blood/lymph flow as well as the dynamics of the associated lymph fiber network were visualized over periods of >10h by using time lapse fluorescence, confocal and 2-photon microscopy.
APC´s were easily detected in lymph nodes. Their dynamics were conserved during the preparation. After injection T cells and DC homed efficiently to the T cell zones of the lymph node, while B cells resided almost exclusively in follicles. There was profound migration of most visible cells within the lymph node parenchyma, which is rather remarkable given the dense packing in the node. Individual short contacts between T cells and B cells were obvious, especially at later time points. Observable contacts between DC and T cells were heterogeneous in duration. DC were unique in being able to establish contacts to far away T cells via long dendrites and to serve multiple T cells simultaneously. In summary, we are able to detect T-APC interactions in vivo, which are very dynamic, as has been shown previously in vitro. The next step is the optimization of the imaging for long-term 4-D reconstruction of large volumes with as much additional information on the surrounding environment as possible.

V14

Macrophage dependent inflammatory skin disease resembling psoriasis in mice with epidermis specific deletion of I kappa B kinase 2

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Nuclear factor kappa B (NF-?B) proteins play an important role in immune responses and cellular survival. Activation of NF-?B is mediated by the I kappa B kinase (IKK) complex. IKK is composed of the IKK1 and IKK2 catalytic subunits and the regulatory subunit NEMO (NF-?B Essential Modulator). Mice with an epidermis specific deletion of IKK2 ($IKK2^{EKO}$) exhibit a severe skin disease shortly after birth that partly resembles human psoriasis and is caused by a tumor necrosis factor-mediated, aß-T cell independent inflammatory response that develops in the skin. We are investigating pathogenic mechanisms involved in the onset of this skin disease. Using Affymetrix gene chip analysis of expression patterns in the skin of $IKK2^{EKO}$ and control mice prior to clinical or histopathological signs of the disease we found a significant upregulation of expression of interferon target genes indicating an early involvement of this class of immune stimulators in the development of the skin condition. Since interferons are known to be primary regulators of macrophage activation, we have investigated the contribution skin macrophages to the pathogenesis of the skin phenotype. We have succeded to eliminate skin macrophages by injecting liposome encapsuled clodronate subcutaneously. We observed that depletion of skin macrophages prevented the formation of an inflammatory infiltrate and correlated with a dramatic improvement of the psoriasis like skin condition. This indicates a crucial function for these cells in this skin disease model and points to a so far underestimated role of macrophages in the pathogenesis of skin inflammation.

V15

Loss of Raf Kinase Inhibitor Protein (RKIP) expression contributes to constitutively acitve Ras-signalling

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Consitutive activation of the Raf signalling pathway is known to play a pivotal role in the progression of malignant melanoma. In this study we provide evidence that the regulatory protein RKIP (Raf-1 kinase inhibitory protein) and its effects on Raf-1 mediated activation of mitogen-activated protein/extracellular signal-regulated kinase kinase (MEK) are important for the progression and metastatic potential of malignant melanoma. Screening 10 melanoma cell lines at mRNA and protein level, we detected significant downregulation of RKIP expression *in vitro* in comparison to normal human epidermal melanocytes (NHEM). Loss of RKIP expression in melanoma cells was confirmed *in vivo* in immunohistochemical analyses of normal skin, primary malignant melanomas, and metastasis of malignant melanomas. In addition, we observed downregulation of RKIP expression in primary melanoma and further decrease or absence of RKIP expression in melanoma metastasis. Stable transfection of the melanoma cell line Mel Im with an RKIP expression plasmid revealed blocking of the Raf-1 kinase pathway and consequently downregulation of ERK1/2 activation via phosphorylation. Proliferation of the RKIP- Mel Im cell clones in comparison to control Mel Im cell clones in *in vitro* proliferation assays was not altered. In very good agreement with the *in vivo* finding that downregulation of RKIP expression is most obvious in melanoma metastasis, re-expression of RKIP in the highly invasive Mel Im cell line leads to a significant inhibition of invasiveness in an *in vitro* assay.
Taken together, our results suggest that loss of RKIP in malignant melanoma is one alteration that contributes to the consitutive activation of Raf/MEK/ERK signalling and enhances the metastatic potential of melanoma cells.

V16

Vanilloid receptor subtype 1 (VR1/TRPV1) is present on sensory nerve fibers and mast cells contributing to nociceptive cutaneous sensations

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The vanilloid receptor subtype 1 (VR1/TRPV1) is a non-selective cation channel that binds the vanilloid capsaicin. Since capsaicin was shown to suppress pruritus effectively, the aim of our present study was to investigate VR1 expression on cutaneous nerve fibers. We studied a series of normal, diseased, and capsaicin-treated human skin. VR1 immunoreactivity could be observed on cutaneous sensory nerve fibers as well as mast cells, while autonomic nerves were consistently negative. This was confirmed upon RT-PCR and Western blot analysis detecting VR1 on primary mast cells and a mast cell line. In pruritic skin diseases such as prurigo nodularis, psoriasis vulgaris, and atopic dermatitis, as well as capsaicin-treated skin, no regulation of VR1-expression was observed while in the latter a decrease of neuropeptides was found. After cessation of capsaicin therapy, neuropeptides re-accumulated in skin nerves. The expression of VR1 on cutaneous sensory nerve fibers supports the notion that vanilloids and their receptors contribute to the induction and modulation of nociceptive cutaneous sensations such as pain and pruritus. VR1 activation leads to a depolarization of nerve fibers as well as to the release of secretory granules containing neuropeptides mediating a neurogenic inflammation. In previous studies it was suggested that neuropeptides released from nerve fibers may act as mast cell liberators while recent studies showed that neuropeptides in vivo are not released at sufficient concentrations to mediate this effect. An explanation for these contradictory results may be our demonstrated presence of VR1 on mast cells which may be activated directly by VR1-ligands. Consequent release of mast cell mediators induces pruritus by binding to histamine-receptors on sensory nerve fibers. In conclusion, our results suggest that VR1 may be part of a direct bi-directional communication network between nerve fibers and mast cells both contributing to the induction and modulation of cutaneous nociceptive sensations.

V17

Protease IV from Pseudomonas aeruginosa induces human beta-defensin 2 and interleukin 8 in keratinocytes

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Living cells as well as culture supernatants of the gram-negative organism Pseudomonas aeruginosa are known to induce the antibiotic peptide human beta defensin 2 (HBD-2) with antibiotic activity against P. aeruginosa in human epithelia originated from lungs, mucous membranes and skin. The pathogen-associated molecules responsible for this phenomenon are still under investigation. P. aeruginosa secrete several exoproteases, which might be involved in the stimulation of keratinocyte reaction.
Culture supernatants of P. aeruginosa strain ATCC 11440 were fractionated by sequential ultrafiltration in steps of 300 kDa, 30 kDa and 3 kDa. Subsequent anion-exchange chromatography of the 30-300 kDa fraction revealed fractions with trypsin-like enzymatic activity and coincident calcium-mobilizing activity in HaCaT keratinocytes. The lysine-specific exoprotease protease IV from P. aeruginosa was purified by sequential ultrafiltration and specific p-amino-benzamidine affinity chromatography from culture supernatants of P. aeruginosa. Identity with protease IV was proven by the specific cleavage of of tosyl-glycyl-prolyl-lysyl-pNA, the typical inhibitor profile and detection in SDS-PAGE. Using single cell fluorescence imaging microscopy, it could be shown, that the purified enzyme induced Ca2+-influx in HaCaT keratinocytes with similar time course and potency as compared with trypsin and thrombin. Pre-stimulation of HaCaT cells with trypsin or thrombin abolished subsequent response to protease IV. The sensivity for trypsin and thrombin points towards a possible involvement of protease-activated receptors on keratinocytes. By real-time RT-PCR analysis it could be shown, that protease IV induced pronounced mRNA-expression of the antimicrobial peptide HBD-2 and the proinflammatory cytokine IL-8 in HaCaT keratinocytes. Protease IV appears to be a pathogen-associated molecule secreted into the environment and able to induce epidermal defense mechanisms such as HBD-2 and IL-8 expression in human keratinocytes. Thus, protease IV may be involved in the recognition of cutaneous P. aeruginosa colonization by keratinocytes followed by the initiation of adaptive innate immune responses.

V18

Experimental photopheresis induces regulatory T cells

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The basis of extracorporeal photopheresis is the reinfusion of leukocytes, which have been exposed to 8-methoxypsoralen (8-MOP) and UVA (PUVA). Besides its beneficial effect in cutaneous T cell lymphoma, photopheresis has shown evidence of benefit for the treatment of autoimmune diseases, solid organ transplant rejection and GvHD. The underlying mechanism for this effect remains unresolved. Since UV radiation of the skin exhibits the capacity to induce tolerance via induction of regulatory T cells, we studied whether extracorporeal treatment of leukocytes with UV radiation had a similar effect. For this purpose we utilized a murine model of contact hypersensitivity (CHS). Splenocytes and lymph node cells of mice which were sensitized with dinitrofluorobenzene (DNFB) were exposed to 8-MOP plus UVA in vitro. 8-MOP/UVA-treated cells were injected intravenously into naive mice which were subsequently skin sensitized with DNFB and ear thickening measured following ear challenge. Animals which had received 8-MOP/UVA-treated cells were significantly suppressed in their CHS response. In contrast, mice which received cells which were untreated or exposed to UVA or 8-MOP alone were not suppressed in their CHS response. Suppression was still observed when T cells were depleted from the 8-MOP/UVA-treated cells. In contrast, induction of suppression was lost when lymph node cells were depleted of CD11c+ cells, suggesting that CD11c+ cells are the primary target cell for photopheresis in this system. Suppression was cell-mediated and antigen-specific as demonstrated by the ability to transfer the tolerance to naive animals which could, however, properly respond to the unrelated hapten oxazalone. Transfer of tolerance was lost when cells were depleted of CD4+ or CD25+ subpopulations, which may indicate induction of regulatory T cells. Taken together, these data suggest that experimental photophoresis induces a state of tolerance which is antigen-specific and mediated via the induction of CD4+CD25+ regulatory T cells.

V19

Early Interleukin 4 Administration Promotes Th1-Driven, Protective Anti-Cancer Immunity

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Identifying agents that boost antigen-specific immune responses is an important goal of researchers involved in cancer immunotherapy. Recently, we found that the co-administration of the polycation poly-L-arginine (pR) and ßgalactosidase (ßgal; thereafter referred to as pR-PV) yields substantially higher numbers of specific, IFN?-secreting, CD8+ T cells than s.c. ßgal-application. In addition, there are studies showing that IL-4 is capable of promoting the activation of TH1 lymphocytes provided that it is applied early after the induction of the immune response and at high doses. Based on these observations, we asked whether IL-4 can be used to augment the immunological as well as clinical efficacy of the pR-PV. To test this hypothesis, BALB/c mice were injected s.c. with the pR-PV either alone or admixed with IL-4. Seven days later, CD8+ T cells producing either IFN? or IL-4 in response to ßgal were quantified by ELISPOT analysis. Results obtained showed that co-administration of IL-4 significantly enhances the number of vaccine-induced, specific CD8+ T lymphocytes that produce IFN?. In addition, we found the IL-4 treatment used to shift the specific immune response towards a TH1 phenotype. To investigate the effect of IL-4 administration on the clinical efficacy of the pR-PV, BALB/c mice were injected on days 0 and 14 with pR-PV +/- IL-4 and challenged on day 24 by the s.c. inoculation ßgal-expressing RENCA cells. None of the naive controls (n=6) rejected the tumor inoculum. S.c. administration of the pR-PV protected 3/6 (50%) animals. The highest protection rate (5/6; 83%) was obtained by the combined use of the pR-PV and IL-4.Together, these results demonstrate that IL-4 has the potential to act as a natural adjuvant capable of inducing a protective TH1-driven anti-cancer immune response.

V20

Targeting of tumor antigens to dendritic cells in vivo induces protective tumor immunity in a B16 melanoma model.

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To facilitate antigen presentation DCs express receptors that guide antigens through the endosomal pathway, resulting in antigen processing and presentation. The antigen receptor DEC-205 (CD205) is expressed by murine DCs and enhances antigen presentation of activated DCs up to 500-fold as compared to pinocytosis. We therefore aimed to use antibodies directed against DEC-205 to load activated DCs in situ with antigens. A fusion protein of the tumor antigen tyrosinase related protein (TRP) 2 and the green fluorescent protein (EGFP) was coupled to anti-DEC-205 antibodies (aDEC) and injected together with CpGs s.c. into mice. Lymph node cells (LNC) were prepared from draining LN 2h to 24 h later. FACS analysis revealed that 8h after injection of the conjugates up to 20% of the $CD11c^+$ DCs displayed EGFP fluorescence. $B220^+$ as well as $CD11b^+$ cells were negative for EGFP fluorescence, indicating that EGFP-TRP2-aDEC conjugates had selectively been taken up by LN DCs in situ. To test whether this targeting resulted in induction of TRP2 specific $CD8^+$T cells, spleen cells from conjugate and CpG injected mice were restimulated with TRP2-peptide and in ELISPOT assays we were able to detect IFN-? producing T cells. In contrast no IFN? producers were detected in control samples. Moreover, we demonstrated vigorous induction of TRP2 specific antibodies in TRP2-aDEC-conjugate injected mice, whereas mice that received uncoupled TRP2 protein and CpGs developed only weak antibody responses. Finally we tested whether immunization with TRP2-aDEC-conjugates and CpG lead to protective tumor immunity in a melanoma model. Therefore mice were immunized with TRP2-aDEC-conjugates and respective control proteins and were challenged for tumor growth by i.v. injection of $4x10^5$ B16 melanoma cells. 14 days later, mice were sacrificed and lung metastases were counted. These experiments revealed that injection of TRP2-aDEC-conjugates prevented tumor growth in 75% of the mice, whereas no tumor free mice could be detected in untreated - or TRP2 injected groups. Notably, when CpG was omitted from the immunization, tumor growth was not prevented, indicating that activated DCs are mandatory for successful immunization. Thus these data show that in vivo targeting of tumor antigens to activated DCs induces protective tumor immunity.

V21

Histone deacetylase inhibitors (HDAI) induce apoptosis, yet relatively little viral replication, in tumor cells infected with Kaposi's sarcoma-associated herpesvirus (KSHV)

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KSHV infects all tumor cells in two diseases: Kaposi's sarcoma (KS) and primary effusion lymphoma (PEL). HDAI, a relatively new class of anti-tumor agents, induce transcription of cellular genes involved in tumor suppression, apoptosis, and maturation (a particular subset of genes that is often ?silenced? in cancers and virally transformed cells). The purpose of this study was to determine whether HDAI could kill tumor cells infected with KSHV. Four HDAI (depsipeptide, MS-275, suberoylanilide hydroxamic acid, trichostatin A) were assessed for their ability to induce apoptosis and viral reactivation in two KSHV-infected tumor cell lines derived from patients with PEL (BCBL-1 and BC-3). Using mAb staining for acetylated histones and direct immunofluorescence, all PEL cells treated with HDAI became hyperacetylated as predicted. As quantified by flow cytometry (Annexin-V and DeadRed® staining) and WST-8 reduction assay, all HDAI induced apoptotic cell death in both cell lines in a time- (1-3 days) and dose- (5-500 nM) dependent manner. To determine whether apoptosis was caused by reactivation of KSHV into lytic cycle, we quantified expression of lytic gene mRNA and protein expression, and examined cells by electron microscopy for evidence of virion formation. Using a range of HDAI doses that invariably induced apoptosis in more than 50% of cells, the number of cells entering and completing lytic cycle was 5-14% and 3-9%, respectively, indicating that cell death caused by viral replication accounted for only a fraction of HDAI-mediated apoptosis. To extend our *in vitro* findings into an *in vivo* animal model, SCID mice were implanted with xenografts of PEL cells and treated with depsipeptide (0.5 or 1 ug/kg i.p. 3x/week). At these doses, depsipeptide was well-tolerated and induced apoptotic cell death within tumors as demonstrated by routine histology. These results suggest that HDAI are a therapeutic option for patients with PEL. Parallel experiments using HDAI in KSHV-infected endothelial cells, which would suggest a treatment role for patients with KS, are underway.

V22

IL-3 induces expression of a lymphatic phenotype

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Factors determining lymphatic differentiation in the adult organism are yet not well characterized. We have made the observation that mixed primary cultures of blood (BEC) and lymphatic (LEC) endothelial cells grown under standard conditions change to a 100 % lymphatic phenotype during subculture. After passage 6 they uniformily express LEC-specific markers Prox-1 and podoplanin. We show that LEC but not BEC constitutively express IL-3 and that IL-3 is responsible for the differentiation of BEC into LEC. Using sorted BEC, IL-3 induces Prox-1 and podoplanin expression and expression persists after subsequent withdrawal of IL-3. Using sorted LEC, blocking IL-3 activity by IL-3 R alpha chain antibodies results in a loss of Prox-1 and podoplanin expression. To analyze the situation in vivo, IL-3 was injected into melanomas subcutaneously grown on the backs of SCID mice. In this model, IL-3 significantly increased numbers of podoplanin positive vessels, which were all positioned within the lymphatic vascular tree, whereas numbers of blood vessels remained constant. In conclusion, IL-3 is a novel factor capable of inducing lymphatic differentiation.

V23

Type 2 segmental Hailey-Hailey disease originating from early loss of heterozygosity: Molecular confirmation of a novel genetic concept

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Hailey-Hailey disease (HHD) (OMIM 169600) is an autosomal dominant trait characterized by erythematous and oozing skin lesions preponderantly involving the body folds in a symmetrical fashion (diffuse phenotype). In the present unusual case, however, unilateral segmental areas along the lines of Blaschko showed a rather severe involvement superimposed on the ordinary symmetrical phenotype. This observation, next to similar ones in various other dominantly inherited cutaneous disorders, prompted one of us to postulate a novel genetic concept for autosomal dominant skin diseases in which two different forms of segmental involvement can be distinguished. The type 1 manifestation reflects heterozygosity for a de novo postzygotic somatic mutation occurring at an early stage of embryogenesis thus representing a true cutaneous mosaicism. The cutaneous lesions within the affected segments reveal a degree of severity that is similar to the clinical expression of the diffuse phenotype caused by a germline mutation. In contrast, the type 2 segmental manifestation would result from loss of heterozygosity (LOH) occurring in a heterozygous embryo at an early developmental stage, giving rise to an area of homozygous or hemizygous tissue clinically showing rather pronounced segmental lesions. Accordingly, the linear lesions of the aforementioned patient would represent a type 2 segmental manifestation of HHD. Using PCR based techniques, we initially detected a splice site mutation in the germline giving rise to the ordinary symmetrical phenotype. Upon studying keratinocytes from the segmental lesions by haplotype analysis, real-time PCR and DNA sequencing we were then able to demonstrate LOH. Our data for the first time confirm the hypothesis for type 2 segmental skin diseases on the cellular and molecular level.

V24

The osmolyte taurine plays a critical role in ultraviolet B radiation-induced immunosuppression

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We have previously proposed that uptake of osmolytes such as taurine is part of the stress response of human epidermal keratinocytes induced by ultraviolet (UV) B radiation. This hypothesis was based on the observation that (i) ultraviolet (UV) B radiation induced the expression of TAUT, the specific transporter for taurine, in longterm cultured normal human keratinocytes (HNK) and that (ii) this upregulation was associated with an increased uptake of taurine into irradiated cells. In support of this hypothesis we now report that taurine uptake protects human skin cells against UVB radiation-induced immunosuppresive effects. Accordingly, preloading of HNK in-vitro with taurine completely prevented both hyperosmotic stress- and UVB radiation-induced gene expression including intercellular adhesion molecule-1 (ICAM-1). These studies indicated that taurine uptake might be critcial in determing the susceptibility of human skin cells towards UVB radiation-induced biological effects such as immunosuppression. In order to further assess this possibility we next compared UVB radiation-induced immunosuppression in wildtype mice versus heterozygous versus homozygous TAUT knockout mice, which had been generated through disruption of exon 1 of the murine TAUT gene. The susceptibility of these three different mouse strains towards UVB radiation-induced immunosuppression was measured by their capacity to mount a contact hypersensitivity response after exposure to increasing doses of UVB radiation prior to sensitization with DNFB. Comparative studies of the ear swelling response revealed that homozygous, but not heterozygous TAUT knockout mice were more susceptible towards UVB radiation-induced immunosuppression than wildtype mice. Taken together these studies suggest that taurine uptake - similar to DNA repair and pigmentation - is critically involved in endogenous photoprotection of skin cells against UVB radiation-induced detrimental effects.

V25

Leukocyte redistribution by systemic administration of imidazoquinolines

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Imidazoquinolines, ligands for TLR 7/8, are known to possess potent immunostimulatory activity and are in clinical use as topical immunotherapeutic agents. To test their potential suitability as adjuvants for systemic immunotherapy, we investigated effects on leukocyte subsets in vitro and on cutaneous immune responses and leukocyte recirculation in vivo. Hapten-induced contact hypersensitivity (ACD) is augmented by the imidazoquinoline R-848 when applied during the sensitization phase of the reaction, which can be attributed to enhanced maturation and migration of dendritic cells. However, when R-848 is given during the effector phase of the immune response, it potently suppresses the elicitation of ACD, even at very high doses of hapten, corresponding to a strongly reduced lesional T cell infiltrate. The immunosuppression was accompanied by a rapid (90%) reduction of peripheral blood leukocytes. Disappearance of leukocytes from the blood corresponded to an enrichment in lymphatic and non-lymphatic organs. Thus, systemic application of imidazoquinolins induced transiently reduced availability of peripheral blood leukocytes, which led to short-term immunosuppression. To investigate whether this effect was due to accelerated extravasation or prolonged tissue residence of T cells, labeled T cells were injected i.v. and allowed to emigrate into tissues. Then, re-entry of the cells into the blood was prevented by blocking E- and P-selectin. Imidazoquinoline treatment prevented tissue-to-blood redistribution of T cells, suggesting that the tissue half-life of T cells was prolonged, which correleated to enhanced expression of ICAM-1 in lymphatic organs of treated mice. Exposure of the murine endothelial cell line BEnd5 to R-848 stimulated expression of E-selectin, P-selectin, VCAM-1 and ICAM-2. In conclusion, imidazoquinolines induce a transient redistribution of leukocytes, resulting in apparently paradoxical suppression of acute inflammatory processes such as ACD. On the other hand, this drug-induced blood-to tissue redistribution of leukocytes may facilitate immunity to tumors or pathogens that evade immune recognition by sequestration into non-inflamed tissues.

V26

Shift from systemic to site specific memory by tumor-targeted interleukin 2

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Interleukin 2 (IL2) has been approved for treatment of patients with cancer. Moreover, it has been used as a component of vaccines against cancer. In this regard, we have recently demonstrated that DC-based peptide vaccination required IL2 to mount an effective immune response against established melanoma metastases. Here, we confirm this observation by use of tumor-targeted IL2. However, the development of a protective systemic memory was substantially impaired by this measure; i.e., animals which successfully rejected subcutaneous tumors of B16-melanoma upon vaccination with TRP-2 peptide-pulsed DC plus a boost with targeted IL2 failed to reject a re-challenge with experimental pulmonary metastases. In contrast, animals which were treated with vaccination alone rejected subsequent intravenous tumor challenges. Kinetic studies on TRP-2 reactive T cells in the peripheral blood demonstrated an increased number of specific T cells subsequent to targeted IL2 therapy. Moreover, additional IL2 fusion protein treatment led to an activation of the TRP-2 specific T cells exemplified by the expression of the alpha-chain of the IL2 receptor (CD25) and the loss of CD62 ligand expression. The loss of CD62 ligand, the homing receptor for secondary lymphoid tissues, was associated with a depletion of such cells from tumor draining lymph nodes as visualized by in situ peptide/MHC class I tetramer staining. Although animals receiving DC vaccination in combination with tumor targeted IL2 lacked a systemic memory, they possessed a site specific memory, i.e., they were protected against subcutaneous tumor re-challenge. Thus, a high IL2 concentration in the tumor microenvironment seems to favor a shift from lymphoid (or central) to non-lymphoid (or effector) memory cells. While non-lymphoid memory cells provide excellent protection to the site in which they reside, lymphoid memory cells provide a systemic memory to sites which did not have prior antigen encounter.

V27

Cockayne Syndrome A and B proteins and repair of oxidative damage to mitochondrial DNA: Mechanism of import and functional relevance *in vivo*.

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Cockayne Syndrome (CS), characterised by neurodegeneration, photosensitivity and premature aging, is caused by mutations in the CSA and CSB gene. We have previously shown that the CSA and CSB proteins are localised in the mitochondrion (mt) and involved in the repair of oxidatively induced mtDNA deletions. However, their mechanism of import into mitochondria, exact type of repaired damage and functional relevance in vivo have not been elucidated. Regarding localisation, both CSA and CSB miss a mitochondrial leader sequence but computer based k-NN sequence analysis predicted 52.2 % and 30.4 % mitochondrial localisation for CSA and CSB, respectively. Furthermore, co-immunoprecipitation showed requirement of CSA/CSB proteins in a heterodimeric state to allow mitochondrial import. Employing a reconstituted in vitro repair assay comprising fapy-glycosylase and endo III as reporter enzymes for oxidative damage in pyrimidines and purines, respectively, we could show that, after repetitive sublethal UVA irradiation, removal of thymine glycol from mtDNA was defective in fibroblasts from CS patients. However, stable transfection of the CSB gene reconstituted this defect. Semiquantitative PCR showed increased levels of mtDNA deletions in CSB knockout mice in vivo compared to wild-type littermates in the brain but not in muscle, cartilage spleen or testis, correlating with the neurodegenerative changes found in aging animals. In aggregate, extending and corroborating previous findings, these results strengthen the role of CS proteins in the repair of oxidative damage to mtDNA and indicate a heterodimeric composition of both proteins as requirement for mitochondrial import. Furthermore, the correlation of neurodegenerative symptoms and defective removal of oxidatively induced mtDNA deletions in CS knockout mice indicates a close relationship of oxidative stress, its repair by CSA and CSB and processes such as neurodegeneration and aging.

V28

Expression of matrix metalloproteases 2, 9, 13 and 14 in skin tumours of human papillomavirus type 8 transgenic mice.

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Human papillomaviruses (HPV) are small DNA viruses that induce a wide variety of hyperproliferative lesions in cutaneous and mucosal epithelia and have been recognized as important carcinogens in human. The most plausible evidence for an association between HPV and extragenital tumours is that proposed for non-melanoma skin cancer (NMSC).
To examine the function of the early genes in vivo, we generated a transgenic mouse model. The complete early region of HPV8 under the control of the keratin 14 promoter, whose activity is mainly restricted to the basal layer of the epithelium, was microinjected into the male pronucleus of fertilized eggs of DBA/Bl6 mice. HPV positive founder mice were then mated with FVB/n mice. These mice spontaneously developed papillomatous, partially ulcerative and erosive skin tumours. Histology revealed epidermal hyperplasia, acanthosis and hypergranulosis and in some cases invasive SCC. In order to gain further insight into the role of the viral proteins in the progression of cutaneous tumours, we investigated expression of matrix metalloproteases MMP2, 9, 13 and 14 in the skin tumours of the K14-HPV8 mice. In situ zymography on frozen specimens displayed a strong gelatinolytic activity suggesting increased amounts of activated enzymes. In vitro zymographic analysis of extracts from dysplastic skin and SCC specimens identified activated gelatinases MMP2 and 9 in these tumours, but not in normal skin. Western blot experiments of tumour extracts also showed that the interstitial collagenase MMP13 and the membrane type-1 MMP (MMP14) are overexpressed in the skin tumours.
These results suggest a role of matrix metalloproteases in HPV induced skin carcinogenesis.

V29

Structure and role of collagen XVIII/endostatin in basement membranes

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Using immuno-electron microscopy with antibodies recognizing specific domains of collagen XVIII/endostatin, we have determined the ultrastructural organization of this collagen in the epidermal and other basement membranes. We provide evidence that collagen XVIII/endostatin is anchored in a polarized orientation into perlecan-containing basement membrane molecular networks. Rotary shadowing electron microscopy demonstrates a non-linear structure of this collagen.
We further examined a potential role of collagen XVIII/endostatin for epithelial cell integrity by investigating epithelial cell layers in mutant mice lacking collagen XVIII/endostatin. Several morphological and functional defects were identified in single epithelial cell layers, particularly in ocular epithelia. Striking structural epithelial defects were observed in the iris pigment epithelium and the retinal pigment epithelium. The changes in ocular epithelia affect visual function with age-dependent loss of vision in mutant mice, due to an abnormal retinoid metabolism in the retinal pigment epithelium. Thus, collagen XVIII/endostatin is essential for the function of ocular epithelia. In contrast, no striking epithelial defects were identified in constantly regenerating stratified epithelia, such as in the epidermis. These findings suggest that the requirements for a proper interaction with the extracellular matrix might differ between non-regenerating epithelia, such as retinal pigment epithelium, and epithelial cell layers with a high regenerative potential, such as epidermis. Our data further suggest a potential role of collagen XVIII/endostatin for cell-matrix interactions of epithelial cells with their underlying basement membranes.

V30

Inhibition of EGF-R/Akt signalling pathway and Induction of Apoptosis in Human Keratinocytes after Treatment with Light Activated *Curcuma longa* Extract

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Curcuma longa (Zingiberaceae family) is a pharmacologically active plant and spice widely cultivated in tropical regions of Asia and Central America. In previous studies we could show that Curcuma longa extract (ZCL8) combined with both, UVA or visible light, induces strong growth inhibition in HaCaT and primary keratinocytes. Now we present data of the underlying mechanisms. It was found that light activated ZCL8 induces apoptotic processes in HaCaT cells. Hoechst staining showed a concentration dependent increase of fragmented cell nuclei after treatment with ZCL8 (2-10 µg/ml) plus UVA (1 J/cm^2). In the absence of UVA, the ZCL8 treated cultures contained similar amounts of apoptotic cells as untreated control cultures. Furthermore, it was demonstrated that light activated ZCL8 induces the release of cytochrome c from mitochondria and the activation of both caspases 8 and 9, respectively. Next, the ability of ZCL8 to modulate signalling pathways that support the cell survival was investigated. It was found that the anti-apoptotic kinase Akt/PKB was inhibited by ZCL8/UVA after treatment with TGF-a. Likewise, an inhibition of the EGF receptor was demonstrated. In contrast the phosphatase PTEN, the major negative regulator of the PI3K/Akt signalling, was activated by ZCL8/UVA treatment. In addition, further examinations indicate a concentration dependent inhibition of the NF-?B activity. These results suggest that ZCL8 may facilitate apoptosis induction by blocking the EGF receptor and inhibiting the activity of Akt/PKB. These findings and particularly the observation that ZCL8 only acts in combination with light (UVA) might contribute to a new photo-therapeutic strategy.

V31

Chemokines Orchestrate Epithelial and Stromal Cell Migration in Cutaneous Wound Healing

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Wound healing represents a dynamic process involving directional migration of leukocytes and structural cells. Chemokines are known to control leukocyte migration and this superfamily of chemoattractive proteins has been suggested to be among the first complete protein families to be identified and characterized at the molecular level. We have performed a comprehensive, "global", time-course analysis of the expression and function of chemokines and their receptors during cutaneous wound healing. Compared to other cytokines, growth factors, adhesion molecules and matrix-metalloproteinases (n=101 different genes), a subset of chemokines are among the most highly regulated genes in wound healing and their expression co-incides with the appearance of matching receptors. Next to leukocytes, resting as well as activated human primary keratinocytes (CCR3, CCR4, CCR6, CXCR1, CXCR3), dermal fibroblasts (CCR3, CCR4, CCR10) and dermal microvascular endothelial cells (CCR3, CCR4, CCR6, CCR8, CCR10, CXCR2, CXCR3) express a distinct and functionally active repertoire of chemokine receptors (showed by time-lapse video-microscopy). Signaling through $G_{(i)}$ alpha protein-coupled chemokine receptors on structural cells markedly enhanced *in vitro* wound repair. Observations in mice and results obtained *in vitro* were confirmed in human wound healing, showing the temporal-spatial expression of chemokine ligands in close proximity to chemokine receptor-positive target cells. Taken together, these findings suggest novel therapeutic strategies for the treatment of wound healing disorders such as chronic ulcers.

V32

Human beta-defensin-2 induction in keratinocytes is controlled by NF-?B and AP1

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Human beta-defensin-2 (hBD-2) was the first inducible peptide antibiotic isolated from human skin. The expression of hBD-2 in keratinocytes is induced by proinflammatory cytokines and bacteria, especially by IL-1ß and *Pseudomonas aeruginosa* (PA). Since little is known about the signal transduction pathways leading to hBD-2 gene induction, we investigated the transcription factors involved in IL-1ß and PA-mediated gene induction of hBD-2.We transfected HaCaT keratinocytes with mutation constructs of hBD-2 promoter-driven luciferase reporter plasmids. Stimulation was performed using supernatants of mucoid PA and IL-1ß. The mutation of three potential NF-?B binding sites resulted in a strongly diminished induction of promoter activity while the mutation of a potential AP-1 binding site reduced promoter activation by more than 50%. A combination of all four mutations completely abolished hBD-2 promoter activity following IL-1ß and PA treatment.To further analyse the influence of NF-?B and AP-1 on hBD-2 gene induction, we investigated the effect of the NF-?B inhibitor Helenalin and inhibitors of the three AP-1 inducing MAP-kinase cascades (ERK1/2 pathway, JNK pathway and p38 pathway). Realtime PCR studies following stimulation of HaCaT keratinocytes with PA and co-incubation with the specific pathway inhibitors confirmed the involvement of NF-?B and AP-1: the specific NF-?B-inhibitor Helenalin almost completely abolished hBD-2 induction; the JNK-Inhibitor SP600125 suppressed hBD-2 induction by approximately 60%; the p38 MAP-kinase inhibitor SB202190 diminished hBD-2 induction by approximately 50%, but ERK1/2-inhibitor PD98059 showed no modulating effect on PA-mediated hBD-2 induction.Our data indicate that NF-?B is essential for hBD-2 induction following IL-1ß/PA stimulation. In addition to NF-?B, AP-1 also seems to be involved in hBD-2 induction following activation of the JNK and p38 pathway. We conclude that both NF-?B and AP-1 play a crucial role in the regulation of hBD-2 induction.

V33

Differentiation of murine embryonic stem cells into keratinocyte- like cells, their isolation and cultivation

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Embryonic stem (ES) cells are omnipotent; they can differentiate into every cell type of the body. The signals that regulate differentiation into squamous epithelial cells are poorly defined. In vitro production of large numbers of epidermal cells from embryonic stem cells could be used in dermatology in order to cover large wounds, e.g. of burn victims. Elimination of immunogenic determinants in ES cells prior to differentiation would eventually allow to generate non- immunogenic epidermal cells that could be universally used for different recipients. In order to understand the signals that regulate differentiation of ES cells into squamous epithelial cells we have used embryoid bodies (EBs) formed from embryonic stem cells as a system for in vitro differentiation. EBs were first cultured in suspension and then plated onto tissue culture dishes. At day 17 of culture cells formed colonies of epithelial cells within sheets of non- epithelial cells. These colonies expressed epithelial cadherin (E- cadherin), a classical type I cadherin and a key regulator of epithelial cell- cell adhesion in embryonic and adult tissues. During subsequent days a subset within the epithelial layer started to express keratin 14, a type I keratin, which is constitutively present in basal keratinocytes of the epidermis and stratified epithelia and in the outer root sheath of the hair follicle. Many of these cells were larger than the surrounding cells and showed filamentous keratin structures. Keratin 14 positive cells subsequently merged to form aggregates. We were able to isolate such cells from the EBs and culture them in vitro for several days. Our results show that embryonic stem cells that form an EB are able to differentiate into keratinocyte like cells and that these cells can be isolated and cultured in vitro.

V34

Impaired Th1 responses in Epstein-Barr virus-induced gene 3 (EBI3)-deficient mice challenged with physiological doses of *Leishmania major*

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Resolution of cutaneous lesions in leishmaniasis is mediated by IFN?-activated, infected macrophages that are capable of eliminating the intracellular parasite. Protective immunity against *Leishmania major* infections is therefore known to strictly depend on the release of IL-12 from *L. major*-infected dendritic cells (DC) which in turn induce IFN?-producing $CD4^+$ Th1 or $CD8^+$ Tc1 cells. IL-27 is part of the IL-12 family and is a heterodimer composed of the p40-related protein EBI3 and p35-related protein p28. IL-27 (EBI3-p28) binds to the WSX-1 receptor and appears to be produced by activated APC. Here, we made use of the EBI3-deficient (C57BL/6) mice to investigate the role of IL-27 released from infected DC in cutaneous leishmaniasis. To do this, we used the more physiologically relevant low dose infection model (1,000 infectious stage metacyclic promastigotes injected intradermally) that closely mimics natural transmissions by the bite of the sandfly. Lesions in EBI3-/- mice were significantly larger between wk 3 and 10 post infection, most dramatic differences were found between wk 6 and wk 8 reaching ~3-fold increased lesion volumes compared to wild type controls (34±4 vs. 12±1 mm^3 at wk 6, n=10, $p<0.002$). In parallel, the lesions of EBI3-/- mice contained greater numbers of parasites reaching a peak load of $9x10^6$ parasites/ ear at wk 6 (wild type mice: $1x10^5$ parasites/ ear, n=10, $p<0.05$). Similar to wild type C57BL/6 mice, lesions in EBI3-/- mice resolved after 10 to 12 wks. The cytokine responses of cells from draining lymph nodes restimulated with soluble *Leishmania* antigen were also determined. At early time points (until wk 6), EBI3-/- mice showed increased levels of IL-4 (195±47 vs. 57±7 pg IL-4/ml) and decreased IFN? production (0.3±0.1 vs. 1.7±0.6 ng IFN?/ml at wk 2, n=10, $p<0.05$) as compared to wild type controls and. In summary, our data suggest that - in addition to IL-12 - IL-27 plays a critical role for the establishment of Th1 immunity in this model of an important human infectious disease.

V35

Epidermal Effects of Peroxisome Proliferator Activated Receptor (PPAR)-Delta Activation

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The peroxisome proliferator-activated receptors (PPARs) comprise a group of nuclear transcription factors that heterodimerize with RXR-alpha. The purpose of this study was to determine the effects of PPAR-delta activation on skin. *In vitro*, using cDNA microarrays, Western and Northern blot analysis, we observed an increase in a variety of differentiation markers in cultured keratinocytes treated with a selective PPAR-delta agonist, including involucrin, transglutaminase I, small proline rich proteins, desmoplakin, envoplakin, and annexin A1. In addition to markers of keratinocyte differentiation, we observed a robust increase in adipose differentiation related protein and fasting inhibitory adipose factor, both of which have been implied in adipogenesis. *In vivo*, in mouse skin, topical PPAR-delta activation increased loricrin and filaggrin expression as assessed by immunohistochemistry, stimulated epidermal lipid accumulation and improved permeability barrier homeostasis, while epidermal proliferation and apoptosis were not changed. The pro-differentiating effects were blunted in mice lacking RXR-alpha, the heterodimerization partner required for DNA binding. Lastly, PPAR-delta mRNA levels were increased in human psoriatic skin as assessed by quantitative PCR and topical PPAR-delta activation reduced ear swelling in a mouse model of TPA induced irritant contact dermatitis, suggesting a role of PPAR-delta in skin inflammation. These results indicate that PPAR-delta activation has pro-differentiating and anti-inflammatory activity. Because it does not cause epidermal atrophy and because there are beneficial systemic effects on serum lipids (increase in HDL cholesterin), PPAR-delta could be both effective for the treatment of skin disease and favorable in its side effect profile.

V36

Evidence for a novel molecular pathway that mediates ultraviolet (UV) B radiation-induced gene expression.

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UVB radiation-induced signaling in mammalian cells has previously shown to involve two major pathways: one that is initiated at the level of the cell membrane and characterized by cell surface receptor clustering and a second one that is triggered through the generation of DNA photoproducts in the nucleous. In the present study we provide evidence for a hitherto unknown pathway that is initated in the cytoplasm and involves trytophan as a chromophore. Accordingly, UVB-, but not UVA- or sham-irradiated trytophan strongly induced gene expression including CYP1A1 in HaCaT cells. In this regard it is of interest that UVB radiation is know to induce trytophan degradation and that trytophan metabolites can act as ligands for the arylhydrocarbon receptor (AhR). The AhR is an intracellualr transcription factor which is localized in the cytoplasm of cells and upon ligand binding translocates into the nucleus where it initiates transcriptional expression of genes including CYP1A1. In order to assess whether UVB radiation can induce AhR translocation, HaCaT cells were transiently transfected with a green fluorescent protein coupled AhR. Indeed, we have found that UVB radiation shuttled the AhR into the nucleus, whereas sham or UVA irradiation did not have this effect. UVB radiation-induced AhR translocation was time-dependent and similar to that observed after treatment of cells with the known AhR ligand benzo(a)pyrene. Most importantly, UVB radiation-induced AhR shuttling was greatly enhanced if HaCaT cells were enriched for trytophan prior, but not after UVB expsoure. Taken together these studies indicate the existence of a 3rd molecular pathwayay involved in UVB radiation-induced signaling and gene expression, which is initiated in the cytoplasm, involves trytophan as a chromophore and is mediated through AhR translocation from the cytoplasm into the nucleus.