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MEETING ABSTRACTS

Transcriptional control in cells and tissues

A1

Expression and activation of T cell receptor dependent transcription factors in regulatory T cells

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T cell receptor signalling and therefore production of IL-2 upon antigen stimulation, has been shown to be impaired in regulatory T (Treg) cells. Whether the expression and activation of the major transcription factors NFATc2, AP-1 and Nf- κ B are affected too, has not yet been determined. We found a strikingly lower expression of all three factors in human Treg cells compared to memory Th cells, but their activation was unharmed. Interestingly, after stimulation with PMA/Ionomycin, thus bypassing upstream signalling events, we found a small Treg cell subset, that was able to overcome its anergic phenotype and produced IL-2. This subpopulation is characterized by higher NFATc2, AP-1 and Nf- κ B and lower FOXP3 levels compared to IL-2 nonproducing Treg cells. Our Data suggests that IL-2 production in Treg cells is not switched off by genetic imprinting, but rather the amounts and ratios of the essential transcription factors NFATc2, AP-1, Nf- κ B and FOXP3 are essential to prevent IL-2 production in Treg cells and thereby support their anergic phenotype despite a very strong stimulation.

A2

Signal transduction studies on single cell level: P38, but not NFATc2, is the main regulator of NFATc1/A expression in human Th cells

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NFATc2 and NFATc1 are the most prominent NFAT factors in T helper (Th) cells. They overlap in their functions for cytokine expression and were commonly activated by T-cell receptor

(TcR)/calcium/calcineurin signaling pathway. However, they differ strikingly in their mode of expression. NFATc2 is constitutively expressed in Th cells, whereas the NFATc1/A, the most prominent NFATc1 isoform in Th cells, is strongly induced by antigen-specific stimulation of T-cell receptor (TcR) and co-receptor(s).

The regulation of NFATc1/A expression is controversially discussed. Single cell analysis of activated transcription factors and signaling molecules enabled us to show that the activated kinase p38 is the main component for NFATc1/A induction. Using specific inhibitors and the existing different modes of activation of signaling pathways we could rule out that activated NFATc2 and NF- κ B play a prominent role in regulating NFATc1/A expression. Furthermore, we clearly demonstrated that NFATc1/A induction does not exhibit a switch-like dependence on calcineurin/NFATc2 activity.

In general, our data and results confirmed the relevance and importance to study cell signaling on single cell level.

A3

With a little help from a friend – CaN cooperates with Bcl-10 to activate NF- κ B

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Antigen-specific stimulation of T helper cells induces activation of the main transcription factors NFAT, NF- κ B and AP1 which are important for expression of cytokines such as IL-2 and IFN γ . It is known that the immunosuppressive drug Cyclosporin A (CsA) blocks the activity of the Ser/Thr phosphatase calcineurin and thereby the activation of the transcription factor NFAT. However, we observed that this drug does not only inhibit the activation of NFAT but also blocks the activation of NF- κ B. In experiments dissecting the NFAT and NF- κ B pathway we identified that CsA and other calcineurin inhibitors interfere with the phosphorylation of Bcl-10 induced by T cell receptor (TcR) or PMA/ionomycin stimulation of primary Th cells. CsA causes a faster degradation of Bcl10 and therefore inhibition of NF- κ B activation. In contrast, CsA did not affect phosphorylation of Bcl10 induced in TNF α stimulated primary Th cells. Using

immunoprecipitation we showed that calcineurin indeed interacts with Bcl-10. We hypothesize that calcineurin interacts with the CARMA/Bcl-10/MALT1 complex and dephosphorylates Bcl-10 and, thus, promotes NF- κ B activation. Therefore, Calcineurin is not only a hub for NFAT but also for NF- κ B activation in TcR-triggered Th cells.

Our data are used to sculpt a mathematical model because we could not cope with the complexity of the interfering signaling pathways and their kinetics. Our model predicted a complexity even higher as assumed before and showed the need of modelling to understand complex signaling processes.

A4

Tfg (Trk fused gene) is a Carma-1/IKK γ interacting protein involved in CD40-induced canonical NF- κ B signaling

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Carma-1 is required for B cell receptor-/CD40- and T cell receptor-/CD28-induced B- and T-cell activation via JNK and NF- β B. In B cells, Carma-1 becomes phosphorylated by PKC β , leading to its oligomerization. Subsequent Bcl10 binding induces IKK β -activation and, thereby, canonical NF- κ B signalling. Despite these findings it is still unknown how exactly Carma-1 is connected to the plasma membrane and to the IKK-complex. Therefore, we purified Carma-1 complexes from mouse CH12 B cells using anti-Carma-1 affinity columns. Mass spectrometric analyses of the column eluates demonstrated the presence of Carma-1 as well as three previously uncharacterized adaptor proteins in B cells, one of which was the Trk-fused gene (Tfg), an adaptor protein containing PBI and coiled-coil domains. Whereas Tfg was originally identified as fusion partner of oncogenic Trk tyrosine kinase mutants, the normal cellular homologue of Tfg has so far not been described in B cells. However, Tfg has been shown in other systems to interact with IKK γ and to enhance TNF-induced NF- κ B activation.

Tfg and Carma-1 co-localized at the plasma membrane and perinuclear structures in B cells. We further corroborated the interactions of Tfg, IKK γ and Carma-1 by Blue Native gel electrophoresis, where Carma-1 and Tfg formed a 0.7–1 MDa complex. Ectopic expression of Tfg increased the molecular mass of IKK γ complexes, fused IKK γ , Bcl10 and Carma-1 complexes to a ~2 MDa complex, and increased basal and CD40-induced canonical activity of NF- κ B and IKK β . In contrast, shRNA-mediated silencing of Tfg decreased CD40-induced IKK β activity. Very interestingly, in primary B cells, highest expression of Tfg was detected in marginal zone and B1 B cells, and Carma-1 and Tfg formed complexes in these B cells. Since Carma-1 is required for marginal zone B cell and B1 B cell development, we suggest that a functional interaction between Carma-1 and Tfg contributes to development and maintenance of these cells by means of canonical NF- κ B signals.

A5

TGF- β signalling in nervous system development

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Transforming growth factor betas (TGF- β) are multifunctional cytokines with widespread distribution. TGF- β s are secreted dimeric proteins that signal via a heteromeric transmembrane serine-threonine tyrosine kinase complex. Phosphorylation of receptor associated Smads leads to the formation of complexes with the common Smad4, which translocates to the nucleus to regulate as a larger transcriptional complex, immediate early gene and target gene expression. However, growing biochemical and developmental evidence supports the notion that alternative or additional, non-Smad pathways also participate in TGF- β signalling. TGF- β s are essential regulators of cellular processes including proliferation, differentiation, migration, cell survival and death during embryonic development, angiogenesis and wound healing. TGF- β actions are quite often described as opposite or distinct effects in context-dependent situations. The explanation for these data may be that TGF- β is cross-talking with numerous other signalling pathways. The presentation intends to describe the complexity of TGF- β signalling on one hand and on the other hand will focus on specific examples of TGF- β signalling during nervous system development.

A6

An atypical NF-kappa B-regulated pathway mediates phorbol ester-dependent Heme oxygenase-1 gene activation in monocytes

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Heme oxygenase (HO)-1 is the rate-limiting enzyme of heme degradation. More recently, HO-1 has been shown to have anti-inflammatory and antioxidant functions, which have been demonstrated in HO-1 knockout mice models and a human case of HO-1 genetic deficiency. Moreover, targeted induction of HO-1 has been shown to have therapeutic effects in various disease models. Here, it is reported that the HO-1 gene is transcriptionally induced by the phorbol ester phorbol myristate acetate (PMA), which is a prototypical activator of PKC, in various monocytic cells. The PMA-dependent induction of HO-1 has a different time-dependent pattern of induction from that of lipopolysaccharide-dependent HO-1 induction in these cells. Activation of HO-1 by PMA was mediated via a newly identified κ B element of the proximal rat HO-1 gene promoter region (-284 to -275). This HO- κ B element was a nuclear target for the NF- κ B subunit p65/RelA as determined by nuclear binding assays and transfection experiments with luciferase reporter gene constructs in RAW264.7 monocytes. Moreover, PMA-dependent induction of endogenous HO-1 gene expression and promoter activity was abrogated in embryonic fibroblasts from

p65^{-/-} mice. PMA-dependent HO-1 gene activation was reduced by an overexpressed dominant negative mutant of I κ B, but not by dominant negative I κ B kinase-2 (IKK2) suggesting that the classical NF- κ B pathway was not involved in this regulation. The antioxidant N-acetylcysteine and inhibitors of p38 MAPK or serine/threonine kinase CK2 blocked PMA-dependent HO-1 gene activation. Finally, it is demonstrated by luciferase assays with a Gal4-CHOP fusion protein that activation of p38 MAPK by PMA was independent of CK2. Taken together, induction of HO-1 gene expression by PMA is regulated via an IKK-independent atypical NF- κ B pathway that is mediated via activation of p38 MAPK and CK2.

A7 **SUMOylation of the transcription factor NFATc1 leads to its subnuclear relocalization and IL2 repression by HDAC**

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The family of NFAT (Nuclear Factor of Activated T-cells) transcription factors plays an important role in cytokine gene regulation. In peripheral T-cells, NFATc1 and c2 are predominantly expressed. Due to different promoter and polyA site usage as well as alternative splicing events, NFATc1 is synthesized in multiple isoforms. The highly inducible NF-ATc1/A contains a relatively short C-terminus whereas the longer, constitutively expressed isoform NFATc1/C spans an extra C-terminal peptide of 246 amino acids. Interestingly, this NFATc1/C-specific terminus can be highly sumoylated. Upon sumoylation, NFATc1/C – but not the unsumoylated NFATc1/A – translocates to Promyelocytic Leukemia-nuclear bodies (PML-nbs). This leads to interaction with HDACs followed by deacetylation of histones, which in turn induces transcriptionally inactive chromatin. As a consequence, expression of the NFATc1 target gene interleukin-2 is suppressed. These findings demonstrate that the modification by SUMO converts NFATc1 from an activator to a site-specific transcriptional repressor, revealing a novel regulatory mechanism for NFATc1 function.

A8 **A role for Fra1 in the control of transcriptional network reorganization following ras transformation**

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RAS proteins act as molecular switches transmitting signals from the cell surface to the nucleus, thereby affecting several downstream signaling cascades. Among these cascades, the mitogenic MEK/ERK pathway affects a network of transcription factors such as SRF, ELK

and API components, largely known to play an important role in regulating cellular proliferation. While the targets of individual transcription factors have been identified, the structure of the transcription factor network down-stream of MEK/ERK signaling mediating transformation is not well understood. In addition, both gene activation and repression are necessary for tumor formation and it is unclear how certain MEK/ERK stimulated transcription factors participate in both processes.

We performed genome-wide gene expression analysis to identify transcription factors differentially regulated via MEK/ERK between immortalized and HRAS-transformed cells. Individual transcription factors such as Fra1, over-expressed in RAS-transformed cells and human tumors derived from lung or bone, were knocked-down using siRNA. A second gene expression profiling was used to determine the target genes of these transcription factors. TRAP (TRanscription factor Affinity Prediction), a biophysical model of transcription factor binding and gene set enrichment analysis (GSEA) was used to screen for genes with conserved binding motifs and for functional gene sets exhibiting similar regulation.

These approaches revealed novel insights into the role of Fra1 upon activation in RAS-transformed cells. We could define a previously unknown involvement of the MEK/ERK-dependent Fra1 transcription factor in governing the alteration of the transcriptional network in tumor cells: Fra1 seems to play a role in chromatin remodeling and in circadian functions. In addition, we observed a Fra1-dependent suppression of interferon target genes, which are known to be regulated via DNA methylation. These data suggest a key role for the API complex and the Fra1 transcription factor in the reorganization of chromatin and the transcriptional network following oncogenic transformation.

A9 **Identification of transcriptional pathways in naturally occurring human regulatory T cells**

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Naturally occurring CD4⁺CD25⁺ Tregs (nTregs) are essential for T cell homeostasis and the maintenance of peripheral tolerance. They prevent the activation of autoaggressive T cells in the context of autoimmune diseases and suppress inadequate allergen specific T cells. On the opposite, nTregs inhibit also effective immune responses against tumors such as melanoma. A detailed understanding of molecular mechanisms that control the functional properties of human nTregs is mandatory for the development of novel immunotherapies against allergy, autoimmunity and cancer. Therefore, we initiated a genomic, proteomic and kinome profiling of human nTregs to identify key molecules in human nTregs associated with their functional activation which are responsible for their state of anergy and/or their suppressive activity. We started with large-scale isolation of nTregs using whole leukapheresis products followed by poly-

clonal stimulation and analysis at different time points. As a result, we identified a distinct molecular activation pattern specific for the activation state of human nTregs. The impact of identified key molecules was tested in functional assays using specific inhibitors and siRNA mediated knockdown of these targets. A general transcriptional network analysis is currently under investigation and will be presented on the meeting. The main objective of our analysis is the identification of novel targets for the immunotherapeutic intervention of dysregulated immune responses in the near future.

Receptor-triggered pathways

A10

Establishment of a specific Real-time RT-PCR protocol to detect human leptin receptor isoforms

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The hormone leptin plays a major role in obesity, but exhibits additional crucial functions as a cytokine in reproduction, immunity and even cancer. In humans, four different isoforms of leptin receptors (ObR1, 219.1, 219.2, 219.3) have been described, which share an identical extracellular domain, but differ considerably in their intracellular domains indicating that they might have different cellular functions. Among the leptin receptor isoforms, the signal transduction pathways originated from ObR1 have been intensively investigated. The activation of STAT3 (*signal transducer and activator of transcription*) is triggered via binding to the ObR1-exclusive box 2 motive, while the MAPK (*mitogen-activated protein kinase*) pathway can be activated through the junxtamembrane box 1 motive, which is present in all isoforms. However, the expression and functions of the 219.1, 219.2 and 219.3 isoform are completely unknown.

To obtain a deeper understanding of the functional role of leptin receptor isoforms, we screened a large number of human cell lines originated from different tissues and organs for their leptin receptor isoform expression pattern. Since reliable protocols for the detection of differential gene expression of human ObR isoforms did not exist, we established and optimized a novel reliable real-time RT-PCR protocol using isoform-specific primers and performed detailed quantitative analyses of the ObR isoform expression. Our results show a ubiquitous expression pattern of all isoforms in several cancer cell lines indicating that ObR can function as an important signalling molecule in many tissues. The insights raised from this analysis might be relevant for understanding the diverse effects of leptin.

A11

Combination of SILAC and *in situ* biotinylation to detect specific protein interactions

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Stable isotope labeling with amino acids in cell culture (SILAC) has emerged as a major technique for quantitative proteomics using cell culture. It has been applied to the investigation of many different biological processes such diverse as the characterization of signaling pathways and the determination of protein interactions.

For investigation of proteins, interacting with the Signal Transducer and activator of transcription 3 (STAT3), we combined SILAC with stringent precipitation of the biotinylated proteins and quantitative mass spectrometry analyses, to detect cellular interaction partners of STAT3 in mammalian cells.

STAT3 is a well-known protein, which plays crucial roles in different biological responses including early embryonic development as well as cell growth and apoptosis. Moreover, STAT3 is constitutively activated in oncogene-transformed cells and various primary tumors.

Although the JAK-STAT signaling pathway is one of the best known, there appears to be STAT3-specific regulatory proteins, which are not established until now. Hence, finding of novel STAT3-interacting proteins can encourage the knowledge of STAT3-protein network.

For metabolic labeling using SILAC, cells were grown in medium containing either only 'light' arginine or only 'heavy' 13C6-arginine, resulting in incorporation of labeled arginine in all sites. In mass spectrometry analyses the peptides containing the 13C6-arginine, has produced a 6 Da difference in mass, relative to the light form.

STAT3 fused with a tag for its biotinylation was expressed and biotinylated in HEK-293 cells, which were fully substituted with 13C-labeled arginine in place of the light arginine. In the parallel approach HEK cells, grown in normal medium, were transfected with STAT3 without the tag for biotinylation. After combining the cultured cells, STAT3 and its interacting proteins were specific precipitated, separated by SDS/PAGE, and analyzed by mass spectrometry. This method bears a strong potential to recover now protein interactions.

A12

The docking protein and proto-oncogene product Gab2 is regulated via a novel negative feedback mechanism mediated by 14-3-3 binding

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In vertebrates, Grb2-associated binder (Gab)1–3 constitute a family of conserved docking proteins. Gab2 is tyrosine-phosphorylated

upon activation of a variety of growth factor, hormone, antigen, cytokine and cell matrix receptors, leading to the recruitment of specific src homology (SH)2 domain-containing effectors, which include the p85 subunit of phosphatidylinositol (PI)3-kinase and the protein tyrosine phosphatase Shp2. These Gab2 effectors potentiate the activation of the PI3-K/AKT and Ras/ERK pathways, respectively. Studies using gene knock-out mice indicate that Gab2 is required for normal mast cell-mediated allergic responses and osteoclast differentiation, and in combination with Gab1, for cardiac function. In addition, Gab2 signals downstream of several oncogenic tyrosine kinases, and are overexpressed in breast cancer, and promotes erbB2-induced mammary tumorigenesis. Therefore, it is critical to define how Gab2 signalling is regulated in normal and pathological states. One critical event in Gab2 signalling is its interaction with the adaptor protein Grb2, which promotes its association with specific receptors and thereby sustains its tyrosine phosphorylation dependent recruitment of the aforementioned effectors. However, the molecular mechanisms that attenuate or limit Gab2 signals have remained unclear.

In the presented study, we have addressed Gab2 regulation using an integrated approach that combines a proteomics-based definition of the Gab2 'phosphomap' with bioinformatics, biochemistry and cell biology. Here we report the discovery of 21 novel phosphorylation sites on human Gab2. Furthermore, we demonstrate that growth factor-induced and PI3K-dependent phosphorylation of Gab2 on two of these novel residues, S210 and T391, leads to recruitment of 14-3-3 proteins. These events mediate negative feedback regulation, since a Gab2 mutant that cannot be phosphorylated on these sites exhibits sustained receptor association and signalling, and promotes cell proliferation and transformation. Importantly, site-specific introduction of constitutive 14-3-3 binding sites into Gab2 renders it refractory to receptor activation, demonstrating that site-selective binding of 14-3-3 proteins is sufficient to terminate Gab2 signalling. Furthermore, this is associated with drastically reduced recruitment of Grb2 to the Gab2 signalosome suggesting a competition between 14-3-3 and Grb2 for Gab2 binding. These findings lead to a model where signal attenuation occurs, because 14-3-3 promotes dissociation of Gab2 from Grb2, and thereby uncouples Gab2 from the receptor complex. This represents a novel regulatory mechanism with implications for diverse tyrosine kinase signalling systems in various cell types.

A13

Compartmentalization of TNF-receptor I signalling: acid sphingomyelinase is activated by Caspase-8 in internalized TNF-R1 receptorsomes
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In a recently identified novel TNF-induced apoptotic signaling pathway the generation of ceramide by acid sphingomyelinase (A-SMase) and the activation of the aspartic protease cathepsin D (CTSD) occur in the same endosomal compartment [1]. CTSD subsequently mediates cleavage of Bid leading to activation of caspase-9 and -3. Since activation of A-SMase is linked to the death domain of TNF-receptor I (TNF-R1) and since the death domain adapter proteins FADD and caspase-8 are recruited

during internalization of TNF-R1 in endosomes (TNF-receptosomes) [2], we addressed the question, whether A-SMase can be activated directly by caspase-8 within this compartment. We here show by confocal laser scan microscopy and in immunomagnetically isolated TNF-receptosome preparations that the active form of caspase-8 colocalizes with A-SMase within TNF-receptosomes. Activation of caspase-8 correlates with cleavage of a 70/72 kDa pro-A-SMase molecule, paralleled by enhanced A-SMase activity, activation of CTSD as well as Bid-cleavage in isolated TNF-receptosomes. The functional link between caspase-8 activity and A-SMase stimulation is revealed by the lack of TNF-induced A-SMase activation in caspase-8 deficient Jurkat cells, which can be restored in vivo after retransfection of caspase-8 as well as in vitro by the addition of exogenous caspase-8 to lysates from caspase-8 deficient cells.

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A14

The epidermal growth factor receptor (EGFR) contributes to efficient entry of influenza A viruses into host cells

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Previous observations suggested that the entry process of influenza A viruses (IAV) is at least in part a signaling-regulated event. Although it is well known that sialic acids on the cell surface serve as direct receptors for IAV by binding to the viral HA, the requirement of early signaling events for viral entry suggests the involvement of signal transmitting receptors. However, the nature of these receptors that could transmit entry relevant signals across the membrane, are so far unknown. Our recent observation that the phosphatidylinositol-3 kinase (PI3K), that is an effector enzyme of growth factor receptors, is involved in IAV entry [1] lead to the hypothesis that receptor tyrosine kinases may play a role as cellular signaling receptors upon virus binding to cells. In this study we introduce the EGFR, a prominent member of the receptor tyrosine kinase family as a novel player to be involved in IAV entry processes. Inhibition of tyrosine kinases in general by small molecule inhibitors as well as specific inhibition of the EGFR by inhibitors, siRNA mediated knock-down or treatment of cells with EGFR blocking antibodies results in reduced viral uptake and subsequently to reduced progeny virus titers. In contrary, over-

expression of the EGFR or treatment with EGF during infection leads to enhanced uptake and increased virus titers. Furthermore, infection results in a redistribution of the EGFR similar to that observed upon stimulation with the ligand EGF. IAV at least in part co-localizes with the EGFR and both, viral particles on the surface and the receptor are localized in lipid rafts. According to our data we propose, that influenza virus is a multivalent agent that induces a clustering of EGFR and other signaling receptors into lipid rafts, by binding to sialic acid coupled proteins. This may lead to a low level induction of the receptor-induced signaling cascades, such as PI3K/Akt that facilitates viral entry. Thus, we could identify for the first time the EGFR as an indirect viral receptor to form a lipid raft-based signaling platform required for efficient IAV uptake.

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A15

Chromatin structure and expression of the AMPA receptor subunit GluR2 in human glioma cells and the role of REST

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The AMPA receptors are postsynaptic ion channels that open following stimulation with glutamate. The regulatory region of the gene encoding the GluR2 subtype of AMPA receptors contains a binding site for the transcriptional repressor REST. The cell-type specific microenvironment, in particular the cell type-specific structure of the chromatin, is crucial for the ability of REST to control target gene transcription. Using antibodies directed against methylated lysine residues 4 or 9 (H3K4 or H3K9) of histone H3, we show that the GluR2 gene has an open chromatin configuration in human U87MG glioma cells, with nucleosomes carrying di- and trimethylated H3K4. In contrast, the GluR2 gene is embedded into a repressed chromatin environment in non-neuronal hepatoma cells and keratinocytes. Chromatin immunoprecipitation experiments revealed binding of REST and histone deacetylase-I to the GluR2 gene under physiological conditions. While overexpression of REST reduced GluR2 mRNA levels, expression of a mutant of REST that contained a transcriptional activation domain enhanced GluR2 gene transcription in U87MG glioma cells. Treatment of the cells with the histone deacetylase inhibitor trichostatin A (TSA) induced an upregulation of GluR2 expression, indicating that the transcription of the GluR2 gene is dependent of the balance between histone acetylation and deacetylation. Transcription of the GluR2 gene was also inducible by TSA following inhibition of Sp1 target gene transcription, indicating that the inhibition of Sp1 is not required for the upregulation of GluR2 expression following histone deacetylase inhibition.

A16

The neuron-specific protein, p42^{IP4} (Centaurin- α 1) is localized in mitochondria, interacts with 2',3'-cyclic nucleotide 3'-phosphodiesterase and is involved in regulation and control of mitochondrial Ca²⁺

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p42^{IP4} is a brain-specific protein also called centaurin- α 1. This protein specifically recognizes two second messengers, the membrane lipid PtdIns (3,4,5)P3 and the soluble inositol phosphate Ins(1,3,4,5)P4. Previously, expression of p42^{IP4} protein in non-neuronal CHO cells stably transfected with pcDNA-p42^{IP4} was shown in cytosol, membranes and nucleus. The membrane fraction in that study also contained mitochondria. The yeast protein Gcs1p, which is structurally and functionally related to p42^{IP4}, is localized in mitochondria and is involved in maintenance of mitochondrial morphology. The program PSORT II had predicted a high probability for mitochondrial localization of p42^{IP4}. Therefore, localization of p42^{IP4} in mitochondria was suggested.

We show here for the first time that p42^{IP4} is localized in mitochondria, isolated from cells transfected with p42^{IP4}, CHO cells and mouse neuroblastoma (N2a) cells. In CHO cells, p42^{IP4} is localized predominantly in the intermembrane space side of the mitochondrial inner membrane. p42^{IP4} is also found in mitochondria isolated from rat brain.

Previously, 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP) has been shown to be associated with mitochondria, but the exact role of CNP in mitochondria is still obscure. We show the localization of CNP in both mitochondrial membrane fractions by sub-fractionation of rat brain mitochondria (RBM). We additionally found interaction of p42^{IP4} with CNP in RBM by pull-down binding assay and by immunoprecipitation.

Cellular Ca²⁺ signals are crucial in the control of most physiological processes, cell injury and programmed cell death. In neurons, mitochondria dampen changes in cytosolic Ca²⁺ loads and sustain cellular Ca²⁺ homeostasis that is required for normal neuronal function. However, mitochondria take up a limited amount of calcium up to a certain threshold. Accumulation of Ca²⁺ above this threshold leads to increased permeability of the inner mitochondrial membrane due to formation of a unselective pore at the contact site between outer and inner membranes. Since permeability transition pore (PTP) opening is important in mitochondrial events leading to cell death, we studied whether p42^{IP4} is involved in Ca²⁺-induced Ca²⁺ release and consequently PTP. We determined the Ca²⁺ capacity and lag-phase for PTP opening in mitochondria isolated from p42^{IP4}-transfected and from control N2a cells. Overexpression of p42^{IP4} led to significant decrease of these functional mitochondrial Ca²⁺ parameters. Thus, we suggest that due to involvement in the regulation of Ca²⁺ transport in mitochondria, p42^{IP4} destabilizes mitochondria by promoting Ca²⁺-induced PTP opening.

A17**Oncostatin M-induced and constitutive activation of the JAK2/STAT5/CIS pathway suppresses CCL1, but not CCL7 and CCL8 chemokine expression**C Hintzen¹, C Haan², J Tuckermann³, PC Heinrich⁴ and HM Hermanns¹¹Rudolf Virchow Center, DFG Research Center for Experimental Biomedicine, University of Würzburg, Würzburg, Germany²Life Science Research Unit, University of Luxembourg, 1511 Luxembourg, Luxembourg³Leibniz Institute for AgeResearch, Fritz Lipmann Institute, Jena, Germany⁴Department of Biochemistry and Molecular Biology, University of Freiburg, Freiburg, Germany

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The recruitment of leukocytes to injured tissue is crucial for the initiation of inflammatory responses as well as for immune surveillance to fight tumor progression. Here we show that oncostatin M, a member of the interleukin-6-type cytokine family and potent pro-inflammatory cytokine stimulates the expression of the chemokines CCL1, CCL7 and CCL8 in primary human dermal fibroblasts at a faster kinetic than IL-1 or TNF. The production of CCL1 and CCL8 is important for migration of monocytes, while specific antibodies against CCL1 additionally inhibit the migration of T lymphocytes. We identify the mitogen-activated protein kinases ERK1/2 and p38 as crucial factors for the enhanced expression of CCL1 and CCL8. Depletion of the ERK1/2 target genes c-Jun or c-Fos strongly decreases CCL1 and CCL8 expression, while p38 MAPK prolongs the half-life of CCL1, CCL7 and CCL8 mRNA through inhibition of tristetraprolin. None of the STAT transcription factors STAT1, STAT3 or STAT5 stimulate transcription of CCL1 or CCL8. However, we identify a negative regulatory function of activated STAT5 for the gene expression of CCL1. Importantly, not STAT5 itself, but its target gene CIS is required for the STAT5 inhibitory effect on CCL1 expression. Finally, we show that constitutive activation of STAT5 through a mutated form of JAK2 (JAK2 V617F) occurring in patients with myeloproliferative disorders similarly suppresses CCL1 expression. Taken together, we identify novel important inflammatory target genes of OSM, which are independent of STAT signaling per se, but depend on MAPK activation and are partly repressed through STAT5-dependent expression of CIS.

A18**Mechanotransduction of pulsatile stretch in a cell culture model of cardiac hypertrophy connexin 43 (Cx43) expression**S Karl¹, D Rojas Gomez², S Dhein² and A Salameh¹¹Herzzentrum, Kinderkardiologie, Universität Leipzig, Germany²Herzzentrum, Herzchirurgie, Universität Leipzig, Germany

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Introduction: In the heart gap junction intercellular coupling represents the fundament of proper electrical propagation. In cardiac hypertrophy up-regulation of Cx43 has been observed and was suggested as being related to sudden cardiac death. It has been found, that expression and distribution of Cx43, which is

the main gap junction forming connexin in the ventricle, is altered in many pathological conditions. We therefore wanted to elucidate whether exposure of cardiomyocytes to chronic pulsatile stretch affects Cx43 and whether mechanotransduction pathways interfere with adrenergic regulation of Cx43, already shown in former experiments.

Results: Neonatal rat cardiomyocytes cultured on gelatine coated Flex Cell cell culture plates were exposed to pulsatile stretch (110% of resting length, 1 Hz) for 24 hours without stimulation of adrenoceptors or in presence of 0,1 µM isoproterenol (Iso) for beta-adrenoceptor stimulation or 0,1 µM phenylephrine (Phe) for alpha-adrenoceptor stimulation. Cx43-expression was determined by Western blotting. Exposure to pulsatile stretch led to a significant increase in Cx43 protein expression (157 ± 6%), as did treatment with Iso (147 ± 6%) or Phe (176 ± 16%). Combined stimulation by stretch and Iso or stretch and Phe lead to only minor further increase in Cx43-levels (Iso+Stretch: 166 ± 16%, Phe+Stretch: 191 ± 17%). To elucidate the further signal transduction cascade we investigated phosphorylation of extracellular regulated kinase (ERK1/2) and glycogen synthase kinase 3-beta (GSK3-beta). Exposure to pulsatile stretch led to a significant increase of phosphorylation of ERK1 (143 ± 6%), ERK2 (159 ± 5%) and GSK3-beta (138 ± 7%) as did treatment with Iso and Phe. Again a significant further increase in these levels by addition of both stimuli could not be observed.

Conclusion: Thus, pulsatile stretch affects cardiac gap junction expression and leads to phosphorylation of MAP kinases (= activation) and of GSK3-beta (= inactivation). There were only small additive effects with adrenergic stimulation indicating a possible ceiling effect in up-regulation of Cx43 expression maybe caused by counter regulative mechanisms.

A19**The G protein-coupled receptor identity of the frizzled proteins**

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Receptors of the Frizzled family initiate Wnt ligand-dependent signal transduction cascades controlling multiple steps in organism development and are highly conserved in animal evolution. Misactivation of the Wnt/Frizzled signaling underlies many cases of cancerogenesis. Frizzled receptors possess seven transmembrane domains and their signaling depends on trimeric G proteins in various organisms. However, as Frizzled proteins constitute a distinct group within the superfamily of G protein-coupled receptors (GPCR), and as Frizzled signaling can apparently be G protein-independent in some experimental setups, the GPCR nature of Frizzled receptors has been questioned. Here we demonstrate that human Frizzled receptors can directly bind the trimeric Go protein in a pertussis toxin-sensitive manner. Furthermore, addition of Wnt ligands elicits Frizzled-dependent guanine nucleotide exchange on Go. An excess of secreted Frizzled-related protein, a known antagonist of the Wnt/Frizzled pathways, inhibits Go activation, as does pretreatment of Go with pertussis toxin. These experiments provide a biochemical proof of the GPCR activities of Frizzled

receptors. They also establish an in vitro assay of monitoring Frizzled activation by Wnt ligands, applicable for the high-throughput agonist/antagonist screening.

A20

Arsenite induces oxidative stress and caspase 3-mediated apoptosis in human neuroblastoma cells involving p53

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Arsenic trioxide (As₂O₃) induces cell death in human neuroblastoma cells. Clinical trials are currently performed to elucidate the use of arsenic trioxide in cancer therapy. Here, we show that arsenic trioxide treatment activates caspase 3 and triggers an upregulation of p53, c-Jun and ATF3 expression in human neuroblastoma cells. Cell death, caspase 3 activity and enhanced expression of p53, c-Jun, and ATF3 was attenuated in neuroblastoma cells that had been treated with the antioxidant N-acetyl cysteine, indicating that arsenic trioxide cytotoxicity is based on the generation of oxidative stress. Experiments involving lentiviral-mediated expression of either a dominant-negative mutant of p53 or a p53-specific short hairpin RNA showed that p53 is essential for arsenite-induced cell death of neuroblastoma cells. In contrast, down-regulation of c-Jun and ATF3 expression by short hairpin RNA interference or inhibition of the transcriptional activity of c-Jun did not rescue neuroblastoma cells from arsenite-induced cell death. Cells were protected from arsenite-toxicity following expression of Bcl-2 or Bcl-xL or mutants of Bcl-2 and Bcl-xL that had been targeted to the endoplasmic reticulum (ER), suggesting that mitochondria and the ER are involved in the apoptotic signaling cascade initiated by arsenite.

A21

Elucidation of the SLP-65 phosphorylation state in activated B lymphocytes

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The SH2 domain-containing leukocyte adaptor protein of 65 kDa (SLP-65) is a central effector for signaling downstream of the B-cell antigen receptor (BCR). Upon phosphorylation on serine/threonine and tyrosine residues, SLP-65 nucleates the formation of multimolecular protein complexes to integrate numerous BCR-signaling events. We have now qualitatively and quantitatively identified phospho acceptor sites of SLP-65 by applying state of the art mass spectrometry. SLP-65 turned out to possess a plethora of phospho acceptor sites. In fact, it turned out to be one of the most phosphorylated proteins described so far. Moreover, by applying stable isotope labelling of amino acids in cell culture (SILAC) we identified several acceptor sites whose

phosphorylation kinetic is differentially regulated upon BCR-stimulation. The functional relevance of some of these sites was subsequently analyzed by mutational analysis of SLP-65 in SLP-65-deficient DT40 B cells. In contrast to the described role of SLP-65 tyrosine phosphorylation for the initiation of Ca²⁺-signaling, serine/threonine phosphorylation of SLP-65 turned out to be a key regulator for BCR-dependent MAP-kinase activation and AP-1 regulated gene transcription. Collectively our data explain several of the SLP-65 controlled biological responses elucidated by genetic means and further support the role of SLP-65 as the key integrator of BCR-signaling. In general (phospho)proteomics combined with reconstitution experiments in gene-targeted DT40 cells proves to be a powerful strategy to uncover post translational modifications and their biological relevance in cell signaling.

A22

Growth factor signaling – cell biology turned into medicine

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A series of novel drugs targeting growth factor signaling – including antibodies targeting growth factors or their receptors, low-molecular weight tyrosine kinase (TK) inhibitors and mTOR inhibitors – are approved as cancer drugs. These drugs differ with regard to target cells and target molecules. Best effects of drugs targeting TKs on malignant cells are, in general, observed in tumors where target TK is activated by amplification, translocation or point-mutations. Mutational activation of down stream signaling proteins, such as PI3 kinase or RAS, is associated with reduced sensitivity to TK-targeting drugs. Determinants of resistance and sensitivity to anti-angiogenic drugs remain mostly unknown. Results from breast cancer suggest that many of these drugs will display highest efficacy when used in adjuvant settings. Our studies on the role of PDGF receptor signaling in cancer have revealed important roles of autocrine PDGF receptor signaling in dermatofibrosarcoma protuberans and glioblastoma. However, in common solid tumors, PDGF receptor signaling is predominantly involved in the regulation of tumor stroma through effects on pericytes and cancer-associated fibroblasts (CAFs). Studies in animal models have demonstrated that inhibition of stromal PDGF-receptors improves tumor drug uptake, and thus suggest a novel rational for combination treatments with PDGF inhibitors and other cancer drugs. Recent analyses of large series of human breast cancer have also identified high stromal PDGF receptor expression as a marker for worse prognosis.

Ongoing studies in our group also aim at identification of novel stroma-derived cancer drug targets. By gene expression profiling of prostate CAFs we have identified CXCL14 as a novel CAF-derived multi-modal stimulator of tumor growth. Ongoing studies also explore how paracrine crosstalk between CAFs and malignant cells, will affect the proliferation, migration and drug response of malignant cells.

A23**Reverse signaling by FasL inhibits primary human T cell activation**

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The death-factor Fas Ligand (FasL) is best known for its capacity to induce cell death in Fas-expressing cells. Besides its death-promoting activity, FasL has been implicated in reverse signaling and might thus also play a role in T cell development and selection and the modulation of T cell activation by acting as a costimulatory receptor. Here we have analyzed the influence of FasL-costimulation on TCR/CD3/CD28-triggered activation of peripheral human T-lymphocytes. Interestingly, FasL engagement inhibited the proliferation of PBMC, CD8⁺ as well as CD4⁺ T cells. Plate-bound but not soluble FasFc fusion protein or anti-FasL pAb blocked CD3/CD28-induced proliferation almost completely. We observed not only less proliferation, but also decreased IL-2 production and reduced expression of the activation markers CD69 and CD25. Importantly, FasFc costimulation also resulted in a dramatic inhibition of TCR internalization, thereby preventing TCR translocation and the formation of signaling platforms essential for optimal T cell activation. Consistent with these findings, various crucial signaling components of the T cell receptor activation pathway were inhibited by FasL triggering and reverse signalling. In this context, the phosphorylation of ERK1/2, p38 MAPK as well as further upstream acting signaling proteins such as PLC γ was markedly reduced. Notably, the inhibition was also observed in the presence of exogenous rIL-2, indicating that a lack of IL-2 is not the cause of the proliferation block. Taken together, our data argue for a negative reverse signaling capacity of FasL on freshly isolated, TCR-triggered human T cells.

A24**CD95 as a negative or positive costimulatory receptor for primary T cell activation**

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The CD95/CD95L- (Fas/FasL-) system is best known for its capacity to induce cell death. However, recent data indicate an additional role for Fas (CD95) in obviously conflicting functions namely activation and proliferation. We therefore investigated the effect of Fas co-ligation during TCR/CD3/CD28-triggered activation of freshly isolated human T-lymphocytes. We noted that plate-bound but not soluble agonistic anti-Fas antibodies led to an accelerated proliferation, suggesting a strong positive costimulatory effect of Fas. Consistent with this observation, more IL-2 and IFN γ was produced and we observed enhanced phosphorylation of STAT5, increased MAPK and caspase activation and strong upregulation of activation markers and cell cycle proteins including CDKs and cyclins. Also, as a consequence of ERK1/2 activation, the phosphorylation of the Retino Blastoma Protein (Rb) at serine 780 and 795 was more pronounced. Moreover, activation-induced TCR internalization

was enhanced upon Fas-costimulation, allowing improved TCR translocation and generation of signal platforms for optimal T cell activation. Much to our surprise, ligation of Fas by plate-bound but not soluble FasL-Fc fusion protein had an opposite effect and blocked TCR-induced proliferation almost completely. In this context, crucial events associated with T cell activation, i.e. tyrosine and ERK1/2 phosphorylation, expression of activation markers, IL-2 production and caspase activation were abrogated. Although we do not have a clear explanation for the opposite effects of the two types of ligation of the very same receptor, we hope to find the answer in ongoing experiments using a number of constructs that allow for differential oligomerization or using FasL-transfectants to replace the fusion proteins and monoclonal antibodies to establish a more physiological situation for primary T cell activation.

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A25**A comprehensive map of the IL-1R signalling network**

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The interleukin-1 (IL-1) signalling pathway represents a paradigm for all IL-1 family cytokines (IL-1A, IL-1B, IL-18, IL-33, IL1F5 to IL1F10) as well as for the toll-like-receptor-induced pathways. Research on IL-1 signalling for more than 20 years has generated a large body of experimental data, which are scattered across many different publications. No signal transduction map exists that covers comprehensively this huge knowledge on IL-1-activated effector molecules. We attempted to improve the publicly accessible IL-1 signalling map currently deposited in Netpath <http://www.netpath.org/pathways>. This map contains 36 molecules and information for a total of 89 reactions including physical interactions, catalytic reactions (e.g. phosphorylations) and subcellular distributions. Netpath includes data from experimental results reported in 46 publications. We created an extended version that now contains 140 molecules and 245 reactions taken from original data out of 146 selected publications. In addition to visualization of molecules involved in IL-1 signal transduction this map enables direct access to the scientific evidence that was used to build up the map. Subsequently we imported the map into GenMAPP, Version 2.1 and mapped mRNA expression data from a time course experiment of human KB cells that were stimulated for 0.5, 1, 3, 16 and 24 h with 10 ng/ml IL-1 α . This analysis revealed that several interconnected parts of the IL-1 signalling network are co-induced or co-repressed during stimulation. By treating the cells with 50 μ M PD 98059 we were able to map the part of the IL-1-signalling network that responds to ERK-inhibition at the level of mRNA. In summary, this type of knowledge-based curated data base serves not only to sort and to store data from biochemical, cell biological and molecular biology experiments but may also help to depict nodes of signal transduction that can be used to design new hypothesis-driven experiments.

A26**Resting and activated states of the B cell antigen receptor**

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Each B cell contains up to 100,000 B cell antigen receptor (BCR) complexes on its surface, which only become fully active on exposure of the B cell to its cognate antigen. Most studies on BCR function aims at a better understanding how the BCR becomes active and transmits its signal to the many signaling pathways inside the cell. Another, albeit related question, is how the B cells ensure that the many BCR complexes stay (in most cases) in a silent inert conformation. This problem of activation control the BCR shares with many of its signaling elements, be it kinases, phosphatases or adaptors, which all have to be regulated tightly in their localization and activity. For the later element autoinhibition plays an essential role in this regulation. In a biochemical study we previously have found that on resting B cells the BCR resides in oligomeric and monomeric forms. We now have conducted experiments that suggest that the BCR also can resume an autoinhibitory conformation and that BCR oligomerization plays an important role in this process. Specifically oligomerization of the BCR in conjunction with the kinase-phosphatase equilibrium at the BCR contributes to the tight control of BCR activation. Our studies resulted in a new model of the resting BCR and its activation.

We also study how the cytosolic protein tyrosine kinase Syk is regulated and activated by the BCR. Syk is also regulated by auto-inhibition and only becomes fully active when recruited to the BCR. We now have identified Syk mutant that are altered in auto-inhibition and tested their activity in the S2 Schneider cell reconstitution system as well as in reconstituted Syk deficient B cell lines. Our data show that a tight Syk regulation is essential for normal B-lymphocytes development.

Tumour biology and cancer therapy

A27**Human calreticulin can act as adjuvant in the maturation of antigen presenting cells**

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Calreticulin (CRT), an endoplasmic reticulum (ER) resident protein, is involved in critical cellular functions, such as protein folding and antigenic peptide cross presentation. Furthermore, this chaperone has been proposed to act as an adjuvant during the activation of dendritic cells (DCs) in vivo. We assessed human eukaryotically expressed CRT for its potential to induce NF-kappa B regulated maturation of monocyte-derived DCs. In order to facilitate eukaryotic expression procedures, we

established and compared three different methods to express recombinant endotoxin-free CRT to be secreted in the supernatant of HEK 293 cells: (1.) the complete, unmodified CRT coding sequence was cloned into the pcDNA3.1V5/His vector (euCRT), (2.) the C-terminal ER-retrieval KDEL amino acid sequence was mutated into KDQL in order to disturb the endoplasmic retention and support the protein secretion (euCRT_KDQL) and (3.) a shRNA was designed to knock down the expression of aminoacyl-tRNA synthetase-interacting multifunctional protein-1 (AIMP-1), which is known to regulate protein retention in the ER. An efficient shRNA sequence specific to AIMP-1 transcripts was delivered to HEK 293 cells, which were afterwards transfected with the CRT-expressing vector (euCRT). No relevant differences between these different approaches were observed in regard to mRNA levels of the transfected CRT determined by Real Time RT-PCR as well as protein expression levels of CRT determined by V5/HIS ELISA in the cell culture supernatants. Thus, for large scale expression of CRT the first strategy with the unmodified CRT sequence (euCRT) was chosen. The functional capability of the expressed calreticulin to induce maturation of DCs was tested. By flow cytometry the translocation of NF-kappa B into the nuclei of the monocytes after stimulation with the recombinant CRT could be demonstrated. Using low-dose CRT (10 µg/ml) the phenotype of the immature DCs changed to a more matured one, as indicated by an increased surface expression of CD40, CD86, CD83. In summary, our first data indicate, that this recombinant CRT can act as an adjuvant for in vitro maturation of DCs and therefore has the potential to assist in T cell stimulation and expansion protocols.

A28**Heat Shock Protein 70 (HSP70) induces cytotoxicity of T-helper cells**

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Stress-inducible heat shock protein 70 (HSP70) has gained plenty of attention because of its potent adjuvant capability to induce antigen-specific CD8⁺ cytotoxic T-lymphocyte (CTL) and CD4⁺ T-helper cell (Th1) responses. In this study, we investigated the behavior of T-cell subsets stimulated with endotoxin-free recombinant HSP70 with respect to proliferation, cytokine expression, cytotoxicity against allogeneic B-lymphoblastoid cell line (B-LCL) and K562 cells as well as target-independent cytotoxicity.

CD4⁺ cells exhibited a strong increase in proliferation after stimulation with HSP70, with rates of up to 29%. In the presence of target cells, a 35-fold up-regulation of granzyme B mRNA was observed after stimulation of CD4⁺ T-helper cells with HSP70 in combination with IL-7, -12 and -15. The target cell-independent secretion of granzyme B by CD4⁺ cells was greatly augmented after stimulation with HSP70 plus IL-2 or IL-7, -12 and -15.

In this study, we have shown that HSP70 is capable of inducing a cytotoxic response of T-helper cells in the absence of LPS or any other PAMPs. The granzyme B secretion and the cytolytic activity of CD4⁺ T cells is induced in a target-independent way, whereas the cytotoxic activity of CD3⁺ and CD8⁺ T cells can be further enhanced in the presence of the target cells. Our data provide novel insights into the role of extracellular HSP70 on T-cell immune response concerning the induction of target-independent T-helper cell cytotoxicity.

A29

NKG2A silencing in effector cells to improve effectivity of cell based therapeutics

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The heterodimeric NKG2A/CD94 receptor delivers an inhibitory signal upon recognition of HLA-E molecules. In several studies it has been demonstrated that signalling via NKG2A/CD94 receptor significantly reduces T or NK cell cytotoxic activity and cytokine production. In hematopoietic stem cell transplantation, expression of NKG2A on NK and T cells has been shown to compromise the graft-versus-leukemia effect. In addition, NKG2A was shown to inhibit tumor-specific T cell responses. In this study, we developed a RNAi-based approach to permanently silence the expression of NKG2A molecules on NK and T-cells. The functional relevance of NKG2A silencing for the cytotoxic potential of genetically engineered NK and T-cells was evaluated. NKG2A⁺ cells were isolated from fresh PBMCs. Lentiviral vectors were designed to express short hairpin RNA sequences (shRNA) targeting NKG2A transcripts. The level of NKG2A suppression was measured by flow cytometry and real-time RT-PCR. The effect of NKG2A receptor silencing on the cytolytic potential of NK and T cells was evaluated in cytotoxicity assays using K562 and B-LCL cells as targets. In addition, granzyme B mRNA transcript levels were detected by real-time RT-PCR.

The transduction of inducible RNAi cassettes containing the sequences for shRNAs targeting NKG2A caused a reduction of protein expression by up to 80% in NK and T cells. In cytotoxicity assays, it was demonstrated that NKG2A silencing was effective to enhance NK and CD8⁺ T cell lysis by up to 40%. In comparison with unmodified cells, granzyme B transcript levels were upregulated by up to 12-fold in NKG2A silenced cells after target exposure. Expression of NKG2A-specific shRNA did not affect the expression of the activating markers NKp44 on NK cells and CD25 on T cells.

Our data suggest that RNAi-mediated silencing of NKG2A in effector cells could improve the effectivity of cell-based immunotherapeutics and might be of particular interest in an autologous approach.

A30

CNK1 controls invasiveness of breast cancer cells via transcriptional regulation of MT1-MMP through the PI3K-Akt-NF-κB signalling axis

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The scaffold protein CNK1 was described to be involved in protooncogenic signalling pathways utilising protein kinases Raf-1 and Src and the small GTPase Rho. Deregulation of these molecules is clearly correlated with cancer. Nonetheless, a direct impact of CNK1 on cancer cell behaviour was not investigated so far. To address this question we analysed the function of CNK1 in a highly invasive breast cancer cell line MDA-MB-231.

Downregulation of CNK1 by retroviral shRNA delivery reduced cell proliferation and this effect was even more pronounced under serum starvation. Furthermore, knockdown of CNK1 impaired the invasion of Matrigel by MDA-MB-231 cells without affecting cell migration, suggesting a defect in proteolytic activity in these cells. In agreement with this observation, expression of several matrix metalloproteinases was diminished in CNK1 knockdown cells. In particular the promoter of membrane type 1 matrix metalloproteinase (MT1-MMP) was shown to be less active upon CNK1 downregulation. Conversely, CNK1 overexpression stimulated the MT1-MMP promoter. This stimulatory effect was sensitive to the IKK inhibitor BAY11-7082 and the PI3K inhibitor LY294002. CNK1 was found to influence the alternative NF-κB pathway through regulation of the processing step from p100 to p52. Moreover, phosphorylation of Akt on Ser 473 was reduced in CNK1 knockdown cells, a result which is consistent with Akt's importance in p100 processing. Importantly, analysis of human cancer samples by immunohistochemistry revealed that CNK1 can be overexpressed in breast cancer samples compared to healthy tissue.

Taken together, these results provide evidence that CNK1 is a part of the invasion-promoting machinery in breast cancer cells and may be considered as a potential therapeutic target.

A31

Cancer/testis antigen 45 is expressed in a nuclear speckles-like pattern in human tumor cell lines

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The cancer/testis antigen (CT) family is defined by its specific expression pattern. In most cases CT antigens are expressed in normal human tissue only in germline cells and some tumors. Because of their restricted expression pattern CT antigens are regarded as potential targets for vaccine immunotherapy. Little is known about the functions of the various CT antigens. Some of these CT antigens (CT 7; MAGE A3/6) seem to be involved in the dysregulation of cell-cycle control and increased cell proliferation. The monoclonal antibody Ki-A10 detects a nuclear antigen with a unique distribution pattern in normal human tissues and tumors. The antigen is now characterized as cancer/testis antigen 45 (CT45).

The accurate localisation of CT45 could provide details about its function. Therefore immunofluorescence stainings with subsequent

confocal laser microscope analysis were performed to deliver precise data about the nuclear localisation of CT45. Different human tumor cell lines (L428, HT1080, WSI-CLS, SW872) and 5-aza-2'-deoxycytidine treated human peripheral blood lymphocytes were stained with mab Ki-A10. All stainings showed an irregular and dotlike pattern, which is wellknown for nuclear speckles (interchromatin granule clusters). Nuclear speckles are dynamic structures known to harbour many proteins of the pre-mRNA splicing machinery. In addition CT45 shows a clear homology to a DEAD-Box protein (DDX26). DEAD-Box proteins are generally believed to be RNA helicases, which in turn are localised in nuclear speckles. Due to these results we believe that CT45 could be involved in pre-mRNA splicing.

A32

Specific effects of Lef-1 splice variants on the regulation of gene expression in pancreatic cancer cells

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The lymphoid enhancer factor (Lef-1) belongs to the nuclear transducers of canonical Wnt-signalling in embryogenesis and cancer. Lef-1 acts, in cooperation with beta-catenin, as a context-dependent transcriptional activator or repressor thereby influencing multiple cellular functions such as proliferation, differentiation and migration.

Here we report an increased Lef-1 expression in human pancreatic cancer, which correlates with advanced tumour stages. As demonstrated by RT-PCR analysis, pancreatic carcinoma exhibit two different transcripts present in pancreatic carcinomas. One transcript was identified as the full length Lef-1 (Lef-1 FL), whereas the second, shorter transcript, lacked exon VI (Lef-1 exon VI) compared to the published sequence. Comparative analysis of these two Lef-1 variants revealed different cellular effects after transient expression in pancreatic carcinoma cells. Forced expression of Lef-1 exon VI in pancreatic carcinoma cells inhibited E-cadherin expression and resulted in reduced cellular aggregation and increased cell migration compared to cells expressing full length Lef-1. Expression of Lef-1 FL, but not the newly identified Lef-1 exon VI, induced expression of the cell cycle regulating proteins c-myc and cyclin D1 and resulted in enhanced cell proliferation.

Thus, our findings implicate that expression of alternatively spliced isoforms of Lef-1 are involved in the determination of proliferative or migratory characteristics of pancreatic carcinoma cells.

A33

Dissecting the molecular pathogenesis of Burkitt lymphoma

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Burkitt lymphoma (BL) is a high grade B cell malignancy (Non-Hodgkin Lymphoma (NHL)) derived from germinal center B cells (GCB) that harbours a chromosomal translocation juxtaposing the protooncogene MYC next to the regulatory elements of one of the immunoglobulin loci. However, the precise contribution of Myc to the pathogenesis of this tumour is poorly understood. Based on the definition of a distinguishing gene expression signature for the molecular BL (mBL) with Myc as one hallmarking signature gene we are interested in getting answers to the questions (i) what are the target genes of Myc in primary human GCB cells and (ii) what is the functional significance of signature genes identified?

We describe a non-viral vector based approach (Vockerodt et al. 2008) to express Myc in primary human GCB cells. Comparative gene expression profiling was performed accompanied by qRT-PCR. In addition elucidation of the function of selected signature genes in BL is accomplished. In a representative cell line with a mBL signature RNAi directed inhibition of elements of the CD40 signaling cascade was conducted. After activating this particular signaling cascade in a BL cell line we analysed respective gene expression profiles of IKKa, IKKb, TRAF2, TRAF6, Jak3, BCL-3 and p38 deficient cells. Based on these different RNAi-mediated GE-profiles we reconstruct the topology of the respective signaling pathway by using the nested effects bioinformatic model, which has been described recently (Markowitz et al. 2005).

Acknowledgements

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A34

New bortezomib-based combination therapy for elimination of myeloma cells

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The clinically approved proteasome inhibitor bortezomib (Bz) represents a promising agent for the therapy of relapsed multiple myeloma. However, long-term remissions are difficult to achieve, in fact myeloma cells often develop secondary resistance to proteasome inhibition. We recently demonstrated that myeloma cells are highly sensitive towards proteasome inhibitors due to their extensive rate of immunoglobulin synthesis, thereby triggering the terminal unfolded protein response (UPR) and apoptosis via endoplasmic reticulum (ER) stress. We want to identify synergistic agents sensitizing myeloma cells towards Bz. The calcium channel blocker verapamil has been shown to inhibit

proliferation of leukemia cells and to interfere with multi drug resistance-based drug elimination. Hence, we analysed the effect of Bz together with verapamil on the viability and ER-stress in different human myeloma cell lines. The combination of Bz and verapamil synergistically decreased cell viability of myeloma cell lines by inducing apoptotic and necrotic cell death. Importantly, Bz-mediated activation of major UPR signalling pathways was enhanced by verapamil. The Bz/verapamil treatment also resulted in caspase activation followed by PARP cleavage. NF- κ B DNA-binding activity markedly declined in myeloma cells treated with both agents. In contrast to Bz, proteasomal activity was not altered by verapamil. However, the amount of ubiquitinated proteins in detergent-insoluble fractions was much higher in the presence of Bz/verapamil compared to Bz alone, suggesting increased formation of protein aggregates within the cell. Beside that, verapamil reduced expression of the multi drug resistance protein 1 and impaired drug-efflux in the myeloma cells. We conclude that verapamil increased the pro-apoptotic effect of Bz by inducing additional ER-stress signals along with inhibiting the NF- κ B activity and modulating drug transport mechanism. Thus, the combination therapy Bz/verapamil may provide a more effective treatment-strategy for multiple myeloma than the Bz-monotherapy and overcome drug resistance.

A35

Correlation of malignancy parameters in colorectal carcinoma with up- and downstream signalling partners of STAT3

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STAT3 (Signal Transducer and Activator of Transcription 3) is persistently activated in about 90% of colorectal carcinoma (CRC) cases. However, CRC cell lines show variability in their degree of constitutive and cytokine-inducible STAT3 tyrosine phosphorylation. We have employed both surgical biopsies from CRC patients and a large collection of permanent CRC cell lines to address (1) which tyrosine kinases are involved in aberrant STAT3 activation in CRC, and (2) in which ways dysregulated STAT3 activity contributes to malignancy-associated cell behavior in CRC.

Western blot data obtained from analyzing CRC tumor specimens as well as vicinal normal tissue were quantified and statistically analysed. These studies revealed correlations between activated STAT3 on one hand and Src and JAK2 activity on the other (in particular in non-tumorous border tissue), and between Src and JAK1 activity (particularly in malignant tumor tissue). STAT3 activation shows a clear decrease in later stages of tumor progression. With regard to potential parameters of malignancy (here: invasiveness), we could show a striking coincidence of STAT3 activation and strong expression of matrix metalloproteinases MMP-1 both by biochemical and histological analysis.

For detailed studies, we chose from an extended panel of CRC cell lines as examples (1) HT-29, in which STAT3 activity is

inducible by IL-6, and (2) C-10, in which a strong constitutive STAT3 phosphorylation is observed. In C-10 cells, blockade of STAT3 dimerization by an inhibitor peptide specifically led to cell death, whereas experimental activation of STAT3 in HT-29 cells via IL-6 resulted in enhanced cell growth, MMP-1 expression and invasiveness.

In HT-29 cells, we investigated the role of IL-6-evoked STAT3 activity in the control of MMP-1 expression. Reporter gene experiments showed a direct influence of STAT3 activation on transcription from the human MMP-1 promoter. By analyzing both protein-protein and DNA-protein interactions within this promoter we obtained evidence for a complex mode a cooperative transcriptional regulation through STAT3 and AP-1. Additional STAT3 dimerization inhibitor compounds and CRC cell lines are currently being analyzed and the results will be discussed.

A36

Adipocytokines – mediators of fat tissue linking obesity and cancer

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Obesity is a dramatically increasing public health problem worldwide. Traditionally, fat tissue was considered to be solely an energy storage depot. However, recent studies have shown that adipose tissue exerts important endocrine functions, which are predominantly mediated by a network of various soluble factors derived from adipocytes. New evidence has come to light elucidating a modulatory role of this adipocytokines in the regulation of cancer development. For example, adipocytokines such as leptin were shown to have an effect on breast cancer progression. In this study we have investigated the impact of leptin on the proliferation and migration of colon carcinoma cells. Treatment of human SW480 colon carcinoma cells with leptin resulted in a significant increase of the proliferation. In parallel, using our unique 3D cell migration assay and time-lapse video microscopy, leptin strongly stimulated the spontaneous migratory activity from 29% locomoting cells to 52%. This leptin-induced migration resulted in an activation of various transcription factors such as Stat-3 and c-Jun. Accordingly the phosphorylation of Stat-3 was accompanied by an increase of SOCS-3, its negative feedback regulator. Furthermore, using a Stat-3 specific inhibitor inhibited the leptin-induced migration. Understanding the impact of different adipocytokines on tumour migration and the underlying signal transduction mechanisms is mandatory for the future development of cancer therapy.

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A37

Unfolded protein response is activated by single application of BMP-2

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Introduction: Tumor formation and progression is characterized by a proceeding degeneration of genetic material. As a consequence a growing number of proteins are misfolded and thus accumulate in the lumen of the endoplasmic reticulum and induce the “unfolded protein response” (UPR). Activation of the UPR can cause elimination of cells through apoptosis or a restabilization and therefore cell survival.

We could previously show that chronic and single application of BMP-2 alters distinct subsets of genes in the breast cancer cell line MCF-7. The group of apoptosis-related genes was predominantly regulated after short-term application of BMP-2. The protein kinase R (PKR) exhibited the most prominent BMP-2 dependent regulation.

Materials and methods: In order to verify the array results, we incubated the breast carcinoma cell line MCF-7 with 50 ng/ml or 100 ng/ml BMP-2 and performed real-time PCR for PKR and selected genes associated with UPR and apoptosis regulation at several time points. The activation of the PKR pathway was analyzed by immunoblotting using phosphor-specific antibodies for PKR and eIF2alpha. UPR is involved in apoptosis regulation. Therefore, the cell cycle status of the cells was studied by FACS analysis using the Cell Cycle Test (Becton Dickinson).

Results: Incubation of MCF-7 cells with BMP-2 induces a 2-fold induction of PKR expression after 24 h independent of the amount of BMP-2 applied. The alterations of PKR expression found on the mRNA level were further investigated on the protein level by western blot analysis. We could show that the BMP-2 dependent up-regulation of PKR mRNA is paralleled by an increase in PKR protein content with a subsequent down-regulation of the total protein content of PKR after 24 hours. Incubation of MCF-7 cells with BMP-2 led to a robust increase of the fraction of phosphorylated PKR after 4 hours suggesting an additional route of BMP-2 dependent regulation of PKR activation. A prominent substrate of PKR is the alpha-subunit of the translation factor eIF2. Phosphorylation of eIF2alpha leads to an inhibition of translation. During incubation of MCF-7 cells with BMP-2 the level of total eIF2alpha is not altered. In contrast, the amount of phosphorylated eIF2alpha is increased in BMP-2 treated MCF-7 cells after 16 h up to 24 h. compared to serum-free controls. Cell cycle analysis revealed a slight reduction of apoptotic cells under the influence of BMP-2 in comparison to controls.

Conclusion: BMP-2 is able to activate the cellular stress response in the breast carcinoma cell line MCF-7 giving a link to a new impact of BMP-2 in tumorigenesis.

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A38

Link between BMP expression and clinical outcome in breast carcinomas

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The expression of various bone morphogenetic proteins, receptors and signalling molecules in several human cancers could be shown, but little is known about the connection between BMP expression and clinical parameters and outcome. In vitro experiments demonstrated that bone morphogenetic proteins exhibit supporting and suppressing actions during tumor formation and progression. We could previously show that BMPs affect different tumor associated processes like apoptosis, cell cycle and differentiation in breast carcinoma cells depending on the time of exposure (Steinert et al., 2008). These results encouraged us to investigate the clinical relevance of BMP in breast cancer patients.

We used tissue microarray technology to determine the BMP-2 expression in over 2000 specimen from breast cancer patients via immunochemistry. Furthermore we analyzed proteins that are involved in tumor-associated processes like apoptosis. The results were tested for significant correlations to histopathological parameters and overall survival.

The immunohistochemical investigations demonstrated a weak to moderate expression of BMP-2 in 60% of the analyzed breast cancer specimen. Small, low-grade tumors exhibited a pronounced BMP-2 expression. The BMP-2 content showed an inverse correlation to the estrogen-receptor ($p = 0.001$) and to the proliferation ($p = 0.001$). The BMP-2 expression is positively correlated to the anti-apoptotic protein bcl2 ($p = 0.024$). The cell cycle regulators cyclin D1 ($p = 0.001$), p27 ($p = 0.029$) and p16 ($p = 0.003$), which inhibit the G1/S-phase transition, are significantly associated with BMP-2. In addition BMP-2 is positively correlated to the expression of SFRP1 ($p < 0.001$), a Wnt-antagonist which is supposed to be a tumor suppressor. The most prominent result was, that BMP-2 expressing tumors exhibited a significant increase in overall survival ($p = 0.001$). Grouping of the specimen according to clinical and immunohistochemical aspects showed a prognostic benefit of patients with a higher BMP-2 level especially for nodal-negative invasive-ductal breast carcinomas.

In conclusion, BMP-2 could be identified as an independent prognostic marker for human breast cancer.

A39

Structural basis for the oncogenic signalling complex formed by Grb2 and Gab2 in Her2 (ErbB2/Neu)-driven breast cancers and CML cells

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The adaptor protein Grb2 and the large multi-site docking protein Gab2 form a complex that is crucial for the oncogenic signalling of some cancer cells. Binding of the C-terminal SH3 (SH3C) domain of Grb2 to Gab2 is essential for the interaction, but molecular details of the complex formation have remained undefined. Using peptide array SH3 overlay blots, isothermal titration calorimetry and protein crystallography, two atypical SH3 domain binding sites in Gab2 (Gab2-1 and Gab2-2) were confirmed and characterised. Gab2-1 has considerable similarity to an epitope in the cell cycle regulator p27Kip1 that also binds Grb2SH3C. The SH3C binding sites in Gab2 differ approximately 5-fold in their affinity and also with respect to the residues important for the interaction apart from a shared core motif RxxK. Both SH3 domain interaction sites in Gab2 are conserved throughout evolution and found, for example, in the Gab homolog *daughter of sevenless* (Dos) of *Drosophila melanogaster*, which binds to the Grb2 homolog Drk. Mutagenesis of the Drk binding sites in Dos *in vivo* impairs fly eye and wing vein development and documents that both sites are functionally important. A high-resolution crystal structure was generated of the Gab2-2 epitope in complex with the Grb2SH3C domain. This reveals a binding mode reminiscent of the interaction of haematopoietic Grb2-relative Mona/Gads with the T-cell adaptor SLP-76 and haematopoietic progenitor kinase I (HPK1). However, subtle differences exist, which could potentially be exploited to generate SH3C domain inhibitors with preference for a specific Grb2 family member.

A40**Interaction of prostate cancer and neurons – the neuro-neoplastic synapse**

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As the angiogenesis is well known for tumor tissue supplemented with nutrients and growth factors, the neurogenesis may play an important role in tumor development, too. Tumor cells respond to cytokines, chemokines, growth factors as well as neurotransmitters in for example increased migration and proliferation. It seems to be crucial for a tumor tissue to interact with its environment and obtain nutrients and signal substances, as well as the tumor release angiogenic factors itself. In our work, we investigate the mutual influences of tumor cells and neural structures to elucidate the complex interaction and effect of cancer and metastasis forming within the body. Cells of the prostate cancer cell line PC-3 release a broad range of signal substances e.g. nerve growth factor, brain derived neurotrophic factor, vascular endothelial growth factor A and C, as well as the cytokines IL-2 and IL-1 beta, which are key regulators of angiogenesis in cancer. We were able to show that IL-2 and IL-1 beta strongly increase the migratory activity of human neuroblastoma cells SH-SY5Y in our well-established 3D cell migration assay from 30% spontaneous locomotion to 60 and 50% increase, respectively. In contrast, the migration activity of prostate cancer cells significant increases when exposed to the neurotransmitter dopamine and neuropeptide substance P up to 25%. This indicates a close relation and impact of prostate cancer cells and neural structures as a complex association, not only as

separate aggregation acting for its own. These cells affect each other by releasing their specific signal substances, which in turn can activate via receptor mediated cell signaling their locomotion and proliferation attitude. Understanding the broad interaction of these cells during cancer and metastasis forming may help us to develop new approaches for cancer therapy in the future.

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Signal alternations induced by pathogens**A41****“Switch to kill, switch to survive” – bacterial toxins modifying Rho GTPases**

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Various bacterial protein toxins act on eukaryotic cells by targeting switches involved in cellular signalling and/or control of the cytoskeleton. Of particular importance as targets are molecular switches of the Rho GTPase family. The GTPase switches have crucial functions in innate and adapted immunity and play pivotal roles in the biology of infection, which explains their preferred targeting by toxins. The toxins activate or inactivate Rho GTPases by different modes of action. Cytotoxic necrotizing factors (CNFs) from *E. coli* and *Yersinia* species activate Rho GTPases by deamidation of a glutamine residue, which is involved in the switch-off mechanism of the GTPases. Activation of RhoA is caused by *Pasteurella multocida* toxin secondary to activation of the heterotrimeric G proteins G_q and G_{12/13}. On the other hand, the *Yersinia* effector protein YopT, which acts as a cysteine-specific protease to cleave the lipid anchor of GTPases, inactivates Rho GTPases. C3 exotoxins inactivate RhoA, B and C by ADP-ribosylation at Asn41 thereby preventing activation of Rho, stabilizing the Rho-GDI complex and inhibiting signalling. Large clostridial toxins, including *C. difficile* toxin A and B, inactivate various Rho GTPases by glucosylation. The mode of action of *C. difficile* toxins and their structure-function relationship will be discussed in detail.

A42**RNA-interference based screen identifies new factors important for NF-kappaB activation and termination**

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The transcription factor NF-kappaB is a key mediator of the innate immune system. Although tremendous research efforts over the past decades have led to a more and more detailed understanding of NF-kappaB signaling, there are still missing pieces in the puzzle, especially upstream of the IKK complex and in the termination of the signaling. To identify more of the factors

important for this signaling pathway we have conducted an RNA-interference based screen.

For this purpose, we have developed an assay for high throughput analysis using a human epithelial cell line stably expressing a p65-GFP-fusion construct. The nuclear translocation of p65-GFP can be quantified by automated microscopic analysis. Three different stimuli were compared: the cytokines TNF-alpha and IL1-beta and the gastric pathogen *Helicobacter pylori*. We chose *H. pylori* as inducer because permanent infection with this bacterium can lead to chronic inflammation, ulceration and cancerogenesis and NF-kappaB is thought to be crucial in the promotion of this pathology. Furthermore, using different time points of the activation, we screened not only for factors important for activation, but also for termination of the signal.

In terms of activation, the screen identified known factors like IKKalpha and IKKbeta as well as factors so far not linked to the NF-kappaB pathway. Interestingly, two factors were identified that are specific for NF-kappaB activation after *H. pylori* infection and not necessary for NF-kappaB activation by the cytokines TNFalpha or IL-1beta. Regarding termination, the screen identified among other factors an ubiquitin E3-Ligase so far not linked to the pathway. Upon down-regulation of this E3-Ligase, p65-GFP resides longer in the nucleus. This correlates with a strong degradation of IkkappaBalpha. The screen was conducted with a library of siRNAs against 646 kinases and associated proteins, and is currently expanded to a genome wide scale.

A43

Influenza A viruses induce PI3-kinase activation by two interdependent mechanisms late in the infection cycle

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We have previously shown that the cellular PI3K/Akt pathway is strongly activated upon influenza A virus infection in later stages of the infection cycle and that this activation was dependent on the expression of the viral non-structural protein 1 (A/NS1) [1]. Later it was demonstrated by us and others that activation occurs upon direct interaction of the A/NS1 to the regulatory subunits of PI3K, p85 alpha and beta [2, 3]. Several reports proposed that two src homology (SH)-binding motifs within A/NS1 (aa 89 [YXXXM] (SH2-binding motif) and aa 164-167 [PXXP] (SH3-binding motif 1)) may mediate binding to p85 beta. Our work confirms that tyrosine 89 within the A/NS1 is required for the interaction of A/NS1 with p85 beta, subsequent PI3K-activation. However, mutant viruses that carry a phenylalanine instead of the tyrosine at position 89 of the NS1 only showed marginal differences to wt viruses with regard to their replication fitness. More detailed analysis revealed that both, wt type and mutant viruses induced similar PI3K activation levels late in infection, suggesting that besides expression of the NS1 there are alternative virus-induced mechanisms to activate the kinase. Here we demonstrate that this additional inducer is viral 5'triphosphate RNA that accumulates late in the infection cycle. Thus, PI3K activity is regulated by a NS1 protein-dependent as well as a vRNA-dependent mechanism, presumably via the RIG-I sensory pathway. Since NS1 is also a negative regulator of RIG-I, we suggest that influenza viruses have

developed multiple mechanisms to achieve a well-balanced PI3K activation at later phases of the infection cycle.

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A44

Pertussis toxin blocks growth factor receptor signalling by attenuating p21ras activity

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Pertussis toxin (PTX), a *Bordetella Pertussis* exotoxin, has been recently shown to prevent growth factor-induced activation of the extracellular signal regulated protein kinases ERK1 and ERK2. Due to its capacity to ribosylate inhibitory GTP-binding proteins of the Gi/o family, the antiproliferative effect of PTX is generally thought to originate from interference with growth factor receptor-induced inhibitory signal transduction pathways. However, in human embryonic kidney (HEK293) cells, we could recently demonstrate that attenuation of inhibitory Gi/o signaling by the regulator protein Goloco failed to affect epidermal growth factor (EGF) receptor-induced ERK1/2 activation. In addition, Goloco also failed to interfere insulin-like growth factor (IGF-1) receptor associated ERK1/2 signaling, indicating that PTX must affect ERK1/2 signalling by mechanism other than inactivation of Gi/o function. The small GTP-binding protein p21ras plays a central role in mitogenic signalling, as it connects a number of growth factor receptors to the raf-1/MEK/ERK1/2 signalling module. Western blot experiments revealed that pre-treatment of HEK293 cells with PTX prevents translocation of p21ras to the plasma membranes. Moreover, immune-precipitation experiments also showed that PTX prevents interaction of p21ras with raf-1 kinase. A similar finding was observed after pre-treatment of the cells with mevastatin, a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor. Mevastatin treatment resulted in a loss of p21ras from the plasma membrane, inhibited EGF-induced p21ras/raf-1 interaction and ERK1/2 activation. These results indicate that, besides of its inhibitory action on Gi/o signalling pathways, PTX may also interfere with growth factor-mediated ERK1/2 activation by attenuating p21ras activation.

A45

Proteomic identification of the tyrosine phosphatase SHP1 as a novel LMP1 interaction partner, which mediates autoregulation of LMP1 signaling

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The Epstein-Barr virus (EBV) oncoprotein LMPI (latent membrane protein 1) mimics a constitutively active receptor molecule. It contributes to viral cell transformation by the activation of NF-kappaB, JNK/API, MAPK, JAK/STAT and PI3-kinase signaling. LMPI recruits TRAF1-3, 5 and 6, TRADD and RIP1, which are also known as signaling mediators of Toll-like and tumor necrosis factor-receptors. Here, we established a functional proteomics approach to identify novel interaction partners of the LMPI signaling domain. This approach led to the characterization of the tyrosine phosphatase SHP1 as a direct binding partner of LMPI. Interaction of SHP1 with LMPI was verified in primary human B-cells, which had been transformed with a recombinant EBV carrying a HA-tagged LMPI allele. The SHP1 binding site of LMPI is located within the membrane-proximal region of the LMPI signaling domain and shows no overlap with known protein interaction domains of LMPI. The unique sequence of this site does not resemble known SHP1 interaction motifs of cellular proteins. Mutation of the SHP1 site caused the loss of SHP1 binding to LMPI in EBV-transformed human B-cells. SHP1 has previously been described as a negative regulator of growth factor or immune receptor signaling by dephosphorylating e.g. tyrosine kinases such as JAKs or SRC kinases. LMPI induction of the NF-kappaB pathway was greatly enhanced in SHP1-knockout DT40 B-cells as compared to wildtype cells. This effect was reverted by reconstitution of SHP1 expression in the SHP1-KO cells. Also mutation of the SHP1 interaction site or the co-expression of a dominant-negative SHP1 caused hyperactivation of NF-kappaB signaling and JAK3 hyperphosphorylation by LMPI. Because the SHP1 interaction site of LMPI mediates inhibitory effects on LMPI signaling, we named this region CTIR1 (C-terminal inhibitory region 1). In summary, the proteomic analysis of the LMPI complex revealed a novel autoregulatory mechanism of oncogenic LMPI signaling, which limits its own activity through the recruitment of a tyrosine phosphatase. This mechanism might be of high relevance for the survival of EBV-transformed cells because LMPI hyperactivity is known to be toxic for the target cells.

A46

SOCS-1 potentiates *Pasteurella multocida* toxin induced cell transformation

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Pasteurella multocida toxin (PMT) is a mitogenic protein toxin that modulates mammalian signalling cascades. In pigs, PMT causes atrophic rhinitis characterized by loss of nasal turbinates. Experimental nasal infection leads to excess proliferation of bladder epithelial cells suggesting it has carcinogenic properties. Recently we showed that PMT induces signal transducers and activators of transcription (STAT) activity through Gαq mediated activation of JAK kinases. Activation of the JAK-STAT pathway is persistent, as PMT does not induce expression of suppressor of cytokine signalling (SOCS) proteins.

We overexpressed SOCS-1 in HEK293 cells to investigate if this would downregulate PMT-induced STAT activation. However, STAT activity was not abrogated; instead, SOCS-1 enhanced

STAT3 activity significantly. To test if this effect was specific for SOCS-1, we expressed SOCS-1, -3 or CIS and monitored STAT3 transcriptional activity. Hyperactivation of STAT3 correlated with the nuclear localization of the SOCS protein and SOCS-1 was a much stronger activator than SOCS-3, while CIS did not enhance STAT activity. However, a CIS mutant containing the SOCS-1 nuclear localisation sequence (NLS) acted as potently as SOCS-1. We next determined the phosphorylation status and expression of the STAT3 activating tyrosine kinase JAK2. Interestingly, JAK2 expression levels were increased in the presence of SOCS-1 eventually leading to hyperphosphorylation of JAK2. It is known that SOCS proteins act as E3 ubiquitin ligases that target proteins, for example JAK kinases, to proteasomal degradation. Oncogenic kinases such as Bcr-Abl can overcome this process through activation of pathways that lead to serine phosphorylation of SOCS-1. It is believed that Pim serine/threonine kinases are crucial for this. Pim kinases are STAT dependent genes and the protein was shown to interact with SOCS-1 directly. Cells stimulated with PMT showed high Pim-1 expression that increased with time and were strongest after over-night stimulation, while IL6-stimulated cells downregulated Pim-1 expression within 3 hours. Incubation with a Pim-1 specific inhibitor reversed the SOCS-1-dependent transcriptional hyperactivity of STAT3 to a great extent. In addition, we found that SOCS-1 is heavily threonine phosphorylated after PMT-stimulation but not after stimulation with IL-6. We hypothesise that persistent expression of Pim-1 leads to phosphorylation of SOCS-1, which protects JAK2 from proteasomal degradation. JAK2 accumulates, leading to hyperactivation of STAT3 and probably enhanced transforming potential. In a colony formation assay using HEK293 cells PMT was able to induce anchorage independent cell growth. This effect was even more pronounced in the presence of SOCS-1. In summary, we show that PMT is able to hijack signalling cascades much in the same way than oncogenic tyrosine kinases and that its capacity to transform cells is enhanced by SOCS-1.

A47

The *Pasteurella multocida* toxin (PMT) induced differentiation of haematopoietic progenitor cells in macrophages and B cells

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The *Pasteurella multocida* toxin (PMT) is a highly mitogenic toxin that mainly affects domestic and wild animals, but can also affect humans through animal bites. PMT induces porcine atrophic rhinitis that is characterised by bone resorption and loss of nasal turbinate bones. PMT acts intracellularly and activates heterotrimeric G proteins by an unknown mechanism. The N-terminus of PMT includes the receptor-binding and translocation domain that supposedly binds to ganglioside type receptors, whereas the C-terminus contains the biologically active domain.

Our data show that PMT induces proliferation and differentiation of haematopoietic progenitor cells into macrophages and B-cells. To investigate the influence of PMT on proliferation of haematopoietic progenitor cells we performed cell proliferation ELISAs that showed a significant increase of growth in PMT-

treated cells compared to the unstimulated control. To identify the cell types that differentiated from the isolated bone marrow cells (BMC) we stimulated BMCs for 7 days with PMT. To exclude the possibility that LPS contaminations triggered the observed cell growth, an N-terminal PMT fragment containing only the translocation domain, or a PMT fragment lacking the complete catalytic domain were used and found to be inactive. BMCs were then analyzed by FACS analysis, which revealed differentiation of the PMT-stimulated cells in populations that were missing in the unstimulated cells. We identified the generated populations using fluorescently labelled surface marker-specific antibodies and found CD45R (B-cells) and CD11b (macrophages) expression significantly increased in stimulated cells. The macrophage population was further characterised in a phagocytosis assay using fluorescent latex beads and found to be as effective as control macrophages generated with L-cell-conditioned media. To investigate whether PMT directly stimulates cell differentiation or induces the secretion of other soluble factors, we assayed the IL6 production that stimulates the differentiation of B-cells and macrophages, in PMT-treated BMCs compared to unstimulated cells. The data show a significant increase of IL6 secretion of PMT-stimulated cells supporting the idea that PMT induces cytokine production in BMCs. Using BMCs from toll like receptor (TLR) -2 deficient mice that are unresponsive to LPS we investigated whether PMT would still be able to cause cell differentiation. However, this was not the case. While LPS stimulates osteoclast activity, it does not allow osteoclast differentiation from progenitor cells. Also PMT mutants without biological activity were unable to stimulate cell differentiation. We therefore propose an LPS-independent mechanism where PMT activity needs TLR2 as an essential cofactor.

A48

Capability of the viral NSI proteins to bind to the cellular adaptor proteins Crk and CrkL determines sensitivity of influenza viruses to Crk/CrkL expression

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The non-structural protein 1 (A/NSI) of influenza A viruses harbors several src homology domain (SH)-binding motifs that are required for interaction with cellular proteins, such as the p85 beta subunit of PI3-kinase. Besides the A/NSI interaction with p85 beta it could be shown, that the SH3-binding motif 2 (aa 212–217 [PPLPPK]) within A/NSI is essential for binding to the cellular adaptor proteins Crk and CrkL. Both regulate diverse pathways in the cell including activation of the MAP kinase JNK, that was previously shown by us to mediate antiviral responses [1, 2]. To elucidate Crk/CrkL functions in the infected cell we knocked-down expression of the adaptor proteins by a siRNA approach. We could demonstrate that only those influenza A viruses that encode a A/NSI-protein harboring the Crk/CrkL SH3-binding motif 2 PPLPPK are attenuated upon downregulation of Crk/CrkL. It could also be observed that the PPLPPK site-harboring candidate strains exhibit a stronger viral activation of the JNK/ATF-2 signaling module compared to other strains and that knock-down of the adaptor proteins resulted in an even

stronger activation of this virus-induced antiviral acting pathway. Consistent with this observation, overexpression of Crk or CrkL resulted in a reduced virus-induced JNK activation. Further analysis revealed that the localization of the A/NSI is altered in Crk overexpressing cells and that the CrkL-phosphorylation pattern is changed upon binding to A/NSI. The data so far suggest that A/NSI binding to Crk or CrkL contributes to the suppression of the antiviral acting JNK/-ATF-2 pathway. The Crk/CrkL binding capability may have only evolved in virus strains that over-induce this antiviral signaling module to suppress its detrimental action.

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A49

Connexin 43 expression is impaired in beginning heart failure in spontaneously hypertensive rats

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Introduction: Arrhythmia is a severe problem in many pathological conditions of the heart such as cardiomyopathy and heart failure. As gap junctions provide the basis for a regular rhythmic beating and regular electrical propagation, connexins forming gap junctions in the heart have been very attractive targets for scientific research in the past.

While in hypertrophy Cx43 is up-regulated, in heart failure Cx43 has been shown to be decreased. We therefore wanted to elucidate whether spontaneously hypertensive rats showing signs of impaired ventricular function have altered Cx43 levels and if so, which pathways are involved in these changes.

Results: Hearts of 6 months old Wistar Kyoto Rats (WKY) and spontaneously hypertensive rats (SHR) showing signs of hypertrophy and impaired ventricular function, i.e. reduced cardiac output (SHR: 72 ± 3,5 ml/min; WKY 81 ± 4,3 ml/min) and impaired diastolic relaxation, were analyzed by western blot. SHR rats showed a marked decrease in Cx43 levels (80,9 ± 4,5%) compared to WKY rats. To elucidate the further signal transduction cascade we investigated phosphorylation of extracellular regulated kinase (ERK1/2) and glycogen synthase kinase 3-beta (GSK3-beta). Examination of MAPK pathways showed enhanced phosphorylation of ERK1 (116,8 ± 1,6%) and ERK2 (139,9 ± 12,6%) while the phosphorylation status of GSK3-beta was markedly decreased (64,4 ± 7,4%). An enhanced phosphorylation of phospholamban (PL) was detected, indicating an impaired Ca²⁺-handling in the failing heart.

Conclusion: Downregulation of Cx43 in failing hearts from SHR rats seems to be related to lower phosphorylation status of GSK3-beta together with phosphorylation of ERK1 (low degree) and ERK2 (modest degree).

The above findings are in contrast to the status of hypertrophy in which GSK3-beta is phosphorylated (= inactive form) and Cx43 is up-regulated.

A50
NF- κ B activation by the viral oncoprotein StpC is enhanced by ERK-mediated p52 and RelB upregulation

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Induction of T-cell lymphomas and T-cell growth transformation by Herpesvirus saimiri strain C-488 depend on the saimiri transformation-associated protein of subgroup C (StpC). Previous studies identified the transcription factor NF- κ B as the major cellular target of StpC. NF- κ B activation relies on a TRAF binding motif in StpC and was enhanced by coexpression of constitutively active Ras or Raf. Concomitantly, StpC repressed Ras-mediated ERK and AP-1 activation. Nevertheless, we now found that specific inhibitors of MEK as well as ERK abrogated cooperative NF- κ B activation. Triggering the ERK pathway by external stimuli, e.g. PMA, also enhanced StpC-induced NF- κ B activity, however, with a significant delay relative to ERK1/2 phosphorylation. These observations suggested that ERK activity regulates the expression of proteins limiting StpC's capacity to induce NF- κ B. Westernblot analyses of proteins representing the classical and alternative pathways of NF- κ B activation revealed that StpC cooperates with Ras and even more with PMA to upregulate the expression and nuclear localization of RelB and NF- κ B2/p52; furthermore, StpC coimmunoprecipitated TRAF2, but not TRAF6, Ras or Raf. In summary, these data suggest that ERK-inducing signaling pathways support NF- κ B activation by StpC through an enhanced expression of NF- κ B proteins utilized by the alternative pathway, which is triggered by StpC:TRAF2 complexes. Future studies will have to address the relevance of the enhancing effect for the proliferation of Herpesvirus saimiri-transformed human T lymphocytes.

A51
The role of STAT6 in human T cell transformation by herpesvirus saimiri

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Herpesvirus saimiri (HVS) induces T-cell lymphoma in New World primates. HVS subgroup C strains growth transform human T-cells *in vitro*. Oncoproteins StpC and Tip are essential for transformation. The lymphocyte tyrosine kinase Lck is the major interaction partner of Tip and phosphorylates Tip at specific tyrosine residues. We find that STAT6 is activated by Tip together with Lck, requiring either Tip residues, Y114 or Y127 for increased activation and both for full activation. Our analysis addresses whether Tip or the Y114F and/or Y127F mutant mediate interactions between STAT6 and Lck or other Src family members. The components are expressed and purified

using the Strep-tag system to identify additional factors involved in complex formation by affinity chromatography. Furthermore, respective fluorescent fusion proteins are made to observe colocalization patterns and interaction within living T cells by fluorescence resonance energy transfer. These studies on the association of Tip, Lck, and STATs can resolve further regulatory mechanisms involved in viral transformation process.

A52
Inverse relationship of TLR/NF- κ B signalling and the Wnt/ β -catenin pathway during inflammation: Deciphering the role of Frizzled1 in *M. tuberculosis* infection

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Wnt ligands are palmitoylated glycoproteins that regulate essential aspects of early development, including embryonic patterning, cell proliferation and cell fate determination. Wnts are secreted and bind to cell-surface heptahelical receptors termed "Frizzleds". To date 10 Frizzled (Fzd) receptors have been identified in mice and men. Depending on the receptor context, Wnt ligands can initiate at least three different intracellular signaling cascades: The Wnt/ β -catenin pathway, the Wnt/Planar cell polarity (PCP) pathway and the Wnt/ Ca^{2+} pathway.

Our previous observation that factors of the Wnt/Fzd signaling pathway are differentially expressed after infection with mycobacteria prompted us to study the interdependence of proinflammatory and Wnt/Fzd signaling processes. We analyzed the regulation of the β -catenin pathway in the context of *M. tuberculosis* infections: After aerosol infection of mice we find an inverse regulation of the TLR/NF- κ B- and the Wnt/ β -catenin pathway: Whereas inducible nitric oxide synthase (iNOS) and IFN- γ formation are increased, β -catenin levels and the transcription of β -catenin dependent target genes are significantly reduced. This demonstrates that Wnt/ β -catenin signaling, which is involved in tissue homeostasis, is switched off under proinflammatory conditions. In murine macrophages, we have then identified Fzd1 mRNA to be upregulated in response to mycobacteria and conserved bacterial structures. Fzd1 has previously been shown to mediate β -catenin dependent signaling in response to Wnt3a and Wnt7b. Microbe-induced Fzd1 transcription depends on the presence of Toll-like receptors (TLR) 2 and 4, the myeloid differentiation response gene 88 (MyD88) and the NF- κ B pathway. Single cell analysis by flow cytometry demonstrates an enhanced Fzd1 expression on macrophages in response to *M. tb.*, as well as LPS, which was synergistically enhanced in the presence of IFN- γ . The analysis of lung homogenates of *M. tb.*-infected mice also shows an enhanced Fzd1 mRNA expression during the course of infection, indicating that Fzd1 upregulation also occurs under inflammatory conditions *in vivo*. Transcripts for Wnt3a are also present in lung homogenates of infected mice. In primary macrophages, Wnt3a restores TLR/NF- κ B induced downregulation of β -catenin signaling to the level of unstimulated cells. This novel Wnt-mediated feedback mechanism may be involved in preserving cell homeostasis during microbe-induced inflammation. Both Toll and

Wnt signaling pathways are evolutionarily highly conserved and have only recently been found to intersect in *Drosophila*. The current data further support the notion that Wnt signaling is involved in orchestrating the immune response in response to microbial stimulation of innate immune cells of vertebrate origin.

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A53

Adhesion molecule expression and cell cycle control in cells of the immune system are sensitive to altered gravity

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Introduction: Life on Earth developed in the presence and under the constant influence of gravity. Thus, it is a fundamental biological question, whether gravity is required for cellular functions at the molecular level in mammalian cells. Their special sensitivity to altered gravity renders cells of the immune system an ideal model system to understand if and how gravity on Earth is required for normal mammalian cell function and signal transduction.

Methods: Experiments have been performed using ground-based facilities such as fast-rotating 2D clinostat and hyper-g-centrifuges, and real microgravity provided by parabolic flights. For parabolic flight experiments on board the Airbus A300 ZERO-G, we developed RP (rapid prototyping)-based experimental equipment, which allows cell culture experiments with living mammalian cells in microgravity. We investigated the influence of altered gravity on T lymphocytes and on monocytic cells.

Results: In experiments with a fast rotating 2D clinostat, we detected strong and rapid initial changes of human T lymphocyte signal transduction within minutes of simulated weightlessness. However, most of the initial alterations returned to "normal" levels after 15 min-simulated weightlessness. Only the expression of p21 protein remained constantly elevated, compared to normogravity controls. In simulated weightlessness, human monocytic cells responded with tyrosine-phosphorylation of several proteins, whereas in PMA-stimulated monocytic cells, tyrosine-phosphorylation was nearly abrogated. Hypergravity of 1.8 g had no effects of the signal pathways investigated. In parabolic flight experiments, we found that 20 s microgravity resulted in distinct changes of expression of cell-cycle regulatory genes such as p21 and p27 on the transcriptional level in primary human T lymphocytes. In human monocytic cells, we detected a distinct downregulation of ICAM-1 (CD54) in non-stimulated and in PMA-stimulated cells.

Conclusion: Thus we conclude that dysregulation of immune function in microgravity might be a consequence of 1) sustained induction of p21 as a cell cycle arrest signal in T lymphocytes and 2) Downregulation of ICAM-1 in monocytes/macrophages, which are then no longer capable of interacting with T lymphocytes in the appropriate way. Since immune cells can respond and adapt to altered environmental conditions very effectively, it is indispensable to investigate whether the observed effects are still active after long-term exposure to altered gravity in the situation of adaptation and steady state. Thus, we are now the phase of preparation of two Space Experiments investigating the function of cells of the innate immunity, one scheduled for autumn 2009 on board of the International Space Station and one as a common Sino-German space life science mission scheduled for January 2010 on board of Shenzhou-8 Spacecraft.

Differentiation, senescence and cell death

A54

Nuclear-mitochondrial crosstalk – role in aging processes

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Numerous diseases share common pathways with aging processes. Therefore, it is important to understand the mechanisms of aging and senescence on a cellular level. Environmental stressors such as sun light and diet are known to induce oxidative stress and advance aging processes. Mitochondrial and nuclear dysfunction and increased oxidative stress contribute to the onset of replicative senescence. We investigated several proteins, which are known to play a role in aging processes: telomerase reverse transcriptase (TERT), matrix metalloproteinase 1 (MMP1) and grainyhead like 3 (GRHL3). TERT is exported from the nucleus under conditions of oxidative stress and replicative senescence. The Src-kinase family via phosphorylation of TERT mediates this export. We identified the tyrosine phosphatase Shp-2 as a negative regulator of this export. Overexpression of Shp2 inhibited nuclear export of TERT. This inhibition was dependent on the catalytic activity of Shp2. Ablation of Shp2 increased tyrosine phosphorylation of nuclear TERT and subsequently led to loss of nuclear telomerase activity. Recently, we also discovered TERT in the mitochondria. There, TERT binds to mitochondrial DNA and protects it against UV light induced damage. TERT also reduced ethidium bromide-induced mitochondrial DNA damage. Mitochondrially targeted TERT, but not nuclear targeted TERT revealed the most prominent protective effect on H₂O₂-induced apoptosis. Mitochondria isolated from hearts of second generation TERT knock-out mice showed reduced respiration demonstrating for the first time a heart phenotype of these mice. A central mechanism in exogenous skin aging is the increased proteolytic degeneration of dermal matrix fibers of connective tissue due to increased MMP1 activity. UVA, UVB and IRA irradiation induced MMP1 expression and activity. After IRA-irradiation reactive oxygen species are generated from the mitochondrial respiratory chain leading to increased MMP-1. The gene regulatory potential of IRA was assessed by microarray analysis. Cluster analysis suggested additional pathways to be involved, e.g. Calcium- and

mTOR-signalling. TNF α is increased in serum of CAD patients, induces senescence and apoptosis of endothelial cells and decreases their migratory capacity. Recently, we discovered the transcription factor GRHL3 as a TNF α regulated gene. GRHL3 deficient mice show defects in cell migration. Therefore, we investigated the role of GRHL3 in endothelial cell migration. Overexpression of GRHL3 increased endothelial nitric oxide synthase activity and cell migration. Moreover, nitric oxide increased endogenous levels of GRHL3, suggesting a positive feedback loop of nitric oxide on GRHL3. Thus, GRHL3 plays an important role in the increase of nitric oxide bioavailability and could therefore serve as a new anti-aging therapeutic. In conclusion, we identified intracellular mechanisms, which regulate three important candidate proteins in aging processes.

A55

The circadian rhythm of primary dermal fibroblasts affects infrared-A-induced gene expression

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Until now the susceptibility of human skin to detrimental effects of solar radiation (e.g. photoaging) has mostly been assigned to the type of radiation and to the skin type. We have raised the question whether the circadian rhythm plays a role in the susceptibility of human dermal fibroblasts (HDF) to IRA (760–1440 nm).

IRA accounts for one third of the solar energy reaching the earth's surface and penetrates deeply into the human skin. Previous studies of our group have shown that IRA in HDF induces a retrograde signaling cascade involving mitochondrial reactive oxygen species (ROS) and, via involvement of the MAP-kinases ERK 1/2, alters gene expression. Among the genes regulated is matrix metalloproteinase 1 (MMP-1), the dominant enzyme in terms of collagen degradation in the dermal extracellular matrix.

The hypothalamic suprachiasmatic nucleus is the centre of circadian rhythms in mammals. It is reset daily by light and regulates the circadian clocks of the peripheral tissues. Clock genes drive both rhythms. In vitro, synchronicity is lost in cell culture but synchronizers such as high serum concentrations can reinduce molecular oscillations of clock genes, resynchronizing the circadian rhythm of cultured cells.

To address the role of circadian rhythm in susceptibility towards IRA induced changes in gene expression HDFs from various donors were treated with a serum shock and irradiated 24 h, 30 h and 36 h later with 360 J/cm² IRA under temperature controlled conditions. Realtime PCR results show an increased susceptibility to IRA peaking 30 h after serum shock compared to control cells. Our findings for the first time show that the susceptibility of skin cells towards a part of natural sunlight is affected by the circadian rhythm. Further analysis of clock genes (PER1, BMAL1) at the time of irradiation will provide insight into the relationship between the circadian rhythm and IRA induced changes in gene expression.

A56

Cellular senescence in human mammary epithelial cells (HMEC) is accompanied by an increase of elastin formation

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Normal human mammary epithelial cells (HMEC) have a finite life span, regulated by a process termed cellular senescence. After an initial stress-associated senescence barrier, some cells are subjected to a self-selection process triggered by the loss of the G₁ cell cycle regulator p16^{INK4a} expression. This effect is associated with an escape from the growth plateau to resume cell cycle progression and to undergo further cell divisions. Thereafter, these so-called "post-selection" HMEC encounter a second senescence barrier, termed agonescence. Our recent work using an siRNA approach revealed an essential impact of the extracellular matrix metalloproteinase-7 (MMP-7) on the aging process of post-selection HMEC. Since MMP-7 is involved in a variety of cellular mechanisms, including processing of inactive precursor proteins, ectodomain shedding of extracellular signaling molecules and remodeling of the extracellular matrix (ECM), respectively, we could observe considerable alterations of the ECM during senescence of HMEC. Thus, the formation of characteristic structures similar to elastin was detectable, which markedly increased in agonescent HMEC cultures after passage 15. Moreover, Western blot analysis revealed an up-regulated expression of tropoelastin, the soluble precursor of the ECM protein elastin, during the aging process of HMEC. This was paralleled by an enhanced activity of the enzyme lysyl oxidase (LOX) which is responsible for cross-linking tropoelastin monomers to form insoluble elastic fibers. Thus, enhanced LOX activity was detectable in cellular homogenates as well as in the culture supernatant during HMEC aging between passage 12 up to passage 16. Together with the down-regulation of MMP-7, the increased formation of elastic fibers supported an essential effect of distinct ECM components and the microenvironment on HMEC aging.[1]

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A57

Mitochondrial TERT enhances mitochondria functions in vivo by protecting mitochondrial DNA integrity from oxidative damage

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Mitochondria are essential for respiration and oxidative phosphorylation. Mitochondrial dysfunction due to aging processes is involved in pathologies and pathogenesis of a series of

cardiovascular disorders. New results accumulate showing that the enzyme telomerase with its catalytic subunit telomerase reverse transcriptase (TERT) has a beneficial effect on heart functions. The benefit of short-term running of mice for heart function is dependent on TERT expression. TERT can translocate into the mitochondria and mitochondrial TERT (mtTERT) is protective against stress induced stimuli and binds to mitochondrial DNA (mtDNA). Because mtDNA is highly susceptible to damage produced by reactive oxygen species (ROS) which are generated in close proximity to the respiratory chain, the aim of this study was to determine the functions of mtTERT *in vivo* and *in vitro*. Therefore, mitochondria from hearts of adult, 2nd generation TERT-deficient mice (TERT $-/-$) and wt littermates were isolated and state 3 respiration was measured. Strikingly mitochondria from TERT $-/-$ revealed a significantly lower state 3 respiration (TERTwt: 987 \pm 72 pmol/s*mg vs. TERT $-/-$: 774 \pm 38 pmol/s*mg, $p < 0.05$, $n = 5$). These results demonstrated that TERT $-/-$ mice have a so far undiscovered heart phenotype. In contrast mitochondria isolated from liver tissues did not show any differences. To get further insights in the molecular mechanisms, we reduced endogenous TERT levels by shRNA and measured mitochondrial reactive oxygen species (mtROS). mtROS were increased after ablation of TERT (scrambled: 4.98 \pm 1.1% gated vs. shTERT: 2.03 \pm 0.7% gated, $p < 0.05$, $n = 4$). We next determined mtDNA deletions, which are caused by mtROS. Semiquantitative realtime PCR of mtDNA deletions revealed that mtTERT protects mtDNA from oxidative damage. To analyze whether mitochondrial integrity is required to protect from apoptosis, vectors with mitochondrially targeted TERT (mitoTERT) and wildtype TERT (wtTERT) were transfected and apoptosis was measured. mitoTERT showed the most prominent protective effect on H₂O₂ induced apoptosis. In conclusion, mtTERT has a protective role in mitochondria by importantly contributing to mtDNA integrity and thereby enhancing respiration capacity of the heart.

A58

The infrared A gene response of human dermal fibroblasts involves several mitochondria dependent and independent signaling pathways

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Infrared A radiation (IRA 760–1440 nm), a major component of solar radiation reaching the human skin, has been shown to alter the expression of matrixmetalloproteinase-1 (MMP-1) in human dermal fibroblasts involving retrograde mitochondrial signaling pathways.

Aim of this study was to examine the gene regulatory potential of IRA beyond MMP-1, the role of mitochondria in initiating IRA-induced signaling responses and the signaling pathways involved. Primary human dermal fibroblasts were irradiated with a single dose of IRA (860 J/cm²).

Gene expression analysis was performed utilizing Affymetrix DNA-microarrays, realtime PCR and Western Blot. To identify differentially regulated genes despite a bias due to interindividual differences in gene expression, we applied a filtering strategy,

which selects genes regulated in at least three of nine independent experiments performed, revealing 599 regulated genes (250 up- and 349 downregulated). From those genes we selected ones that relates to skin aging, based on functional Gene-Ontology clustering. Thirteen genes (BAX, BAD, FASTK, TNFRSF6B, PIK3R3, PIP5K1B, ITPR3, ITPR2, ATP1B1, FNI, VCAM1, IL6ST, STAT3) were further analyzed in additional experiments by realtime PCR and Western Blot analysis, results confirmed the findings from microarray analysis. In a third step inhibitors of retrograde and other signaling pathways as well as mitochondria targeted and whole cell distributed antioxidants were applied to investigate the role of mitochondria in the cellular response to IRA. Mitochondria dependent and independent pathways were found to be involved. Our results underline the impact of IRA on gene expression in skin, with the mitochondria being the major player in IRA-induced signaling.

A59

In brain mitochondria, calcium and cell death-associated permeability transition are controlled by possibly associated proteins, 2',3'-CNPase, Centaurin-alpha and peripheral benzodiazepine receptor, and their substrates/ligands

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Mitochondria play a central role in calcium homeostasis and cellular calcium signaling. During cellular calcium overload, mitochondria take up cytosolic calcium, which, in turn, induces opening of the permeability transition pore (PTP), disruption of mitochondrial membrane potential and cell death. PTP is a protein complex changing according to the needs of the cell and responding to different external and internal stimuli. The identity of the PTP is still unresolved. 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP) and p42(IP4) (centaurin-alpha) have been shown to be associated with rat brain mitochondria (RBM), but the exact role of these proteins in mitochondria is still obscure. Localization of p42(IP4) and CNP within the inner membrane and contact sites indicates further functions for these proteins. We found interaction of p42(IP4) with CNP by pull-down binding assay and by immunoprecipitation.

Since PTP opening is important in mitochondrial events leading to programmed cell death, we studied whether p42(IP4) and CNP are involved in calcium-induced calcium release and consequently PTP. Simultaneous measurements of the respiratory rate, trans-membrane potential and calcium transport in the mitochondrial suspension were performed. We also developed the method of isolation of functionally active mitochondria from several cell types. We determined the calcium capacity and lag-phase for PTP opening in mitochondria isolated from p42(IP4)-transfected and from control neuroblastoma cells. Overexpression of p42(IP4) led to promotion of calcium-induced PTP opening. The enzymatic activity of CNP was reduced under PTP opening, whereas the level of CNP detected in RBM before and after PTP opening were unchanged. Involvement of CNP in PTP operation was confirmed in further experiments using

mitochondria isolated from CNP-knock-down oligodendrocytes (OLN93 cells). In mitochondria isolated from OLN93 cells transfected with CNP-targeting siRNA, CNP reduction was correlated with facilitation of calcium-induced PTP opening. The CNP substrates, 2',3'-cAMP and 2',3'-cNADP, induced PTP opening in RBM. The peripheral-type benzodiazepine receptor (PBR) is an 18 kDa mitochondrial membrane protein with still elusive functions. A release of pro-apoptotic factors, AIF and cytochrome c, from RBM was shown at threshold calcium load. Anti-PBR antibody blocked the release of AIF but did not affect the cytochrome c release. The endogenous PBR ligand, protoporphyrin IX, facilitated PTP opening and phosphorylation of the mitochondrial proteins, thus, inducing effects opposite to anti-PBR antibody. This study provides evidence for PBR involvement in PTP opening, controlling the calcium-induced calcium efflux, and AIF release from mitochondria. In summary, our results provide evidence that PBR, CNP and p42 (IP4) are involved in regulation of calcium-induced PTP opening, important stage of initiation of programmed cell death.

A60 Nuclear Shp-2 keeps telomerase reverse transcriptase in the nucleus – new potential anti-aging target

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Vascular diseases are associated with cellular aging, which is accompanied by telomere shortening counteracted in the nucleus by telomerase reverse transcriptase (TERT). Under conditions of oxidative stress TERT is exported from the nucleus and this export is mediated by Src-kinases via tyrosine phosphorylation of TERT. Nuclear export of TERT resulted in accelerated aging of endothelial cells. Therefore, the aim of this study was to determine a counterplayer for nuclear export of TERT.

In embryonic fibroblasts deficient in Src, Fyn and Yes, TERT nuclear export induced by oxidative stress is abolished. Fyn does not seem to play a role, because unlike Src and Yes it is not found in the nucleus. A putative regulator of this export is the tyrosine phosphatase Shp-2, which can regulate the activity of the Src-kinase family. We demonstrated that Shp-2 is localized in the nucleus and associated with TERT in endothelial cells. Overexpression of Shp-2 inhibited oxidative stress induced nuclear export of TERT and ablation of Shp-2 by siRNA reduced nuclear telomerase activity. This inhibition was dependent on the enzymatic activity of Shp-2 and on tyrosine 707 in TERT because overexpression of the dominant negative Shp-2 mutant (C459S) led to a reduction of TERT protein and telomerase activity, whereas telomerase activity in TERTY707F overexpressing cells was not altered by Shp-2. Thus, tyrosine 707 seems to be a critical target for regulation of TERT localization by Shp-2 mediated dephosphorylation. To establish a causal link between Shp-2, nuclear TERT and oxidative stress, we determined reactive oxygen species (ROS) formation in endothelial cells. Overexpression of Shp-2(C459S) (2.45 fold +/- 0.34 of Shp-2 wt) or ablation of Shp-2 by siRNA increased ROS levels

(2.23 fold +/- 0.54 of scrambled siRNA). In contrast, keeping TERT in the nucleus by mutating tyrosine 707 or overexpressing Shp-2 wt reduced ROS formation (0.73 and 0.75 fold, respectively).

In summary, these data indicate that TERT is associated with nuclear Shp-2, Shp-2 acts as a negative regulator for nuclear export of TERT probably via regulating tyrosine 707 dephosphorylation of TERT and reducing ROS formation. Thus, increasing nuclear Shp-2 activity could be a useful tool to delay vascular aging processes.

A61 Gadd45β-induced prolonged activation of p38 kinase defines a novel pathway mediating negative selection of thymocytes

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The clonal deletion of thymocytes by negative selection is an important process to ensure immunologic tolerance, even though the underlying molecular mechanisms are poorly understood. Here, we show that Gadd45β, a regulator of mitogen-activated protein kinases, is critically involved in triggering negative selection. Gadd45β expression was inducible in different models of negative selection. Strikingly, only TCR-ligating peptides resulting in negative selection, but not positively selecting ligands or dexamethasone, a TCR-independent apoptosis agonist, induced Gadd45β expression. Expression of Gadd45β maintained a sustained activation of p38 kinase and thereby promoted TCR-mediated apoptosis. In contrast, inhibition of Gadd45β expression or p38 activity impaired cell death. Moreover, thymocytes from Gadd45β-deficient mice revealed only transient p38 activation, reduced caspase activation and cell death. Thus, we provide evidence that Gadd45β and a resulting persistent activation of p38 constitute a novel apoptotic pathway involved in negative selection.

A62 Fungal GPCR signaling in pheromone response

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The mushroom *Schizophyllum commune* shows several phenotypes linked to pheromone signaling and Ras-dependent development. The genes involved in MAPK signaling, cAMP dependent signaling and Rho signaling all turned on after pheromone recognition in a Ras-dependent manner have been analyzed using the genome sequence. Receptor-pheromone recognition leads to mating and is dependent in mated dikaryons for nuclear migration and clamp cell fusion. In vitro mutagenesis could show domains in the protein involved in ligand recognition. Receptor

localization and expression studies could elucidate the involvement in clamp cell fusion. The signal is transmitted through a MAPK cascade and via Ras. Ras constitutive mutant alleles transformed into a wildtype recipient as well as deletion of a RasGap lead to enhanced intracellular signaling. By this approach phenotypes in clamp cell fusion, mushroom development and meiosis, as well as hyphal directional growth can be linked to Ras signaling.

The genome sequence allowed us to identify components, which are used for expression studies during pheromone response and mating. A proteome analysis additionally is used to define components involved in pheromone response in this easily tractable fungus.

A63

Grainyhead like 3 – a newly identified TNFalpha regulated transcription factor – is regulated by the Src kinases & NO

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One major independent risk factor of cardiovascular diseases (CAD) is aging. Therefore, it is important to understand vascular aging on a cellular level. It is known that TNFalpha is increased in serum of CAD patients, induces senescence and apoptosis of endothelial cells (EC) and decreases their migratory capacity. Recently, we discovered the transcription factor Grainyhead like 3 (GRHL3) as a TNFalpha regulated gene. GRHL3 $-/-$ mice demonstrated defects in cell migration. Therefore, the aim of our study was to investigate the role of GRHL3 in EC migration and the underlying mechanisms.

After verifying expression of GRHL3 in EC, GRHL3 was cloned and overexpressed in EC. GRHL3 induced migration to a similar degree as vascular endothelial growth factor (VEGF). To get insights in the underlying mechanisms, we analyzed a known VEGF-induced migration activator, endothelial nitric oxide synthase (eNOS). eNOS phosphorylation on S1179 was increased upon overexpression of GRHL3, suggesting that GRHL3 increased EC cell migration through a nitric oxide (NO) dependent pathway. To determine whether a potential positive feedback loop of nitric oxide (NO) and GRHL3 exists, we next tested the effect of NO on endogenous GRHL3 expression by real time PCR. Incubation with the NO donor papanonoate dramatically increased GRHL3 mRNA expression (papanonoate: 3.75-fold \pm 1.34 of control). Furthermore, decreased migratory capacity of senescent EC concomitant with an increase in Src kinase activity has been shown. To analyze whether Src has an influence on GRHL3, we determined the nuclear levels of this promigratory transcription factor in mouse embryonic fibroblasts (MEF), deficient for Src, Fyn and Yes (SFY $-/-$). A higher concentration of GRHL3 protein was observed in nuclear extracts of SFY $-/-$ MEF. To demonstrate that the Src kinase family is indeed a negative regulator of GRHL3 expression in EC, we treated the cells with the Src kinase family inhibitor PP2 and analyzed expression of GRHL3. Indeed, expression of GRHL3 was dramatically increased by PP2 (PP2: 4.50-fold \pm 1.72 of control).

In conclusion, GRHL3 is an important inducer of EC migration mediated through activation of eNOS/NO. NO in turn induces

GRHL3 expression, suggesting a positive feedback loop. In addition, the Src kinase family negatively regulates endogenous GRHL3.

A64

Infrared A-radiation alters the intracellular calcium homeostasis in human dermal fibroblasts

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Infrared-A radiation (IRA; 760–1440 nm), a major component of natural sunlight, accounts for one third of the energy reaching human skin. We have shown in previous studies that IRA irradiation of human dermal fibroblasts (HDF) leads to an increase in expression of matrix metalloproteinase 1 (MMP-1) via an increase in mitochondrial reactive oxygen species (ROS) and subsequent activation of the stress kinases ERK1/2.

The aim of this study was to elucidate which retrograde signaling pathways are induced by IRA. Microarray analysis of the IRA response revealed the regulation of several genes related to calcium homeostasis and this together with the known influence of mitochondrial impairment on cellular calcium fluxes prompted us to focus on the effect of IRA on the cellular calcium homeostasis.

Our results show for the first time that IRA irradiation (100 J/cm²) of HDF leads to a biphasic increase of the cytosolic calcium level ([Ca²⁺]_c) in the cell. Experiments utilizing EGTA to complex all extracellular calcium confirmed, that the source of the cytosolic calcium increase are intracellular calcium storages. We previously showed that IRA induced MMP-1 expression is dependent on reactive oxygen species (ROS), so we tested whether use of an antioxidant affects the IRA-induced increase of [Ca²⁺]_c. The treatment of HDF with N-acetyl-L-cysteine (NAC) mitigates the IRA-induced rise in [Ca²⁺]_c. To address the question of functional relevance of the IRA-induced calcium flux for IRA-induced gene regulation, we treated the cells with the calcium chelator BAPTA-AM. The complexation of intracellular calcium lowers the IRA-induced increase of MMP-1 expression, demonstrating the involvement of calcium in IRA-induced signaling.

In conclusion, IRA irradiation leads to a ROS dependent calcium flux from intracellular storages into the cytosol. Interception of the calcium signaling by a calcium chelator affects the IRA induced gene regulation.

A65

Functional consequences of mitochondrial DNA deletions in human skin fibroblasts: increased contractile strength in collagen lattices is due to oxidative stress-induced lysyl oxidase activity

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Deletions of the mitochondrial DNA (mtDNA) are thought to contribute to extrinsic skin aging, because they are found at increased amounts in photoaged skin and are induced through

chronic UV irradiation in human skin fibroblasts *in vitro* and *in vivo*. In order to study how the presence of mtDNA deletions translates into functional and structural changes in the skin we have seeded human skin fibroblasts into collagen gels in order to generate human dermal skin equivalents. These cells, which were matched for donor age, and passage number, were either derived from Kearns-Sayre syndrome (KSS) patients, which constitutively carry large amounts of the UV-inducible mitochondrial common deletion, or normal human volunteers (NHF). In the present study we have focused on the analysis of changes that occur in this system within the first four days of culture. We have found that KSS fibroblasts – in comparison to NHF – contracted the gels faster and stronger. This effect was dependent on reactive oxygen species (ROS) as the contraction difference was reduced in an oxygen-deprived atmosphere and in the presence of the antioxidant N-tert-butyl- α -phenylnitrone (PBN), respectively. Gene expression and Western blot analysis revealed significant upregulation of lysyl oxidase (LOX), an enzyme required for crosslinking of collagen fibers, in KSS fibroblasts. Treatment with the specific LOX inhibitor β -aminopropionitrile (BAPN) decreased the contraction difference between KSS and NHF equivalents to a similar degree as PBN, and both BAPN and PBN diminished LOX activity. These data suggest a causal relationship between mtDNA deletions, ROS production, and increased LOX activity, which then leads to increased contraction of collagen gels. They support the concept that mtDNA deletions in human skin fibroblasts lead to functional and structural alterations of the skin. Accordingly, increased LOX expression was also observed *in vivo* in photoaged human and mouse skin.

A66

Gene regulation and deregulation by MAL/MRTF coactivators

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Proteins of the MRTF family play a key role in regulated gene expression downstream of Rho family GTPases. MAL/MKLI/MRTF-A is a transcriptional coactivator of serum response factor (SRF) and is bound and inhibited by G-actin in the non-induced state. In acute megakaryocytic leukaemia in infants, a recurrent translocation t(1;22) results in the OTT-MAL/RBM15-MKLI fusion oncoprotein. How it contributes to the malignancy is unknown. We tested by biochemical analysis whether OTT-MAL is functionally deregulated. We showed that OTT-MAL is a constitutive activator of SRF and target gene expression. This requires the SRF binding motif and the MAL-derived transactivation domain. OTT-MAL localises to the nucleus and is not regulated by upstream signalling. OTT-MAL deregulation reflects its independence from control by G-actin, which fails to interact with OTT-MAL in co-immunoprecipitation experiments. OTT-MAL also caused a delayed induction of the MAL-independent, TCF-dependent target genes *c-fos* and *egr-1*, and the MAPK/Erk pathway. In addition, RBPJ/CBF-1 regulated gene expression was activated by OTT-MAL but not by MAL. Our data suggest that the deregulated activation of MAL-dependent and independent promoters results in tissue-specific functions of OTT-MAL.

When tested in heterologous tissue culture systems, however, we observed strong anti-proliferative effects of OTT-MAL. Similarly, overexpression of MAL exhibited antiproliferative and pro-apoptotic effects requiring transcription through SRF. To gain insight into the molecular mechanisms involved, we performed gene expression analysis. By using a combination of actin binding drugs, which specifically interfere with the actin-MAL complex, we identified on a genome wide basis 210 genes primarily regulated by G-actin. We found many known MAL-dependent SRF target genes, as well as novel directly regulated genes. Several putative antiproliferative target genes were newly identified. We showed that a group of MAL regulated genes negatively interferes with the EGFR-MAPK pathway, thereby reducing proliferative signalling. Our results show the existence of negatively acting transcriptional networks between pro- and antiproliferative signalling pathways towards subsets of SRF target genes.

A67

Increased levels of large scale deletions of mtDNA of skin fibroblasts result in increased collagen degradation in dermal skin equivalents

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Aged tissues contain increased levels of large-scale deletions of the mitochondrial (mt) DNA. Especially extrinsically aged skin has been shown to carry a significant burden of mtDNA deletions along with several other structural and functional impairments. Until now the functional role of mtDNA mutations for functional and structural changes of aged skin is not understood.

Therefore we studied how human skin cells harbouring mtDNA mutations affect their microenvironment by comparing normal human fibroblasts (NHF) to dermal fibroblasts derived from patients suffering from Kearns-Sayre syndrome (KSS) in 3D collagen gels resembling human skin (dermal equivalents, DE). KSS fibroblasts carry a 10,000 fold higher amount of mtDNA deletions exemplified by quantitative measurement of the 4977 bp Common Deletion. We here examined the degradation of components of the extracellular matrix in DE during cultivation. During six weeks we detected more fragmented collagen measured by the marker hydroxyproline (HYP) via GC-MS. Moreover we observed a less robust collagen lattice structure in dermal equivalents with KSS fibroblasts in comparison to NHF by picosirius red staining of histological sections. In line with that, we could measure higher mRNA expression levels of the dominant collagen degrading enzyme matrixmetalloproteinase-1 (MMP-1) in KSS DE using realtime PCR and increased MMP-1 protein amounts in histological sections over the whole cultivation time.

We conclude that increased levels of photo-inducible mtDNA deletions are functionally relevant for intensified matrix degradation and are therefore responsible for detrimental changes in photoaged skin.

Signalling in immune cells**A68****Inhibition of dendritic cell maturation and activation is mediated by STAT3**K Bauer¹, S Binder¹, C Klein², JC Simon² and F Horn¹¹University of Leipzig, Department of Molecular Immunology, Leipzig, Germany²University of Leipzig, Department of Dermatology, Venerology and Allergology, Leipzig, Germany

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Dendritic cells (DC) are the most potent antigen presenting cells, because they can internalize, process and present antigens in order to activate T-cells. Accordingly, DC has the ability to activate tumour-specific T-cell response. However, this does not occur in most types of cancer; in opposite DC even help maintaining tumour tolerance. Instead of being eliminated by the immune response, tumours progress and metastasize. This failure of the immune system is caused by an abnormal DC differentiation and activation mediated through several tumour derived factors like IL-6 and IL-10. It is well known, that IL-6 and IL-10 signalling is mainly supported by signal transducer and activator of transcription 3 (STAT3). The mechanisms how STAT3 affects the maturation and activation of DC and which STAT3 target genes are involved in this process is still unknown.

To understand the modulation of dendritic cell function by IL-10, gene expression profiling was performed by using Affymetrix technology in human monocyte-derived DC treated with IL-10, alone or in combination with LPS. The regulation of selected genes was validated by real-time PCR. IL-10 regulated in DC the expression of a limited number of genes, including IL-7, CD200, DGKA and signal transduction elements. We have compared the cell phenotype and functional properties of monocyte/macrophage derived dendritic cells which were preincubated with IL-10 followed by activation through LPS. This combined treatment modulated a number of genes comparable to LPS alone, but differ in their amplitude of regulation. As expected, IL-10 suppressed the expression of several LPS-inducible proinflammatory molecules.

Our results deliver insight into, how STAT3 interferes with maturation and activation of DC and generates immunotolerance towards tumours.

A69**Drug induced modulation of T cell activation and differentiation in atopic dermatitis patients**C Brandt¹, A Radbruch¹, M Worm² and R Baumgrass¹¹DRFZ, AG Signaltransduktion, Berlin, Germany²Charité, Allergie-Centrum, Berlin, Germany

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Atopic dermatitis (AD) is a T cell dependent chronic relapsing inflammatory skin disorder. Cyclosporine A (CsA) has been shown to be an effective treatment for severe AD. We studied the effect of low-dose CsA therapy on T cell activity and T cell differentiation in AD patients. Using a whole blood assay we demonstrated that TcR signalling in peripheral blood T cells of CsA treated AD patients is reduced to 42% ± 18 but not totally blocked. Such partial inhibition of TcR signalling allowed

regulatory T cell induction under *in vitro* conditions. Therefore we asked is there an *in vivo* regulatory T cell induction, too. Indeed AD patients under low-dose CsA therapy have higher numbers and frequencies of functional regulatory T cells compared to untreated AD patients. To evaluate the causal connection between low-dose CsA treatment and higher regulatory T cell numbers we studied individual patients before and during CsA therapy. The data clearly indicate increased numbers and frequencies of regulatory T cells after onset of CsA therapy which remained stable during the therapy. The therapeutic effect of low-dose CsA therapy in AD patients could be due to both, inhibition of T cell hyperactivity and induction of suppressor T cells.

A70**Mast cells secrete IL-15 by microvesicles shedding upon P2X7 receptor stimulation**E Bulanova¹, V Budagyan¹, Z Orinska¹, M Klinger² and S Bulfone-Paus¹¹Research Center Borstel, Immunology and Cell Biology, Borstel, Germany²Institute of Anatomy, University of Lübeck, Lübeck, Germany

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Mast cells are recognized as the key cells of allergic inflammatory reactions. They express and secrete a number of pro-inflammatory cytokines and chemokines. Interleukin-15 (IL-15) is a potent anti-apoptotic cytokine and a regulator of T, B and NK cells differentiation and proliferation. Bone marrow-derived mast cells (BMMCs) constitutively express IL-15 mRNA and this expression is further upregulated by LPS stimulation. However, there is no evidence for IL-15 cytokine secretion from activated mast cells. P2X7 receptor is one of the purinoceptors, which is activated by ATP. Mast cells highly express P2X7 on the cell surface. Recently, we have reported that ATP induces P2X7-mediated apoptosis of BMMCs, as well as triggers pro-inflammatory cytokine secretion, presumably in the time period between commitment to apoptosis and actual cell death. ATP triggers rapid but transient phosphorylation of multiple signaling molecules in BMMCs, including extracellular-signal regulated kinase (ERK), Jak2, and STAT6. Moreover, BMMCs release annexin-V-positive microvesicles upon P2X7 receptor stimulation. These vesicles contain biologically active IL-15, which later appears in the vesicle-free supernatant and stimulates the proliferation of the IL-15-dependent CTLL cell line. The IL-15-containing microvesicles were also found in the supernatants from THP-1 monocytic cell line and bone marrow-derived dendritic cells upon agonistic P2X7 stimulation. Thus, the microvesicle shedding mechanism constitutes one of secretory pathways for a release of IL-15 in mast cells, dendritic cells and monocytes, which might play an important role in regulation by IL-15 of diverse physiological processes or their pathological deviations.

A71**Regulation of B cell entry into the cell cycle**EA Clark
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B cells are induced to enter the cell cycle by ligation of the B-cell receptor (BCR) complex and by Toll-like receptor (TLR) agonists. B-cell activation is regulated by molecules with several distinct modes of action: A first example, the adapter molecule Bam32 (B-lymphocyte adapter of 32 kDa), helps promote BCR-induced cell cycle entry, while the secondary messenger superoxide has the opposite effect. Bam32 and superoxide may fine tune BCR-induced activation by competing for the same limited resources including Rac1/2 and/or the plasma membrane phospholipid PI(3,4)P2. A second example, the BCR-associated co-receptor CD22, inhibits BCR-induced proliferation by binding to novel CD22 ligands on B cells and dendritic cells. Regulators of B-cell survival and death also influence B-cell transit through the cell cycle. Caspase 6 normally is simply classified as an effector caspase in cell death pathways; but in B cells caspase 6 negatively regulates CD40- and TLR-dependent G1 entry, in part by controlling levels of phosphorylated retinoblastoma (Rb) protein. Caspase 6 deficiency predisposes B cells to differentiate rather than proliferate after stimulation. The Bcl-2 family member, Bim, is normally classified as a 'pro-apoptotic' protein; but in B cells, it exerts a positive regulatory effect on cell cycle entry, which is opposed by Bcl-2. New insights into how B-cell transit through the cell cycle is controlled may lead to thoughtful design of drugs that selectively target pathogenic B cells.

A72**Real time monitoring of B cell antigen receptor-proximal events by fluorescence lifetime imaging**

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Many molecular events downstream of B cell antigen receptor engagement have been elucidated by different genetic and biochemical approaches. These studies revealed that a hallmark of BCR signalling is the assembly of a multiprotein complex comprising at least the SH2 domain-containing adapter protein of 65 kDa (SLP-65), Bruton's tyrosine kinase (Btk) and Phospholipase C- γ 2 (PLC- γ 2). Only in context of this so called Ca²⁺ initiation complex PLC- γ 2 is activated and produces the second messengers Diacylglycerol and Inositol-1,4,5-trisphosphate. Hence, SLP-65 provides a molecular platform that links BCR engagement to the regulation of important transcription factors and the reorganization of the cytoskeleton. However, little is known about the kinetics and subcellular dynamics of Ca²⁺ initiation complex assembly. Here we report a real time imaging approach to monitor BCR-induced changes in the three dimensional structure of SLP-65. For this aim different dichroic fluorescent SLP-65 variants were expressed in *slp65*^{-/-} DT40 B cells. In resting cells the conformation of SLP-65 allows for fluorescence resonance energy transfer (FRET) between both fluorophors that was monitored by measuring the fluorescence lifetime of respective donor fluorophors. BCR engagement

significantly attenuated the efficiency of FRET indicating an induced conformational change in SLP-65. The kinetics of these changes correlated with that of BCR-induced Ca²⁺ mobilization. Hence, this novel approach makes it possible to analyze the spatial and temporal dynamics of BCR-induced Ca²⁺ initiation complex assembly.

A73**B cell antigen receptor-induced plasma membrane recruitment of the SH2 domain-containing inositol phosphatase is mediated by the protein tyrosine kinases Lyn and Syk**

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Signals transduced by the B cell antigen receptor (BCR) are essential for B cell development and activation. Precise regulation of BCR signals is required to provide antigen-specific humoral immunity on one hand and tolerance of self proteins on the other hand. The SH2 domain-containing inositol 5' phosphatase (SHIP) is an important component for limiting antigen-induced signals in B cells. SHIP hydrolyzes the 5' phosphate of phosphatidyl-3,4,5-trisphosphate (PIP3) at the inner leaflet of the plasma membrane thereby disrupting binding motifs for the plextrine homology domains and attenuating the activities of Bruton's tyrosine kinase and phospholipase C- γ (PLC- γ 2), respectively. Initially SHIP activation was believed to depend on inhibitory coreceptors like the Fc- γ R1IB. However, studies using *ship*^{-/-} DT40 cells or mice revealed that SHIP is activated downstream of BCR engagement in absence of Fc- γ R1IB also. The mechanism of BCR-induced SHIP activation and its relocalization towards the substrate PIP3, however, remains obscure to date. Here we report a real time imaging approach to analyze the molecular mechanism of BCR-induced SHIP relocalization. Interestingly, neither Fc- γ R1IB nor the SHIP SH2 domain contributed to this process. Using genetic variants of DT40 B cells we could show that SHIP plasma membrane recruitment occurs upstream of PLC- γ 2 activation. Our studies revealed that two apparently independent mechanisms are involved. First the Lyn-dependent assembly of a trimolecular complex comprising SHIP, the SH2 domain-containing adapter protein (Shc) and the growth factor receptor-bound protein 2 (Grb2) supports the SHIP relocalization. Second, the protein tyrosine kinase Syk is required for efficient SHIP plasma membrane recruitment.

A74**The immunoglobulin tail tyrosine of membrane-bound IgG and IgE provides antigen receptor-intrinsic costimulation to class-switched memory B cells**

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Improved antibody responses by class-switched memory B cells require enhanced signaling from their antigen receptor (BCR) in a coreceptor-independent manner. However, all BCR classes on newly generated and antigen-experienced B cells utilize the canonical Ig α /Ig β subunit for signaling via the immunoreceptor tyrosine-based activation motif (ITAM) in their cytoplasmic domains. We have now identified the signal amplification mechanism of the activated IgG- and IgE-BCR on class-switched B cells. An evolutionary conserved tyrosine residue in the cytoplasmic segments of membrane-bound IgG and IgE heavy chains, named Immunoglobulin Tail Tyrosine (ITT), becomes phosphorylated and recruits the adaptor protein Grb2 in order to prolong activation of protein kinases and sustain the generation of second messengers. Exchange of the ITT for phenylalanine phenocopies the reduced signaling profile of the IgM-BCR expressed on naïve B cells. Hence membrane-bound IgG and IgE not only recognize antigen but also exert BCR-intrinsic costimulation to render memory B cells less dependent on T cell help for activation. Moreover, our finding of a signaling competent phospho-ITT confutes the paradigm of BCR tyrosine phosphorylation being confined to ITAM-containing subunits.

A75**A novel mechanism for the regulation of Gab1 recruitment to the plasma membrane**

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Adapter proteins involved in signal transduction fulfil their cellular functions by bringing signalling molecules together and by targeting these signalling components to defined compartments within the cell. Furthermore, adapter proteins represent a molecular platform from which different signalling pathways are initiated. Gab1 is an adapter, which recruits the p85 subunit of the phosphatidylinositol 3-kinase, the adapter Grb2, the adapter and phosphatase SHP2 and the GTPase-activating protein Ras-GAP. By this, Gab1 contributes to the activation of the PI3K cascade and the MAPK cascade by many growth factors and cytokines. The recruitment of Gab1 to phosphatidylinositol-3,4,5-tris-phosphate within the plasma membrane by its pleckstrin homology domain is regarded as a major regulatory step for the activation of Gab1. Here, we present a novel and more complex mechanism for Gab1 translocation, which involves and depends on the activation of ERK. We demonstrate that the presence of PI3K activity in the cell is not sufficient for binding Gab1 to the plasma membrane. Instead, additional MAPK-dependent phosphorylation of serine 551 in Gab1 is crucial for the recruitment of Gab1 to the plasma membrane. This mechanism represents a new mode of regulation for the function of PH domains.

A76**Calcium signals in lymphocyte activation and disease**

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Calcium ions function as universal second messengers in virtually all eukaryotic cells including cells of the immune system where they are crucial for the function of T and B cells, mast cells and dendritic cells. The predominant mechanism regulating intracellular Ca²⁺ levels in cells of the adaptive immune system is store-operated Ca²⁺ influx through so-called Ca²⁺-release activated Ca²⁺ (CRAC) channels. We identified ORAI1 (also named CRACM1) as a pore subunit of the CRAC channel essential for the function of T cells and mast cells. ORAI1/CRAC channels are activated when intracellular Ca²⁺ stores are depleted. The resulting decrease in the ER Ca²⁺ concentration is sensed by stromal interaction molecule 1 (STIM1) that is required for activation of ORAI1/CRAC channels. We showed that murine T cells lacking STIM1 exhibit severely impaired store-operated Ca²⁺ influx. T cells from mice lacking STIM1 or its paralogue STIM2 both showed significantly reduced cytokine production *in vitro* and a defect in regulatory T cell development as well as lympho- and myeloproliferation *in vivo*. Mutation of ORAI1 in humans is associated with severe combined immunodeficiency (SCID), increased susceptibility to infections and a failure to thrive. A similar defect is found in mice transgenic for the equivalent R93W mutation in murine ORAI1, which all but abrogates CRAC channel function and T cell activation. Taken together STIM1, STIM2 and ORAI1 are essential regulators of store-operated Ca²⁺ entry in cells of the immune system and other tissues.

A77**Neuroprotection and enhanced neurogenesis by extract from the tropical plant *Knema laurina* after inflammatory damage in living brain tissue**I Häke¹, S Schönenberger¹, J Neumann³, K Paulsen-Merker², K Reymann³, G Ismail⁴, L bin Din⁵, I Said⁵, A Latiff⁵, F Zipp⁶ and O Ullrich²¹Institute of Immunology, Medical Faculty, Otto-von-Guericke-University Magdeburg, Germany²Institute of Anatomy, Faculty of Medicine, University of Zurich, Switzerland³Leibniz-Institute for Neurobiology, Magdeburg, Germany⁴Malaysia University of Science and Technology, Kuala Lumpur⁵Faculty of Science and Technology, University Kebangsaan Malaysia, Bangi, Malaysia⁶Cecilie-Vogt-Clinic for Neurology, Charité – University Medicine Berlin, Germany

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Inflammatory reactions in the CNS, resulting from a loss of control and involving a network of non-neuronal and neuronal cells, are major contributors to the onset and progress of several major neurodegenerative diseases. Therapeutic strategies should therefore keep or restore the well-controlled and finely-tuned balance of immune reactions, and protect neurons from inflammatory damage. In our study, we selected plants of the Malaysian rain forest by an ethnobotanic approach, and investigated them in cell-based-assay-systems and in living brain tissue cultures in order to identify anti-inflammatory and neuroprotective effects. We found that extracts from the tropical plant *Knema laurina* (Black wild nutmeg) exhibited highly anti-inflammatory and neuroprotective effects in cell culture experiments, reduced NO- and IL-6-release from activated

microglia cells dose-dependently, and protects living brain tissue from microglia-mediated inflammatory damage. On the intracellular level, the extract inhibited ERK-1/2-phosphorylation, I- κ B-phosphorylation and subsequently NF- κ B-translocation in microglia cells. *Knema laurina* belongs to the family of Myristicaceae, which have been used for centuries for treatment of digestive and inflammatory diseases and is also a major food plant of the Giant Hornbill. Moreover, extract from *Knema laurina* promotes also neurogenesis in living brain tissue after oxygen-glucose deprivation. In conclusion, extract from *Knema laurina* not only controls and limits inflammatory reaction after primary neuronal damage, it promotes moreover neurogenesis if given hours until days after stroke-like injury in levels comparable to the best neurotrophic factors known today.

A78

TLR2 and TLR4 signaling in macrophages is negatively regulated by a Lyn-PI3K module and promoted by SHIP1

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We demonstrate here that the Src family kinase Lyn negatively regulates Toll-like receptor (TLR) signaling in bone marrow-derived macrophages (BMM ϕ s) and *in vivo*. Lyn^{-/-} BMM ϕ s produced and secreted significantly more IL-6, TNF- α , and IFN- α/β compared to WT BMM ϕ s, indicating that Lyn is able to control both MyD88- and TRIF-dependent signaling pathways downstream of TLR4. CD14 was not involved in this type of regulation. Moreover, Lyn attenuated proinflammatory cytokine production in BMM ϕ s in response to the TLR2 ligand, FSL-1. In agreement with these *in vitro* experiments, Lyn-deficient mice produced higher amounts of proinflammatory cytokines than WT mice after *i. v.* injection of LPS or lipopeptide. Though Lyn clearly acted as a negative regulator downstream of TLR4, it did not, different to what was proposed previously, alter the process of LPS tolerance. Stimulation with a low dose of LPS resulted in reduced production of proinflammatory cytokines after a subsequent stimulation with a high dose of LPS in both WT and Lyn^{-/-} BMM ϕ s as well as *in vivo*. Mechanistically, Lyn interacted with PI3K and in correlation, PI3K inhibition resulted in increased LPS-triggered cytokine production. In this line, SHIP1-deficient BMM ϕ s, exerting enhanced PI3K-pathway activation, produced less cytokines compared to WT BMM ϕ s. In conclusion, Lyn is a negative regulator of TLR-induced cytokine production *in vitro* and *in vivo* and acts, at least in part, via PI3K.

A79

Swiprosin I – regulator of proximal BCR signaling

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The adaptor protein Swiprosin-1/Efhd2 that we identified recently in B cell lipid rafts exhibits its highest expression in immature B cells. Swiprosin-1/Efhd2 consists of a proline rich domain, two EF-hands and a C-terminal coiled-coil domain. Knock-down of Swiprosin-1/Efhd2 in an immature B cell line with a shRNA directed against the 3'-UTR (WEHI231shSwI) impaired spontaneous and BCR-elicited apoptosis. Due to co-clustering of a Swiprosin-1-EGFP fusion protein with the B-cell receptor we expected Swiprosin-1 to be a player in proximal BCR signaling. Indeed, downregulation of Swiprosin-1 attenuated total tyrosine phosphorylation and diminished BCR induced intracellular calcium flux. In contrast, WEHI231 cells ectopically expressing Swiprosin-1 showed increased, but shortened total tyrosine phosphorylation at early time points, as well as increased BCR induced calcium flux. Concomitantly, transient re-expression of Swiprosin-1 in WEHI231shSWI cells restored BCR-induced calcium flux.

These data suggested interactions of Swiprosin-1 with central elements of the BCR signaling cascade. In fact, GST-pulldown experiments showed that Swiprosin-1 can interact with tyrosine-phosphorylated proteins of ~145 and 70 kDa. With this assay we identified the protein tyrosine kinase Syk in its phosphorylated form as interaction partner of Swiprosin-1. The interaction of pSyk with Swiprosin-1 is mediated by the C-terminal part of Swiprosin-1 because a deletion mutant of Swiprosin-1 lacking the EF-hands and the coiled-coil domain of Swiprosin-1 did not show any interaction with pSyk. We next tested the kinase activity of Syk as a function of Swiprosin-1 levels. Interestingly, immune complex kinase assays showed that BCR-induced Syk activity was reduced in WEHI231shSwI cells.

Hence, Swiprosin-1 could regulate proximal BCR signaling through maintenance of Syk activity.

A80

T Cell transformation by herpesvirus saimiri requires STAT5 pathways

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Aberrant growth stimulation by Signal Transducers and Activators of Transcription (STAT) is implicated in human carcinogenesis. The viral effector oncoprotein Tip, which binds to and is phosphorylated by the T-cell lymphocyte kinase Lck, is crucial in the activation of the cellular STAT5 signalling pathway in Herpesvirus-transformed T-cell lines, a model for malignant T-cell proliferation. While Tip-Lck interaction is required for transformation by HVS, mutation of Tip tyrosine residues had distinct effects. Two defined mutations of Tip can direct the phosphorylation and activation of either STAT3 or STAT5 by Lck. Tip Y114 mutation to phenylalanine (TipY114F) abolished the constitutive STAT3 activation observed in HVS-wildtype transformed T cells. Conversely, TipY114F enhanced the efficiency of human T cell transformation in absence of exogenous interleukin-2 (IL-2). In contrast, mutation of the major phosphorylation site, Y127, in Tip is compatible with viral transformation

only when IL-2 is supplemented. This growth factor requirement correlated with STAT5 activation by Lck and Tip. Our current work focuses on the synergy among Tip, Lck, STAT5 and JAK family kinases. Interaction analysis will address whether Tip or the Y127F mutant mediate interactions between STAT5 and Lck or other Src family members. The role of the JAK-STAT pathway was elucidated using specific JAK inhibitors. We assume that, for viral transformation, Tip preferentially targets and reprograms the STAT5 pathway, which is central for T-cell growth and homeostasis. Comparable modulation of the STAT5 signalling may be involved in other forms of malignancy and cell differentiation; their analysis may therefore be a useful model for the development of new therapeutics.

A81

The PCH family member CIP4 is released from T cells upon activation-induced cell death

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The 'Pombe Cdc15 homology' (PCH) family of adaptor proteins gained much attention only very recently. PCH proteins link membrane trafficking events to the actin cytoskeleton. Structurally, a so-called F-BAR domain characterizes all members of the PCH family and recent studies indicate that this domain enables PCH proteins to bind to and deform cellular membranes resulting in membrane curvature and tubulation. Accordingly, PCH proteins have been functionally associated with endo-/exocytosis and the trafficking of vesicles. As several PCH proteins interact with the death factor FasL, we were interested in the expression and function of PCH proteins in T cells in this context.

Employing a new monoclonal anti-CIP4 antibody generated in our laboratory, we initially observed that the PCH family member CIP4 is expressed in T cells only after activation indicating a specific role in T cell maturation or effector function. Surprisingly, staining activated T cells for CIP4 followed by laser scanning microscopical inspection revealed that CIP4 seems to be somehow released from the cells in vesicular structures resembling exo- or ectosomes. The vesicle release can be visualized as a direct plasma membrane "budding". Moreover, CIP4-microvesicle formation and release proved to be enhanced during the initial phase of activation-induced T cell death triggered e.g. by exposure to staphylococcal superantigen. To us this indicates that this process might be relevant during T cell death to provide additional danger signals to neighboring cells. We are currently analysing the molecular content of these vesicles, the role of CIP4 in their generation and their impact on T cell biology.

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A82

Analysis of knockout/knockin mice that express a mutant FasL lacking the intracellular domain

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Fas ligand (FasL; CD178; CD95L) is a type II transmembrane protein belonging to the tumour necrosis factor family; its binding to the Fas receptor (CD95; APO-1) triggers apoptosis in the receptor-bearing cell. Signalling through this pathway plays a pivotal role during the immune response and in immune system homeostasis. Similar to other TNF family members, the intracellular domain has been reported to transmit signals to the inside of the FasL-bearing cell (reverse signalling). Recently, we identified the proteases ADAM10 and SPPL2a as molecules important for the processing of FasL. Protease cleavage releases the intracellular domain, which then is able to translocate to the nucleus and to repress reporter gene activity. To study the physiological importance of FasL reverse signalling in vivo, we established knockout/knockin mice with a FasL deletion mutant that lacks the intracellular portion (FasLDeltaIntra). Co-culture experiments confirmed that the truncated FasL protein is still capable of inducing apoptosis in Fas-sensitive cells. Preliminary immune histochemistry data suggest that, in contrast to published data, the absence of the intracellular FasL domain does not alter the intracellular FasL localization in activated T cells. We are currently investigating signalling and proliferative capacities of T cells derived from homozygous FasLDeltaIntra mice to validate a co-stimulatory role of FasL reverse signalling.

A83

Compartmentalized Ras signaling in lymphocytes

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Ras proteins are peripheral membrane proteins targeted via post-translational modification of a C-terminal CAAX sequence. Although the plasma membrane (PM) is one organelle to which Ras proteins are targeted, this compartment is no longer considered the only platform from which Ras can signal. The palmitoylated isoforms, Nras and Hras, cycle between the Golgi apparatus and PM as a consequence of a palmitoylation/depalmitoylation cycle. We have studied dual compartment signaling in T lymphocytes. Ras activation as a consequence of antigen receptor (TCR) engagement on T lymphocytes is required for T-cell development, selection and function. Lymphocyte function-associated antigen-1 (LFA-1) mediates lymphocyte adhesion, stabilization of the immune synapse and bidirectional signaling. Using a fluorescent biosensor we found that TCR activation with or without co-stimulation of CD28 led to activation of Ras only on the Golgi apparatus, whereas co-stimulation with LFA-1 induced Ras activation on both the Golgi and the PM. Ras activation on both compartments required RasGRP1, an exchange factor regulated by calcium and diacylglycerol (DAG), but phospholipase C activity was required only for activation on the Golgi. Engagement of LFA-1 increased DAG levels at the PM by stimulating phospholipase D (PLD). PLD2 and phosphatidic acid phosphatase were required for Ras activation on the PM. Thus, LFA-1 acts through PLD2 to reshape the pattern of Ras activation downstream of the TCR.

A84**Identification of interaction partners of the adapter protein Nck in T cells**J Pieper¹, B Lengl-Janßen¹, M Voss¹, C Gelhaus², M Leippe², O Janssen¹ and M Lettau¹¹University Hospital Schleswig-Holstein, Campus Kiel, Institute of Immunology, Kiel, Germany²Department of Zoophysiology, Zoological Institute, Christian-Albrechts University, Kiel, Germany

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The adaptor protein Nck almost exclusively consists of one SH2 domain and three SH3 domains and plays an important role in linking receptor tyrosine kinases (and associated proteins) to modulators of the actin cytoskeleton. In T cells, Nck thereby connects T cell receptor signaling to the machinery of actin reorganization thus initiating changes in cell polarity required for T cell effector function. We previously showed that Nck interacts with the death factor FasL and is required for the recruitment of FasL to the cytotoxic immunological synapse upon recognition of a target cell (Lettau et al., PNAS 2006). Given the importance of Nck in T cell effector function, we now performed a systematic screening for interaction partners of the four individual interaction modules of Nck in primary and leukemic T cells. To this end, we expressed full length Nck, the three SH3 domains and the individual SH3 and SH2 domains of Nck as GST fusion proteins to precipitate binding partners from untreated or pervanadate-treated PHA blasts, Jurkat and HUT78 cells. Candidate binding proteins were cut from gels after Coomassie, Silver, Sypro Ruby or Flamingo Pink staining and processed by tryptic in gel digestion for mass spectrometrical analysis. Of several candidate-binding partners identified by the pulldown assays, we confirmed direct interaction by Far Western Blotting. As might be expected, we observe major differences in Nck binding proteins from resting versus activated T cells. Interestingly, the list of interactors not only points to known functions including recruitment of the WASP/Arp2/3 complex and the actin cytoskeleton but also to the recently described nuclear translocation and function of Nck, e.g. in damaged cells. Therefore, we are currently focusing on the verification of the latter Nck interactions in nuclear extracts to unravel this new aspect of Nck biology.

A85**Proteomic profiling of secretory granules of different T cell subpopulations**H Schmidt¹, C Gelhaus², M Nebendahl¹, M Leippe² and O Janssen¹¹UK-SH Campus Kiel, Institute of Immunology, Molecular Immunology, Kiel, Germany²Department of Zoophysiology, Zoological Institute, Christian-Albrechts-University, Kiel, Germany

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In cytotoxic T lymphocytes and Natural Killer (NK) cells, effector molecules including granzymes, perforin, granulysin and FasL are stored in specialized granules termed secretory lysosomes (SL). These vesicles represent dual-functional organelles that obviously combine degradative and exocytotic properties [1]. We previously established an enrichment protocol to define the

proteome of SL from NK cells. We found that the protein content of SL very much depends on the function of a given cell type or clone, best reflected by crucial differences in functionally relevant proteins in transformed NK cell lines [2].

In order to compare the lysosomal content of different T cell subpopulations, we enriched SL from alpha/beta (CD4 or CD8) and gamma/delta (Vdelta 1 or Vdelta2) T cell lines and clones. To this end, the T cell lysates were separated by density gradient centrifugation on Iodixanol gradients. As described before, for the differential proteome analysis we focused on the fraction that contained most FasL, Lamp1 and Lamp3 (as specific SL or general lysosomal markers) and compared the isolated lysosomal fractions by 2D-DIGE. We found that the protein content of SL of *in vitro* expanded CD4 and CD8 cells as well as Vdelta1 and Vdelta2 cells is more similar than for example gamma/delta cells compared to alpha/beta cells. A detailed MALDI-based profile of individual SL proteomes based on more than 1000 picked spots from several DIGE experiments will be presented with a focus on the functionally relevant proteins mentioned above. The observed differences might reveal new aspects of population-specific dynamics of activation/maturation and effector function in the T cell compartment.

Acknowledgements

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A86**The inducible transcription factor NFATc1 controls the survival of germinal center B lymphocytes**E Serfling¹, R Rost¹, C Wen¹, A Khalid¹, A Avots¹, F Berberich-Siebelt¹, S Klein-Hessling¹ and E Kondo²¹Universität Würzburg, Pathologisches Institut, Abtlg. Molekulare Pathologie, Würzburg, Germany²Department of Pathology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan

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In almost all lymphocytes the three members of NFAT family of transcription factors, NFATc1, c2 and c3, are expressed. While the nuclear transport, the DNA binding and, therefore, the activity of all three NFATc members is commonly controlled by the Ca²⁺-dependent phosphatase calcineurin, their transcriptional regulation differs from each other. In the majority of lymphocytes both NFATc2 and c3 are constitutively expressed, whereas immunoreceptor and co-receptor signals enhance rapidly the levels of NFATc1 in effector T and B lymphocytes. This results in the rapid synthesis of NFATc1/alphaA, a short NFATc1 isoform, which differs mainly by the lack of a C-terminal stretch of approximately 245 amino acid residues from other NFAT proteins. By inactivating the endogenous NFATc1 gene and

re-introducing human NFATc1/alphaA into chicken DT40 B cells we show here that high NFATc1/alphaA levels protect B cells against B cell receptor-mediated AICD. Prominent target genes of NFATc1/alphaA in DT40 B cells are the Bcl-6, PKC-theta and Bag-2 genes whose expression is strongly enhanced in DT40 cells expressing human NFATc1/alphaA. The anti-apoptotic activity of NFATc1/alphaA appears to be due to an enhancement of NF- κ B signals and of binding of NFATc1 to promoters of anti-apoptotic genes. Similar to the HIV-1 LTR and IL-8 promoters, the promoters of numerous anti-apoptotic genes contain composite kappaB/NFAT sites to which NFATc1 can bind (as homodimers, similar to NF-kappaB) in human NFATc1/alphaA expressing DT40 cells. Since NFATc1/alphaA suppresses the RNA synthesis of secreted IgM and Blimp-1 but enhances Bcl-6 and Bach-2 RNA levels, NFATc1/alphaA appears to control the affinity maturation of Ig genes in germinal center (GC) B cells. Immuno-histochemical stainings with an Ab raised against NFATc1/alpha protein shows that – in striking contrast to the cytosolic localisation of NFATc1 in the majority of lymphoid cells – NFATc1/alpha is constitutively expressed in nuclei of a subset of GC B cells, as well as in Burkitt's lymphomas which originate from GC B cells. Most of these GC B cells expressing nuclear NFATc1/alpha do not express IgM and apoptotic markers suggesting NFATc1/alpha contributes to the survival of a subset of matured GC B cells. This hypothesis is currently tested by more immunohistochemical stainings and the use of genetically modified mice carrying NFATc1 flx/flx alleles and AID- and CD23-Cre for the inactivation of NFATc1 gene in GC B cells, or just before GC formation. All our data indicate that in T and GC B cells the inducible synthesis of short NFATc1/alphaA isoforms contributes to the survival of lymphocytes while – as we have shown earlier (see Chuvpilo et al., *Immunity* 2002) – the NFATc proteins NFATc2 and c3 support the Activation Induced Cell Death (AICD) of T (and B) lymphocytes upon TCR (BCR) stimulation.

A87

RCAN1C is differentially expressed in T helper cell subsets

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The Ser/Thr-phosphatase calcineurin is a key enzyme of T cell receptor (TcR)-dependent signaling. Endogenous regulatory proteins regulate its activity in T cells. Here, we show that the transcription of *RCAN1*, coding for the calcineurin inhibitory protein calcipressin 1, is calcineurin/NFAT-dependently upregulated after TcR stimulation. This is mainly due to an increase in the expression levels of the splice variant *RCAN1C*, as the splice variant *RCAN1A* remains unchanged. *RCAN1C* expression is differentially regulated in various CD4⁺ T helper (T_H) cell subpopulations: *RCAN1C* is stronger upregulated in CD45RO⁺ memory T_H cells compared to CD45RA⁺ naïve T_H cells or regulatory T cells. Additionally, memory T_H cells show an elevated baseline expression and a prolonged upregulation of *RCAN1C* transcription upon stimulation. We are discussing how *RCAN1* is regulated and which signal transduction pathways and factors might be involved. It remains to be clarified which

differentially activated signaling molecules in the selected T_H cell subsets cause the diverse *RCAN1C* expression pattern.

A88

Impact of norepinephrine, dopamine and substance P on the activation and function of CD8 lymphocytes

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During the past 30 years it became evident that neurotransmitters are important regulators of the immune system. The presence of nerve fibers and the release of neurotransmitters within lymphoid organs represents a mechanism by which signals from the central nervous system influence the immune cell functions. Neurotransmitter per se cannot induce any new function in immune cells but they are mainly responsible for the “fine-tuning” of an immune response. There is already a broad knowledge on the influence of neurotransmitter on B cells or CD4 lymphocytes/T helper cells, but with regard to CD8 positive cytotoxic T lymphocytes (CTLs) there are still many open questions. Therefore, we conducted the present study in order to complement the picture of neurotransmitter action on this leukocyte subset. We investigated the influence of norepinephrine, dopamine and substance P on the key functions of CTLs in vitro: activation, extravasation, migration and cytotoxicity. The activation of CTLs via CD3/CD28 cross-linking was inhibited by all of the three investigated neurotransmitters via mechanisms that involve PKA as investigated by the use of specific cAMP analogs. Furthermore, these cells showed a decrease in ERK1/2 phosphorylation and their level of IL-2 mRNA was reduced. Addition of high dose IL-2 was able to reverse the inhibiting effect of the neurotransmitters. With regard to extravasation we found dopamine to be a strong inducer of the adhesion of naïve CD8 lymphocytes to endothelium. In contrast, norepinephrine induced the adhesion of activated/effector T cells. Norepinephrine increases the IL-8 release from endothelium thereby leading to an increased adhesion of CXCR1 positive cells. We found CXCR1 to be mainly expressed on activated, perforin positive CTLs. All of the investigated neurotransmitters increased the spontaneous migratory activity of naïve CTLs with dopamine being the strongest inducer. But activated CTLs showed a reduced migratory activity in the presence of norepinephrine and substance P which seems to be mediated via the Epac/Rap1 pathway as investigated by the use of specific cAMP analogs. Dopamine had no effect on activated CTLs since dopamine receptors are down-regulated during the activation process. The ability of activated CTLs to release their cytotoxic granula in response to CD3 cross-linking was analyzed by measuring the beta-hexosamidase release. This process was not influenced by any of the neurotransmitters used. In conclusion, there is no general scheme for neurotransmitters to act either stimulatory or inhibitory on leukocytes. In contrast, the effect of a neurotransmitter depends on the leukocyte subtype and its activation state or phenotype. Thus, neurotransmitters are specific modulators of certain immune functions.

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A89**Investigating immunomodulatory mechanisms of cannabinoids: the role of MMP-9**S Tauber¹, R Scheider-Stock² and O Ullrich¹¹University of Zurich, Institut for Anatomy, Department for Cell- and Neurobiology, Zurich, Switzerland²University of Magdeburg, Institut for Pathology, Department for Molecular Genetics, Magdeburg, Germany

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The endocannabinoid system, which comprises endogenous ligands and various receptors, plays an important role in immune control. Endocannabinoid signalling impacts crucial immune cell functions such as migration and chemotaxis and acts as an auto-protective and compensatory system under inflammatory conditions. The inflammatory mediator Matrix-metalloproteinase 9 (MMP-9) helps immune cells to migrate to the sites of inflammation by degrading the extracellular matrix.

The aim of our study was to determine the effect of cannabinoid signalling on the secretion of MMP-9 by macrophages. U937 monocytes were differentiated to macrophages by incubation with PMA (100 nM) for 48 h, and primary human macrophages were isolated from whole blood. Treatment with the synthetic cannabinoid WIN 55, 212-2 (2 or 4 µM) induced a dose dependent inhibition of MMP-9 secretion as shown by activity assay and western blot analysis of conditioned media. Kinetic analysis of cell lysates by western blot showed intracellular accumulation of MMP-9 going along with the inhibited secretion, proposing inhibition of the secretion process as a mechanism. Quantitative real-time-PCR was used to measure MMP-9 mRNA and revealed a possible negative feedback loop on the transcriptional level which involves MAP-kinases as shown by phospho-specific western blot analysis. The effect could not be blocked by pharmacological inhibition of the known cannabinoid receptors CB1, CB2, and TRPV1, but was stereospecific, as it could not be reproduced with the enantiomer WIN55, 212-3. The effect therefore seems to be mediated by a yet unidentified cannabinoid-binding site.

Our results suggest that cannabinoid-induced inhibition of MMP-9 secretion presents a new mechanism of anti-inflammatory action of the cannabinoid system and helps to provide a basis for the development of cannabinoid-based drugs for inflammatory diseases.

A90**Decision making in NK cells**D Urlaub¹, S Mesecke², H Busch², R Eils² and C Watzl¹¹Institute for Immunology, University Hospital Heidelberg, Heidelberg, Germany²Division of Theoretical Bioinformatics, DKFZ, Heidelberg, Germany

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A balance of positive and negative signals that are transmitted by different surface receptors controls the effector functions of NK cells. To date our understanding about the integration of positive and negative signals and the decision-making process inside NK cells remains poor.

With the help of bioinformatic modelling we try to understand how NK cells first integrate antagonising signals and then

compute a reliable killing decision. Gradual signal input through activating and inhibitory receptors is integrated to come to a "yes or no" decision by the NK cell to kill an attached target cell. Triggering of activating receptors leads to Src kinase activation and Vav-1 phosphorylation, whereas inhibitory receptors dephosphorylate Vav-1 via the phosphatase SHP-1. Therefore, we proposed in a first hypothesis, that Vav-1 is the decision making point in the signal transduction network. With this hypothesis we created a family of simplified models describing NK cell activation upon various stimuli. The predictions derived from these models were compared with experimental data. Our experiments showed that increased clustering of activating receptors lead to a rapid switch-like increase in Vav-1 phosphorylation. Similarly, titrating the engagement of inhibitory receptors resulted in switch-like dephosphorylation of Vav-1. Testing NK cell activity after various amounts of activating and inhibitory receptor engagement revealed a functional dominance of inhibitory receptors. Our current model is consistent with a central role of Vav-1 in the decision making process of NK cells and enables a novel insight into the integration of positive and negative signals during lymphocyte activation.

A91**Human S100A8 and S100A9 activate phagocytes via Toll-like receptor 4 independent of RAGE**T Vogl¹, M Wolf¹, B Petersen¹, C Ehrhardt², MAD van Zoelen³, D Foell¹ and J Roth¹¹Institute of Immunology, University of Münster, Münster, Germany²Institute of Molecular Virology, University of Münster, Münster, Germany³Center of Infection and Immunity Amsterdam, University of Amsterdam, The Netherlands

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Endogenous Damage Associated Molecular Pattern (DAMP) proteins are known as important pro-inflammatory factors of the immune system, which are released during cellular stress. Recognition of DAMPs involves the multiligand Receptor for Advanced Glycation End products (RAGE) and Toll-like receptors (TLRs) in sensing not only Pathogen Associated Molecular Patterns (PAMPs) but also endogenous proteins. Members of the fast growing family of DAMP proteins are besides heat shock proteins, HMGB1 or defensins also some members of the family of S100 proteins, which promote inflammatory processes. It was claimed that RAGE is involved in almost all S100 protein activities.

We here investigated the capacity of human S100A8 (MRP8, myeloid related protein 8) and human S100A9 (MRP14) on activation of human phagocytes. S100A8 and S100A9 form homodimers as well as heterodimers and belong to the S100 family of EF-hand calcium-binding proteins. Both proteins are the major cytoplasmic proteins of phagocytes and are released at sites of inflammation by activated or necrotic phagocytes.

While human S100A8/S100A9-complexes did not show any phagocyte activation, S100A8 homodimers as well as S100A9 homodimers induce strong pro-inflammatory mechanisms in these cells. Human S100A9 induces intracellular translocation of MyD88 and activation of IRAK-1 as shown recently already for murine S100A8. Finally NF-κB activation results in elevated expression of TNF-alpha as well as other pro-inflammatory

genes. In blocking experiments with TLR4-specific monoclonal antibodies we demonstrate that both S100 proteins specifically signals via TLR4 receptor complex on human phagocytes. Using TLR4/MD2/CD14 transfected HEK293 cells and RAGE transfected HEK293 cells we clearly can exclude the involvement of RAGE for at least these two S100 proteins.

Our *in vitro* results could be further confirmed *in vivo*. Mice lacking S100A8/S100A9 are protected against abdominal sepsis induced by *E. coli*. Our present data clearly demonstrate the importance of TLR4 by which phagocytes promote their own activation via expression and secretion of endogenous ligands of this receptor.

A92 **Identification of SH3 domain interaction partners of FasL using a human SH3 domain phage display library**

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Unlike other members of the tumour necrosis factor superfamily, Fas ligand (CD95L) contains a unique polyproline region (aa 37–70) as part of its N-terminal intracellular tail. We already described several SH3 or WW domain proteins that proved to interact with FasL via this proline-rich domain (PRD). We defined distinct adapter proteins that are involved in the regulation of FasL sorting and trafficking and identified ADAM10 as the FasL sheddase (see [1] for review). Given that ADAM10-mediated ectodomain shedding regulates the surface expression of FasL and that FasL is subsequently released into the cytosol by regulated intramembrane proteolysis (RIPing) through the γ -secretase-like enzyme SPPL2a, we are interested in defining interactions involving the generated intracellular fragment of FasL. Interestingly, so far we failed to co-immunoprecipitate either the FasL N-terminal membrane fragments generated by ADAM10 cleavage or the FasL intracellular remainder generated by SPPL2a activity with previously described interaction partners, while full length FasL was co-immunoprecipitated. From precipitates with a new mAb directed against the intracellular portion of FasL, however, we can readily detect processed FasL in T cell blasts. In order to identify other SH3 domain proteins that potentially (and selectively?) interact with the RIPed FasL PRD, we used a SH3 domain phage display library containing all 288 SH3 domains expressed in humans. We are thus confident to be able to present the complete "SH3 interactome" for the FasL PRD in Weimar. The identification of interactors will give us some hints on the still open function of the intracellular FasL fragments.

Acknowledgements

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A93

Model cell systems representing expression regulation and signal transduction of thymic stromal lymphopoietin (TSLP) in the development of asthmatic symptoms

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Thymic stromal lymphopoietin (TSLP) is a novel interleukin-7-like cytokine, which triggers dendritic cell-mediated inflammatory responses ultimately executed by Th2 helper cells. TSLP is a central player in the development of allergic, especially asthmatic symptoms, and has been suggested to represent a missing link in the information flow from antigen-exposed epithelial cells via dendritic cells (DCs) to T helper cells.

We have challenged the lung epithelial cell line A549 with various allergens such as extracts from dust mites, pollen and fungi, and could show by real time PCR for the first time an allergen-dependent specific transcriptional upregulation of TSLP.

To approach the as yet poorly defined TSLP-induced signal transduction in dendritic cells, we established a cellular model system on the basis of the murine pro-B cell line Ba/F3. The heterodimeric human TSLP receptor (hTSLPR) consisting of the novel TSLP receptor chain and the IL-7 receptor alpha chain was functionally reconstituted in factor-dependent Ba/F3 cells. We could demonstrate by phosphotyrosine protein analysis and by STAT type-specific reporter gene assays, that the ligand-stimulated TSLPR triggers activation of STAT3 and STAT5 as well as of STAT1. Employment of specific inhibitors proved the functional involvement of Janus kinases (JAKs) in TSLP-dependent, receptor-mediated cellular responses.

These achievements provide tools for research towards a better molecular understanding of atopic asthma and for the future development of new therapeutics interfering with TSLP function.

A94

Regulation of T cell chemotaxis by CXCL4

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Directed migration of cells along a chemotactic gradient is a fundamental cellular process involved e.g. in host defense, tissue development, and wound repair. Surprisingly, although a broad spectrum of different mediators are able to induce a chemotactic response, only very few regulators or inhibitors of this function are known. CXCL4 (platelet factor 4; PF4), a platelet-derived CXC-chemokine, modulates long-term immunoregulatory functions in T cells but lacks the capacity to induce chemotaxis in these cells. However, in the current study we are able to show for the first time, that CXCL4 acts as a potent inhibitor of T cell chemotaxis induced by CXCR3 ligands CXCL11 and CXCL9, but not CXCL10. CXCL4 did neither interfere with ligand binding to CXCR3 nor with ligand-induced internalization or calcium signaling of CXCR3. By several lines of evidence we could rule

out the participation of known CXCL4-receptors, like proteoglycans or CXCR3-B, in CXCL4-mediated inhibition of chemotaxis. We, thus, claim the presence of a further, so far unidentified receptor for this chemokine, which is present on T cells. Intriguingly, CXCL4 also reduces the chemotaxis of T cells and neutrophils induced by the CXCR3-independent ligand CXCL12 (SDF-1 α). Taken together, our results identify CXCL4 as the first chemokine, which acts as an inhibitor rather than an inducer of chemotaxis on T cells and neutrophils in vitro.

Adhesion, motility, morphology

A95

Glucocorticoids cause VE-cadherin upregulation and cytoskeletal rearrangements in the blood-brain barrier endothelial cEND cell line

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The demonstration that glucocorticoid-mediated activation can control occludin expression in brain capillary endothelial cells has provided a new avenue of research in the field of blood brain-barrier (BBB) permeability regulation. In order to identify more key genes involved in glucocorticoid-mediated regulation of BBB permeability, we used cDNA microarrays to study changes in gene expression in dexamethasone-treated brain capillary endothelial cEND cells: Primarily, we observed changes in expression of the VE-cadherin gene involved in cell adhesion. Vascular-endothelial-cadherin (VE-cadherin) is an endothelial cell-specific adhesion protein localized in cell-cell contacts. It is known as an important determinant of vascular architecture and endothelial cell survival. Quantitative real-time PCR showed an upregulation of VE-cadherin expression exclusively in cEND cells, in accordance to previous observations made for the occludin gene. Subsequently, we verified divergent transcriptional activation of the VE-cadherin gene by dexamethasone. Furthermore, we measured the change in protein levels of VE-cadherin and demonstrated a transactivation of the VE-cadherin promoter in cEND cells via dexamethasone. Dexamethasone was further shown to induce cellular differentiation into a cobblestone cellular morphology and reinforcement of adherens junctions concomitant with the increased anchorage of VE-cadherin to the actin cytoskeleton. We thus propose that glucocorticoid effects on VE-cadherin protein synthesis and organization are important for the formation of both adherens and tight junction, and for improved barrier properties in microvascular brain endothelial cells.

To find cell- or tissue-specific ligands that could be used in therapeutic regime of barrier disorder diseases it is necessary to study and understand the molecular mechanisms of beneficial effects of glucocorticoid action on BBB-genes involved in BBB-permeability regulation.

A96

Glucocorticoids regulate the human occludin gene through a single imperfect palindromic glucocorticoid response element

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The 65 kDa protein occludin is an essential element of the blood-brain barrier. This integral membrane protein represents an important part of the tight junctions, which seal and protect the blood brain barrier against paracellular diffusion of solutes to the brain parenchyme and are therefore responsible for the high resistance and low permeability between cerebral capillary endothelial cells. However, the molecular basis for the regulation of occludin gene expression is only incompletely understood. In former projects we showed that treatment of a brain microvascular cell line, cEND, with glucocorticoids resulted in increased occludin expression in cell-cell-contacts. Induction of occludin expression by glucocorticoids was shown to be dependent on the glucocorticoid receptor. This study aims to identify the underlying molecular mechanism of gene expression and to identify potential glucocorticoid receptor binding sites within the occludin promoter, the glucocorticoid response elements (GRE). We identified one candidate GRE within the distal part of the occludin promoter that differs from the consensus GRE by the presence of a 4-basepair instead of a 3-basepair spacer between two highly degenerate halfsites (5'-ACATGTGTTTACAAAT-3'). Chromatin immunoprecipitation assay and site-directed mutagenesis confirmed binding of the glucocorticoid receptor to this site. We synthesized plasmid containing four copies of this GRE in the vector pGL-3 basic. After stimulation of the cells with hydrocortisone we could observe up to 8-fold increased activity in luciferase reporter assay. The need for glucocorticoid receptor dimerization to induce gene expression was further confirmed by transfection studies using wild type and glucocorticoid receptor dimerization-deficient expression vectors, indicating that transactivation of occludin occur through the GRE.

A97

Chemokine activity induced by interleukin-6

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Macrophages contribute to the innate immune response by eliminating bacteria, viral particles and apoptotic bodies. They develop from circulating monocytes. In case of an infection monocytes attach to the endothelial cells of the blood vessels, migrate along the endothelial cells, leave the circulatory system

to enter the inflammatory tissue and differentiate into macrophages. Cell migration is frequently induced by chemokines which act through G-protein coupled receptors. Only a few cytokines, signaling through single transmembrane domain receptors have been shown to induce cell migration. Often, this potential depends on the induction of classical chemokines and is not a direct cellular effect. Here we discovered interleukin-6 as a potent stimulant for monocytic cell migration. Furthermore, we present data on interleukin-6-induced integrin activation, cell attachment, actin polymerization, fibronectin-dependent migration, and trans-migration through a layer of endothelial cells. Our results show that IL-6 fulfils all biological properties to mediate cell migration of monocytic cells, which may contribute to the pro-inflammatory potential of IL-6.

A98

TCDD deregulates contact inhibition in rat liver oval cells via Ah receptor, JunD and cyclin A

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The aryl hydrocarbon receptor (AhR) is a transcription factor involved in physiological processes, but also mediates most, if not all, toxic responses to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Activation of the AhR by TCDD leads to its dimerization with ARNT and transcriptional activation of several phase I and II metabolising enzymes. However, this classical signalling pathway so far failed to explain the pleiotropic hazardous effects of TCDD such as developmental toxicity and tumour promotion. Thus, there is an urgent need to define genetic programmes orchestrated by AhR to unravel its role in physiology and toxicology. Treatment of rat liver oval cells with TCDD leads to a release from contact-inhibition. Loss of contact-inhibition is one characteristic hallmark in tumourigenesis. We have recently shown that TCDD-exposure leads to an elevation of JunD protein levels and to transcriptional activation of Cyclin A in an AhR-dependent, and probably ARNT-independent way. Ectopic expression of Cyclin A in confluent cultures overcomes G1-arrest indicating that increased Cyclin A levels are indeed sufficient to bypass contact-inhibition. Elevation of JunD precedes that of Cyclin A suggesting a role of JunD in Cyclin A induction. Indeed, down-regulating JunD by siRNA blocks TCDD-induced expression of Cyclin A. DNA affinity purification assays and reporter gene analysis indicate that JunD binds to an ATF/CRE consensus sequence in the rat Cyclin A promoter. Using in vitro DNA affinity purification assays, we also revealed binding of ATF2, but not Fra- or Fos-proteins, to the ATF/CRE consensus sequence. Down-regulating ATF2 by siRNA blocks TCDD-dependent Cyclin A induction indicating that ATF2 is the interaction partner of JunD mediating Cyclin A expression. In summary, we have discovered in rat liver oval cells a novel AhR-dependent and probably ARNT-independent signalling pathway involving JunD/ATF2 and Cyclin A, which mediates deregulation of contact-inhibition by TCDD.

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A99

Proteinase-activated receptors 1 and 2 activate protein kinase D1 in human melanoma cells

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Proteinase-activated receptors (PARs) are G-protein coupled receptors with seven transmembrane domains that are stimulated by a unique activation mechanism. The extracellular N-terminus is specifically cleaved by certain serine proteinases, which exposes a formerly latent peptide sequence. This “tethered ligand” is able to bind to the second extracellular loop of the receptor, leading to the transduction of signal events. Four PARs have been cloned so far. PAR₁, PAR₃ and PAR₄ are stimulated by thrombin, whereas PAR₂ can be activated by trypsin. *In vitro*, PARs can also be stimulated by synthetic peptides, which mimic the tethered ligand sequence. PAR₁, but not PAR₂, is expressed by primary melanocytes. It is well known that PAR₁ is overexpressed in malignant melanomas, enhancing migration and metastasis. PAR₂ also seems to play a role in melanoma progression and metastasis. The underlying mechanisms, however, still remain elusive. Stimulation of both receptors on the the human melanoma cell line WM9 led to rearrangement of integrin $\alpha v \beta 3$ and phosphorylation of protein kinase D1 (PKD1). PKD1 is known to control persistent cell migration by regulating the intracellular integrin $\alpha v \beta 3$ recycling pathway. Stable knockdown of PKD1 in WM9 cells led to an inhibition of cell proliferation, changes in cell shape and a downregulation of integrin $\alpha v \beta 3$ on the cell surface. Moreover, cell migration was impaired. Taken together, we could show for the first time that both PAR₁ and PAR₂ are involved in melanoma cell migration and proliferation via activation of PKD1.

A100

Analysis of matrix-dependent cell migration by a barrier migration assay

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Background: Cell migration plays a pivotal role in many biological processes. During embryogenesis, the orchestrated movement of cells is required for organ development. Wound healing depends on the migration of fibroblasts and endothelial

cells. Contrariwise, misregulation of migratory processes may lead to serious consequences including metastasis and mental retardation.

A variety of cell migration assays are applied to investigate the molecular mechanisms that contribute to migration. An important objective of cell migration assays is to approach the situation in the body in order to get physiologically meaningful information. In vivo, cells are embedded in an extracellular matrix (ECM). Matrix proteins are linked to the cellular cytoskeleton via integrins, which convert information about the substrate to the inside of the cell thereby providing essential signals for cell migration.

Methods and results: The wound healing or scratch assay classically investigates migration of adherent cells. Thereby, scratching a confluent cell layer with a pipet tip creates a cell-free gap. The subsequent migration of cells into the newly created gap is analyzed. However, the scratch leads to cell injury, which may affect cell migration. And, most importantly, protein coating is scratched off implicating that cells migrate into the gap on an unphysiological surface such as glass or plastic.

To overcome these inherent problems of the scratch assay, we developed a barrier cell migration assay. After substrate coating with a matrix protein, a rigid barrier is carefully placed in the dish. Cells are seeded around the barrier to form a monolayer. Removal of the barrier generates a cell-free gap, and the subsequent cell migration into the gap is analyzed. In contrast to the scratch assay, surface coating is preserved in the barrier assay. To show the effect of ECM proteins on cell migration we analyzed the migration of human tubular epithelial cells (HKC-8) on coverslips, which were either uncoated or coated with collagen IV and fibronectin. Collagen IV constitutes the main ECM protein of the renal basal membrane, whereas fibronectin accumulates during tubulointerstitial fibrosis. Coating with fibronectin increased migration velocity in comparison to uncoated glass coverslips, whereas collagen IV exerted an inhibitory effect. To exemplify the role of cytokines we analyzed migration of HKC-8 cells on different substrates upon treatment with transforming growth factor beta (TGF-beta). TGF-beta enhanced cell migration on uncoated glass and on collagen IV. However, there was no increase in cell motility on fibronectin-coated coverslips.

Conclusion: The novel barrier cell migration assay offers the possibility to analyze cell migration on defined ECM proteins, and thus represents a simple device to investigate the role of cell-matrix interactions in migration of adherent cells under more physiological conditions.

A101

Endocytosis, cell signalling, adhesion and motility

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Endocytosis and exocytosis play a key role in how cells respond to their environment. Endocytosis directly (and indirectly) affects responses to guidance cues and growth factors, the uptake of nutrients and even pathogens; it modulates cell signalling in response to receptor activation and is essential for cell migration. In this talk I will consider the endocytic repertoire of a cell and

show how distinct mechanisms may differently affect function. I will describe the characterisation of new endocytic mechanisms and show how a molecular understanding can lead to a better view of function. I will round up with a simple view of how exocytosis can be regulated by calcium mediated signalling.

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A102

Increased migration of colorectal cancer cells induced by TNF-alpha-treated stromal fibroblasts from human liver metastases

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Background: Inflammation plays key roles in invasion, angiogenesis and metastasis. Given the multifaceted roles of tumor-necrosis-factor-alpha (TNF-alpha) in these processes, its effects on the stromal fibroblasts that constitute the desmoplastic stroma in colorectal metastases are of interest.

Methods: Primary cultures of cancer-associated stromal fibroblasts (CAFs) were generated from human tissues harvested during hepatic resection. TNF-alpha expression in tissue was examined by immunohistochemistry. Activation of nuclear-factor-kappaB (NF-kappaB) activity was measured by gel mobility shift assay. The effect of TNF-alpha on migratory capacity and gene expression of CAFs was tested in presence/absence of parthenolide, an herbal inhibitor of NF-kappaB. Gene expression in tissues and cell cultures was examined by Northern blot analysis. Protein measurements in the cell culture supernatant were performed with cytometric capture beads.

Results: The colorectal metastases display immunoreactivity for TNF-alpha in tumor cells and leukocyte cells, whereas stromal fibroblasts are negative. To investigate transcriptional effects of TNF-alpha on CAFs, we analysed the expression of potential inflammatory target genes that are involved in tumor progression. CAFs that were exposed for 24 hours to TNF-alpha (10 ng/ml) showed a dramatic increased expression of interleukin-6 (IL-6), monocyte-chemotactic protein-1 (MCP-1) and intercellular cell adhesion molecule-1 (ICAM-1). Increasing

concentrations of parthenolide (1, 5, 10 microM) dose-dependently inhibited the activation of NF-kappaB by TNF-alpha exposure for 30 min, as well as the TNF-alpha effect on IL-6 and MCP-1 mRNA and protein expression. Exposure of CAFs with TNF-alpha significantly increased the chemotaxis of HT29 colon carcinoma cells towards these cells in a coculture migration chamber system. This migratory effect activated by paracrine TNF-alpha was inhibited by co-incubation with parthenolide.

Conclusion: CAFs are an important target for inflammatory signaling mediated by TNF-alpha/NF-kappaB in the context of tumor-stroma interaction and may play an important role in metastasis progression. Our results suggest that the inhibition of NF-kappaB activation may be an interesting strategy in antitumor therapy targeting hepatic colorectal carcinoma metastasis.

A103

Crosslinking of microtubules and actin filaments by S100A8/S100A9

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Microtubules (MT) and actin filaments of phagocytes exhibit highly dynamic properties. The underlying molecular mechanisms are not entirely known. Their exceptional dynamic cytoskeleton is the prerequisite of many phagocyte related functions like exocytosis, phagocytosis, and in particular migration. Cell dynamic/migration is a highly complex process strictly regulated by numerous signaling cascades often initiated by changes in cytosolic calcium concentrations. Therefore, calcium-binding proteins are key elements in signal transduction. The major calcium-binding molecules expressed in granulocytes and monocytes are myeloid-related protein 8 (MRP8 [S100A8]) and MRP14 (S100A9), two members of the S100 protein family. MRP8 and MRP14 form heterodimers in the absence and tetrameric complexes composed of two heterodimers in the presence of calcium.

We demonstrate that MRP8/MRP14 tetramers promote MT polymerization as well as MT bundling; moreover, the complex crosslinks MTs to F-Actin in a strictly calcium dependent manner. HEK293 cells transfected with MRP8/MRP14 contain significantly more polymerized tubulin in comparison to mock-transfected cells. These results are in line with reduced level of polymerized tubulin in phagocytes isolated from MRP14 $-/-$ mice. Furthermore, phagocytes of MRP14 knockout mice show altered migration rates compared to wildtype cells. In addition to calcium-induced activation the MRP8/MRP14 complex can be specifically phosphorylated by p38 mitogen-activated protein kinase (MAPK), which abrogates MT/F-Actin crosslinking. Thus MRP8/MRP14 integrates signals of at least two independent signaling pathways. Our results provide evidence, that the MRP8/MRP14 complex fulfils a pivotal role in remodeling cytoskeletal structures necessary for migration of leukocytes.

Methods and miscellaneous

A104

Drosophila GoLoco-protein Pins as a target of G α -mediated G protein coupled receptor signaling

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Heterotrimeric G-proteins are molecular switches that regulate numerous aspects of cellular physiology by transducing the signals from G protein-coupled receptors (GPCRs). In the basal state, the G α -subunits of the heterotrimeric G proteins are GDP-liganded (the inactive form) and bind to the $\beta\gamma$ -complex. GPCRs can activate guanine nucleotide exchange on the G α -subunits to produce the active, GTP-bound state. GoLoco domains present in many proteins play important roles in multiple heterotrimeric G protein-dependent activities, physically binding the G α -subunits of the G $\alpha i/o$ class. In most cases GoLoco binds exclusively to the GDP-loaded form of the G α -subunits. Our biochemical and genetic experiments as well as structural modeling show that the poly-GoLoco protein Pins binds to both the GDP- and GTP-forms of *Drosophila* G αo . We identify the Pins GoLoco domain I as necessary and sufficient for the unusual interaction with G αo -GTP. We further pinpoint the central Lysine residue present in this domain as responsible for the interaction. Molecular modeling suggests that the side chain of this Lysine points directly into the guanine nucleotide-binding pocket of G αo , stabilizing the extra negative charges of the γ -phosphate group of GTP. Such a positively charged amino acid is unique in the *Drosophila* GoLoco proteome, but is conserved in several GoLoco domains of other organisms. We conclude that Pins, through its GoLoco domain I, is a target for G αo -mediated GPCR signaling.

A105

Identification of composite promoter modules in inflammation-regulated genes

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In the context of the collaborative research centre SFB566 entitled "cytokine receptors and cytokine-dependent signalling pathways as therapeutic target structures" we have performed more than 1100 DNA microarray experiments under highly standardized experimental conditions with respect to RNA isolation, cRNA synthesis, cRNA labelling, cRNA hybridization and raw data acquisition. The expression of 90 inflammatory genes including 6 housekeeping genes were assessed in a large variety of human cell systems and stored in our microarray database CytoBASE <https://microarray.med.uni-giessen.de/base/index.phtml>. A set of 102 experiments comprising 18 human cell types, 59 experimental groups and treatment with 36 different

stimuli/inhibitors in 47 different combinations was analyzed by hierarchical clustering using MultiExperiment Viewer (MEV, version 4.1, <http://www.tm4.org>) and Significance Analysis of Microarrays (SAM, <http://www-stat.Stanford.EDU/~tibs/SAM>). As a result 20 consistently coregulated inflammatory genes with different biological functions such as cytokines and chemokines (IL-6, CCL2, CXCL3, CXCL8, CXCL10), proteases (MMP-1, MMP-15), metabolic enzymes (COX-2, MnSOD) and intracellular signalling molecules (BIRC2, Ikbalpha, IRF1, JUNB) were identified. By genome-wide microarray analysis we also compiled a control set of 48 genes that were not regulated under eight different inflammatory conditions. We searched 1.1 to 2.1 kb of the promoter regions of these genes for enriched transcription factor binding sites using F-Match <http://www.biobase.de>. We further identified combinations of binding sites of enriched transcription factors that correlate with coregulation of inflammatory but not of control genes by using the composite module analysis tool <http://www.biobase.de>. Several promoter modules containing 3 to 10 transcription factor binding sites within a promoter region of 200 bp were identified that distinguished the inducible 20 inflammatory from the 48 control genes. This information can be used in future experiments designed to reveal novel mechanisms of coregulation of different functional classes of inflammatory genes at the level of DNA.

A106 **Role of extracellular signal-related kinase (Erk) I in the regulation of neuroinflammation**

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We have previously shown that the 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor (HMGCR) atorvastatin is therapeutic in experimental autoimmune encephalomyelitis (EAE) while also inducing a sustained phosphorylation of the MAPK Erk1 that is important for inducing T cell energy. However it is also known, that HMGCR also influence the antigen presenting cell compartment including dendritic cells (DC). This led us to investigate the role of Erk1 in DC biology in more detail. Indeed bone-marrow derived dendritic cells from Erk1 deficient mice had an increased migratory capacity when compared with DC isolated from wildtype littermate mice. As a likely consequence to cytoskeletal regulation, Erk1^{-/-} DC had an increased surface expression of costimulatory molecules and were more potent to prime T cells in vivo. To investigate the implications of these findings in an inflammatory scenario, we induced EAE (experimental autoimmune encephalomyelitis) in Erk1^{-/-} and Erk1^{+/+} mice with myelin oligodendrocyte glycoprotein peptide 35–55 (MOG_{35–55}) and could show that a deficiency of this MAPK results in a moderate increase in disease severity. To differentiate the role of Erk1 between peripheral immune system and the brain compartment we induced EAE in Erk1^{+/+} mice harboring Erk1^{-/-} immune cells by applying bone marrow chimeras (Erk1^{-/-} → Erk1^{+/+}). We report that Erk1 has an important regulatory function in the immune system as shown by

pronounced disease severity in Erk1^{-/-} → Erk1^{+/+} bone marrow chimeras. All together these results indicate the significance of Erk1 in regulating DC functions that are relevant for T cell priming and neuroinflammation, and thus signal the importance of therapeutically targeting this MAPK for the treatment of autoimmune diseases.

A107 **Induction of CCL13 expression in synovial fibroblasts underlines a significant role of oncostatin M in rheumatoid arthritis**

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Rheumatoid arthritis (RA) is a severe, chronic disease characterized by a profound inflammatory response that leads to both joint destruction as well as extraarticular symptoms with a significant impact on both morbidity and mortality. The cause and pathologic process underlying RA have not yet been fully elucidated. However, it is clear that both the cellular immune system and the cytokine network are subject to profound dysregulations. The success of an anti-proinflammatory cytokine therapy directed against either tumor necrosis factor alpha (TNF α) or, to a lesser extent, interleukin-1 (IL-1), has highlighted the significance of these cytokines in the pathological progression of RA. Nevertheless, these therapeutic strategies are not beneficial to all patients, suggesting that other cytokines like IL-6, oncostatin M, IL-4, IL-13, IFN γ are also present in the synovial fluid of chronically inflamed joints. Here we demonstrate that among a number of cytokines present in the synovial fluid of RA patients, only OSM induces a prolonged expression of the chemokine CCL13. The expression of CCL13 appears to be tissue-specific since neither in dermal nor lung or cervix fibroblasts CCL13 expression could be observed. We identify human synovial fibroblasts (HSF) as well as synovial fibroblasts of RA patients (RASFs) as producers of CCL13. The expression of CCL13 is mediated through STAT5 activation and p38 MAPK-dependent pathways and results in increased migration of monocytic cells.

A108 **Modulatory function of PI3-kinase γ in nociceptive neurons**

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Phosphoinositid 3-Kinase γ (PI3K γ), the only known member of class Ib PI3K's, is activated by G-Protein coupled receptors (GPCRs), producing the second messenger phosphatidylinositol-3,4,5-trisphosphate (PIP3). In addition, PI3K γ possesses an intrinsic protein kinase activity and might express a scaffold function in the signaling network of different cells. PI3K γ is expressed in hematopoietic cells acting as a main mediator of proliferation, differentiation, migration and survival of leukocytes. Recent studies expand these well-established cell specific functions of PI3K γ to other cell types like cardiomyocytes. In heart PI3K γ exhibits a negative regulatory function on the contractility by controlling the intracellular cAMP-level in concert with Phosphodiesterase 3B (PDE3B).

In the present study we describe for the first time PI3K γ expression and functional pattern in cell types of neuronal origin. PI3K γ -protein is expressed in small diameter neurons of dorsal root ganglia (DRG), which form widely unmyelinated C-fibers into the body's periphery. These primary sensory neurons exhibit nociceptive functions and can lead to a slow, dull and long lasting pain perception. These neurons are heavily involved in the processes of neuropathic and inflammatory pain accompanied by molecular modifications in the interplay of extra- and intracellular milieus – the playground of PI3K γ . Now we take advantage of the PI3K γ -knockout mice to evaluate the impact of PI3K γ in behavioral studies and primary dorsal root ganglia cultures. Analysis of nociceptive behaviors and studies on DRG-cultures suggest an involvement of the signaling protein in the μ -opioid-receptor path. An important regulation of the opioid-receptor system is the sensitization and desensitization of the receptors. Effects of different μ -receptor-agonists point to a differential involvement of PI3K γ in antinociceptive signaling in primary sensory neurons.