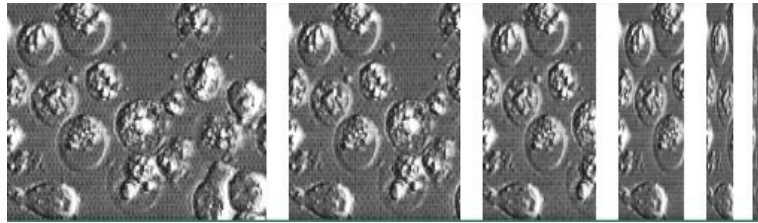


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Targeting Class I_A Phosphoinositide 3-Kinase Isoforms in Glioblastoma

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Background Glioblastoma (GBM) is characterized by poor patient survival rates, resistance to radiotherapy and chemotherapy, as well as a highly invasive phenotype. It represents the most common type of tumor of the central nervous system. Phosphoinositide 3-kinases (PI3K) are a class of lipid kinases, which play an important role in intracellular signaling events and cell survival. Furthermore, the pro-survival PI3K/Akt signaling pathway is often deregulated in solid tumors, including GBM. The most prominent deregulations include mutations and/or deletions of the tumor suppressor gene phosphatase and tensin homologue deleted on chromosome ten (*PTEN*) and activating mutations in the oncogene *PIK3CA* (encoding class I_A PI3K p110 α). This study further investigates the role of class I_A PI3K isoforms (p110 α , p110 β , and p110 δ) in respect to signaling pathway activation, cell proliferation, and resistance to chemotherapeutic agents in human GBM cell lines, as well as in *ex vivo* cultures.

Material and Methods Individual PI3K isoforms were targeted by either isoform-specific pharmacological inhibitors or RNA interference. Furthermore, treatments combining chemotherapeutic agents, such as cisplatin, doxorubicin, and temozolomide, with isoform-specific inhibitors were performed. Cell proliferation (MTS assay), colony formation (soft agar assay), and induction of apoptosis (caspase activity by Caspase Glo 3/7 assay; PARP cleavage, caspase 3 and ICAD expression by Western blot) were analyzed after treatment. Activation of the PI3K/Akt signaling pathway was investigated by using phosphorylation site-specific antibodies of downstream elements (Western blot).

Results Two different p110 α -specific inhibitors led to a concentration dependent decrease in GBM cell proliferation and impaired anchorage-independent growth. Additionally, down-regulation of isoforms p110 α or p110 β using RNA interference induced apoptosis in GBM cells. In agreement with these observations, treatment of GBM cells with p110 α -specific inhibitors led to decreased activation of Akt and decreased levels of phosphorylated ribosomal protein S6.

Conclusions Even though targeting individual class I_A PI3K isoforms has an impact on cellular responses, it might be necessary to simultaneously target more than one isoform. This study aims at providing a better understanding of the specific functions of class I_A PI3K isoforms in human GBM cell biology and will thus help in developing new targeted therapies to cure this common malignant brain tumor.

Role of TRAIL and the pro-apoptotic Bcl-2 homolog Bim in acetaminophen-induced liver damage

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Abstract

Acetaminophen (APAP, paracetamol) is a commonly used analgesic and antipyretic agent. Although considered safe at therapeutic doses accidental or intentional overdose frequently causes acute liver failure characterized by centrilobular hepatic necrosis with high morbidity and mortality. While many molecular aspects of paracetamol-induced cell death have been described no conclusive mechanism has been proposed. We recently identified TRAIL and c-Jun kinase-dependent activation of the pro-apoptotic Bcl-2 homolog Bim as an important apoptosis amplification pathway in hepatocytes. In this study we thus investigated the role of TRAIL, c-Jun kinase and Bim in APAP-induced liver damage. Our results demonstrate that TRAIL strongly synergizes with APAP in inducing cell death in hepatocyte-like cells lines and primary hepatocyte. Furthermore, we found that APAP strongly induces the expression of Bim in a c-Jun kinase-dependent manner. Consequently, TRAIL- or Bim-deficient mice, or mice treated with c-Jun kinase inhibitor were substantially protected from APAP-induced liver damage. This study identifies the TRAIL-Jun kinase-Bim axis as a novel target in the treatment of APAP-induced liver damage and substantiates its general role in hepatocyte death.

Serum Biomarkers of Cell Death for Monitoring Therapy Response of Gastrointestinal Carcinomas

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During epithelial tumour cell apoptosis, cytokeratin (CK)-18 is cleaved by caspases into specific fragments that are released into circulating blood and can be detected by a specific ELISA (M30). The combination of the M30-ELISA with an ELISA (M65) that detects both caspase-cleaved and uncleaved CK-18 allows the discrimination between apoptotic and non-apoptotic (necrotic) cell death.

In this study we have investigated the use of CK-18 fragments and total CK-18 as potential biomarkers for the treatment response in 35 patients with gastrointestinal (GI) cancers.

While both cleaved and total CK-18 levels were intrinsically elevated in tumour patients, they were further increased during 5-fluorouracil-based therapy. Importantly, the increased levels of CK-18 could discriminate between patients with different clinical response. Cancer patients with a partial response or stable disease revealed a significantly higher increase of cleaved CK-18 during chemotherapy as compared to patients with progressive disease. Moreover, patients responding to chemotherapy showed a significantly lower M65/M30 ratio compared to non-responding patients, indicating that in responding patients apoptotic cell death predominates whereas patients with progressive disease mainly showed non-apoptotic cell death. Our results suggest that detection of circulating caspase-cleaved CK-18 might be a useful serum biomarker for monitoring treatment response in GI cancer patients.

NO. 04

Defects in endoplasmic reticulum stress and interferon signaling in Newcastle disease virus resistant PC12 cells

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Newcastle disease virus (NDV), an avian member of the Paramyxoviridae family was previously shown to have oncolytic effects in vivo and in cultured tumor cells. An attenuated NDV strain, MTH-68/H, was found to cause apoptosis in mammalian tumor cells, with a wide range of cytotoxicity. The reason for differences in NDV susceptibility of tumor cell lines is unknown, since the mechanism of oncolysis is poorly understood. In order to establish a suitable model system for the identification of NDV-susceptibility markers in tumor cells, NDV-resistant PC12 clones were isolated, and the effect of MTH-68/H treatment on wild-type and NDV-resistant PC12 cells was compared. While MTH-68/H induced apoptosis in wtPC12 cells, this response was completely abolished in the NDV-resistant cell lines. To identify key signaling proteins involved in NDV-sensitivity, two major signaling pathways were analyzed in this study: (1) the endoplasmic reticulum stress pathway (its stimulation by MTH-68/H infection leads to cellular stress and apoptosis); (2) the interferon pathway (that protects cells from NDV-induced cytotoxicity). Results of the present study indicate that mutational alterations in these pathways affect PC12 susceptibility to MTH-68/H infection.

This work is supported by Science, Please! Research Team on Innovation (SROP-4.2.2/08/1/2008-0011) and GVOP-362.1-2004-04-0172/3.0.

SerpinB1 is critical for neutrophil survival and the maintenance of the bone marrow reserve of mature neutrophils *in vivo*

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Neutrophils are short-lived leukocytes with a crucial role in host defense. SerpinB1 is one of the best inhibitors of the three neutrophil serine proteases: elastase, cathepsin G and proteinase-3. Here, we report that the bone marrow reserve of mature neutrophils is considerably reduced in *serpinB1*^{-/-} mice. Neutrophil mobilization from the bone marrow during acute lung injury caused the bone marrow reserve pool to be completely exhausted in *serpinB1*^{-/-} mice. Numbers of myeloid progenitors were normal in *serpinB1*^{-/-} bone marrow, coincident with the absence of target protease expression at these developmental stages. Upon overnight culture, apoptosis and necrosis were greater in purified bone marrow neutrophils from *serpinB1*^{-/-} compared to wild-type mice. The profound neutropenia of *serpinB1*^{-/-} mice was reproduced in lethally irradiated wild type mice transferred with *serpinB1*^{-/-} bone marrow cells. Furthermore, the defect of *serpinB1*^{-/-} mice was rescued by bone marrow transfer of wild type cells. Collectively, these findings indicate that the neutropenia of *serpinB1*^{-/-} mice is due to a defect in the hematopoietic compartment and is confined to mature neutrophils. Analysis of the bone marrow of mice deficient in both *serpinB1* and target proteases showed a rescued phenotype, suggesting that serpinB1 sustains neutrophil survival by regulating neutrophil serine proteases.

NO. 06

Poly(ADP-ribose)glycohydrolase is an upstream regulator of Ca²⁺ fluxes in oxidative cell death

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Oxidative DNA damage to cells activates poly(ADP-ribose)polymerase-1 (PARP-1) and the poly(ADP-ribose) formed is rapidly degraded to ADP-ribose by poly(ADP-ribose)glycohydrolase (PARG). Here we show that PARP-1 and PARG control extracellular Ca²⁺ fluxes through melastatin-like transient receptor potential 2 channels (TRPM2) in a cell death signaling pathway. TRPM2 activation accounts for essentially the entire Ca²⁺ influx into the cytosol, activating caspases and causing the translocation of apoptosis inducing factor (AIF) from the inner mitochondrial membrane to the nucleus followed by cell death. Abrogation of PARP-1 or PARG function disrupts these signals and reduces cell death. ADP-ribose-loading of cells induces Ca²⁺ fluxes in the absence of oxidative damage, suggesting that ADP-ribose is the key metabolite of the PARP-1/PARG system regulating TRPM2. We conclude that PARP-1/PARG control a cell death signal pathway that operates between five different cell compartments and communicates via three types of chemical messengers: a nucleotide, a cation and proteins.

MG-2477, a new tubulin inhibitor with potent antitumor activity *in vitro* and *in vivo* induce autophagy and delayed apoptosis in A549 cells

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We have previously demonstrated that MG-2477 (3-Cyclopropylmethyl-7-phenyl-3*H*-pyrrolo[3,2-*f*]quinolin-9(6*H*)-one) remarkably inhibited the growth of several cancer cells *in vitro*. Here we identified MG-2477 as an antimitotic agent since it inhibited tubulin polymerisation bound to colchicines site, induce arrest of cells in metaphase and disrupted the tubulin network as assessed by immunofluorescence staining of A549 cells. Treatment of A549 cells with MG-2477 indicate that the drug induce an arrest of the cell cycle in G2/M phase, confirmed also by accumulation of cyclin B. Western blot analysis also reveal increased levels of p53 and p21 protein in response to treatment with MG-2477. As other microtubule interacting agents the compounds induces phosphorylation of Bcl-2 and Bcl-xL proteins.

Interestingly the drug induces autophagy followed at later times of incubation by apoptotic cell death. Autophagy was early detected (12 h) by the conversion of LC3-I into its cleaved and lipidated form (LC3-II). This effect was also confirmed by appearance of large acidic vacuoles that were detected by flow cytometry after acridine orange staining. At longer times of exposure (48 h) phosphatidylserine externalization on the outer membrane along with caspase-3 and PARP activation take place revealing that apoptotic cell death occur. Notably pharmacological inhibition of autophagy with 3-Methyladenine, Bafilomycin A1 increase apoptotic cell death suggesting that the induced autophagy by MG-2477 plays a protective role that delay apoptosis. Treatment of A549 cells with MG-2477 resulted, just after 12 h of incubation, in a marked decreased of the phosphorylation of the mTOR targets S6 protein and 4EB-P1. Interestingly MG-2477 also induce dephosphorylation of AKT at Ser473 but at Thr308 suggesting that autophagy is controlled by the Akt-mTOR pathway.

Altogether our results show that death signals activated by the novel antimitotic agent MG-2477 are dependent on mTOR and this effect could be attributed to damaged microtubules. In addition these findings may have implications for cancer therapy and provide new clues for anticancer drug design and development.

NO. 08

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The Bcl-2 family plays a major role in the regulation of the cellular apoptotic response. Bcl2L12 (Bcl2-Like 12) is a novel member of the Bcl-2 family which, like BFK and Bcl-G, contains the conserved BH3 and BH2 domains. Its protein sequence is evolutionary conserved but an additional longer form was described in humans previously. However, the role of Bcl2L12 in cellular events including apoptosis or cell cycle remains poorly understood.

Here we characterize Bcl2L12 as an approximately 34 kDa protein which plays a pivotal role in the response of HeLa cells to Taxol. We investigated its role for taxol-induced cell death generating cells stably expressing shRNA to knock down Bcl2L12. Bcl2L12 KD cells were highly protected against Taxol induced cell death. Interestingly, the reduction of Bcl2L12 protects from caspase dependent and independent cell death induced by Taxol. Furthermore, cell cycle analysis revealed that BCL2L12 knock down cells fail to arrest in M-Phase after Taxol treatment indicating a role of Bcl2L12 in cell cycle progression. We observed a phosphorylation of Bcl2L12 in M-phase of the cell cycle in several cell lines and identified Bcl2L12 as a substrate of the cell cycle dependent kinase CDK1 *in vitro*.

Non-steroidal anti-inflammatory drugs (NSAIDs) decrease cell viability and induce apoptosis in cutaneous T cell lymphoma (CTCL) cell lines

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Cutaneous T cell lymphomas (CTCL) form a heterogeneous group of non-Hodgkin lymphomas with primary involvement of the skin. Its most frequent forms are Mycosis fungoides (MF) and the leukemic counterpart Sézary syndrome (SzS). Even though early stages of CTCL are often indolent over long periods of time, advanced stages are refractory and difficult to treat. Death ligands (CD95L and TRAIL) critically contribute to lymphocyte homeostasis due to induction of apoptosis and may further represent safeguard mechanisms to prevent lymphoma development. In previous studies, we characterized CTCL cell lines as resistant to TRAIL-mediated apoptosis which was correlated to high c-FLIP expression. In the present study, we investigated the effects of non-steroidal anti-inflammatory drugs (NSAIDs) as acetylsalicylic acid, sodium salicylate and diclofenac in CTCL cell lines (HH and MyLa) as well as in tumor T cells from SzS patients. NSAIDs decreased cell viability and induced apoptosis, associated by caspase-3 processing. Decreased mitochondrial membrane potential and cytochrome c release were indicative for an involvement of intrinsic pathways. Downregulation of c-FLIP and caspase-8 processing clearly indicated an activation of extrinsic pathways. Finally, NSAIDs sensitized CTCL cells for TRAIL-induced apoptosis.

In conclusion, the study provides a rationale for the use of NSAIDs as a potentially new therapeutic option for cutaneous T cell lymphomas.

Identification of Puma as a potential mediator of SFV-induced apoptosis

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SFV (Semliki Forest Virus) is an alphavirus triggering Bak-mediated apoptosis in mammalian cells, but the exact mechanism of apoptosis induction has not yet been revealed. Here we demonstrate a potential role of the BH3-only protein Puma (p53-upregulated modulator of apoptosis) to mediate SFV-induced cell death.

SFV-induced apoptosis of 3T9-immortalized Mouse Embryonic Fibroblasts (3T9-MEFs) lacking Puma in response to SFV was delayed in comparison to wildtype cells, deletion of several other BH3-only proteins did not impair cell death significantly. Puma-deficient MEFs also showed decreased activation of both caspase-3 and caspase-8 as well as reduced sensitivity to poly I:C induced cell death. On the other hand antiviral signaling, as measured by interferon- β (IFN- β) production, remained intact. Consistent with these observations infection of wildtype MEFs resulted in the upregulation of Puma at two hours post-infection.

Our results strongly suggest an important role of Puma during SFV-infection in 3T9-MEFs. The ability of Puma to bind all known Bcl-2 pro-survival proteins and its importance to mediate various apoptotic stresses including DNA damage or cytokine deprivation make its implication in our system likely. How exactly Puma expression is enhanced by the virus and whether other proteins are required in the process needs to be further investigated.

NO. 11

Autophagy, apoptosis and necrosis in oxidative stressed neuroblastoma cells

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Hydrogen peroxide (H_2O_2) is an extremely reactive oxidoradical that, when in excess, determines an oxidative stress in the cell. Autophagy, a lysosomal degradation pathway, is triggered by oxidative stress as a defensive response. Yet, hyperactivation of autophagy may lead to cell death. In human neuroblastoma SH-SY5Y cells 200 μM H_2O_2 rapidly induced the formation of LC3-positive autophagic vacuoles and of beclin1-Vps34 double-positive macro-aggregates. The transgenic expression of a dominant negative Vps34 or the post-transcriptional knock-down of beclin1 abolished the induction of autophagy. H_2O_2 provoked cell death associated with permeabilization of lysosomes and of mitochondria. Caspase inhibition by ZVAD-fmk did not trigger an alternative cell death pathway, but rather afforded complete protection from oxidative toxicity, despite the cellular accumulation of autophagic vacuoles. On long term incubation with H_2O_2 , necrotic cell death became apparent in LC3-positive cells. These data highlight a hierarchy of cellular responses to H_2O_2 in which autophagy precedes apoptosis and necrosis.

Ambra1 is a haploinsufficient tumor suppressor gene regulating autophagy and cell proliferation

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Macroautophagy is a lysosomal pathway used by eukaryotes for degrading and recycling various cellular constituents. Autophagy occurs during nutrient deprivation to replenish depleted energy store, but is essential for the maintaining of cellular homeostasis in basal conditions, too.

Furthermore, defects in this degradative process lead to a wide range of disorders, including cancer. In this context, autophagy has been identified as a crucial process in both oncogenesis and tumor progression.

Similarly to what observed for *Beclin1*, here we identify a new haplo-insufficient tumor suppressor gene that is a positive regulator of autophagy: *Ambra1* (Activating molecule in Beclin 1-regulated autophagy).

In particular, *Ambra1*^{+gt} mice are more prone than wt mice to develop a malignancy, showing an approx. 50% increase of spontaneous tumorigenesis in several organs.

We have previously demonstrated that *Ambra1* deficiency during embryogenesis both *in vivo* and *in vitro* induces an increase in cell proliferation. Therefore, we are also investigating whether the observed tumors are related to a direct impairment of cell growth control by autophagy. In this context, we observed an increase in the amount of a number of positive cell cycle regulators.

In principle, the demonstration of a haploinsufficient tumor suppressor phenotype for *Ambra1* reduced function in mice may have direct implications for analysing the molecular pathogenesis of human cancer.

Cathepsin D directly activates caspase-8 by multiple intra-chain proteolysis and stabilization of its active form

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Although it has already been suggested that cathepsins may act in concert with caspases,

our finding that, in neutrophils, cathepsin D acts proximal to caspases and activates directly the initiator caspase-8 was unexpected (1). Most current data supports the hypothesis that caspase-8 is activated by dimerization, not proteolysis – reviewed in (2, 3), although some authors dispute this – reviewed in (4). Interchain proteolysis, but not enforced dimerization of caspase-8 by Fas-associated protein with death domain (FADD), was proposed for its activation by granzyme B or caspase-6 (5, 6). Therefore, proteolytically processing of caspase-8 by cathepsin D might influence its activation.

We report here a new activation mechanism of caspase-8 in which, during neutrophil apoptosis, cathepsin D induces activation of the initiator caspase-8 by intra-chain proteolysis. At acidic pH, cathepsin D cleaved the recombinant human caspase-8 protein at several sites and significantly increased its activity. This increased activity was completely blocked by the pharmacological inhibitor of cathepsin D, pepstatin A. Under non-dimerizing conditions, the caspase-8 fragment generated by incubation of the recombinant human caspase-8 protein with cathepsin D could be affinity labelled with the biotinylated caspase substrate VAD-fmk arguing that the 21-kD fragment of caspase-8 is enzymatically active. When cathepsin D was incubated together with the recombinant human proteins of the initiator caspase-9 and -10, no significant increase of their activities was detected suggesting that cathepsin D could selectively activate the initiator caspase-8, but not the initiator caspase-9 and -10. In an *in vitro* cell-free assay using cytosolic extracts of freshly isolated blood neutrophils, we observed that the addition of recombinant human caspase-8 protein incubated with cathepsin D was followed by the activation of caspase-3 and consequently the induction of apoptosis. Thus, we demonstrated that cathepsin D is able to launch neutrophil apoptosis by directly and selectively inducing the intra-chain proteolysis of the initiator caspase-8.

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Mitochondrial fission and cristae disruption increase the response of cell models of Huntington's disease to apoptotic stimuli.

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Huntington's Disease (HD), a genetic neurodegenerative disease caused by a polyglutamine expansion in the Huntingtin (Htt) protein is accompanied by multiple mitochondrial alterations. Here we demonstrate mitochondrial fragmentation and alterations in cristae morphology in cell lines from patients and from a knock-in mouse model of HD as well as in primary striatal neurons from the YAC128 HD mouse model. In HD cells, elevated intracellular Ca^{2+} stores correlate with increased basal activity of the phosphatase calcineurin, which in turn dephosphorylates the pro-fission protein Drp1, leading to its mitochondrial translocation and activation, and ultimately to mitochondrial fragmentation. Changes in the shape of the cristae and disruption of the Opa1 oligomers that stabilize them are faster in these fragmented HD mitochondria upon apoptotic stimulation. Consequently, cytochrome c release and apoptosis are faster in HD models. Enforced expression of Opa1 or prevention of Drp1 activation ameliorate mitochondrial morphology and ultrastructure, and ultimately cell death in all the HD models tested. In conclusion, a feed forward mechanism of mitochondrial fission and cristae remodelling explains the hypersensitivity of Huntington's disease mitochondria to apoptotic damage.

The relationship between secreted frizzled-related protein 4 (sFRP4) and endothelial cell apoptosis.

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sFRP4 is one of a family of five secreted glycoproteins that antagonise the Wnt signalling pathway. sFRP4 has been shown to have a pro-apoptotic and anti-proliferative role in tissues such as corpus luteum, placenta, mammary gland and prostate. We investigated the effect of sFRP4 on angiogenesis and found it to be a potent inhibitor of this process both in vitro and in vivo. sFRP4 was able to increase reactive oxygen species production in endothelial cells (EA.hy926) but this ability was blocked by the addition of superoxide dismutase. Early apoptotic events, evidenced using a JC-1 assay, showed sFRP4 was capable of inducing apoptosis in HUVEC but did not affect SKOV-3 cells (a human cell line derived from ovarian serous cystadenocarcinoma). Further in vitro investigations using the cysteine-rich domain (CRD) and netrin-like domain (NLD) of sFRP4 indicated that the CRD was responsible for antagonising endothelial cell proliferation and ring formation while the NLD induced apoptosis. The NLD was able to increase intracellular calcium levels in a dose-dependent manner. In summary, the NLD of sFRP4 is able to induce endothelial cell-specific apoptosis by the induction of reactive oxygen species, which is partly driven by increased intracellular calcium levels.

Molecular pathways involved in neutrophil apoptosis under hypoxia

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Hypoxia occurs under various conditions and it can be either sustained (SH) or intermittent (IH). Neutrophil apoptosis is inhibited by hypoxia. We demonstrated that IH significantly increased neutrophil survival compared to SH and normoxia as assessed by morphology, caspase-3 activation and CD16/Annexin-V expression. Both IH and SH were shown to activate NF- κ B and p38MAPK pathways, by increasing NF- κ B translocation to the nucleus and enhancing p38MAPK phosphorylation. Utilizing NF- κ B and p38MAPK inhibitors abolished the hypoxic survival of neutrophils and decreased IL-8 expression. However, only IH, but not SH, stimulated ERK1/2 phosphorylation. Additionally, both IH and SH up-regulated anti-apoptotic Mcl-1, down-regulated pro-apoptotic Bax protein expressions and prevented Bax translocation to the mitochondria. The effect of IH was more prominent. Finally, inhibition of p38MAPK, but not ERK1/2, significantly decreased Mcl-1 expression under SH. In contrast, IH-induced Mcl-1 up-regulation was abrogated by both ERK1/2 and p38MAPK inhibitors. In conclusion, IH can modify neutrophil apoptosis via activation of NF- κ B, ERK1/2 and p38MAPK, which are involved in regulation of extrinsic and intrinsic mitochondrial apoptotic pathways. The survival effect of SH is mediated via NF- κ B and p38MAPK, but not ERK1/2 activations. Thus, Bcl-2 protein function in IH may be regulated by different signal transduction pathways than SH.

Analysing the Function of the Bcl-2 Family Member Bok

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Bok (Bcl-2 related ovarian killer) is a poorly characterized pro-apoptotic multidomain member of the Bcl-2 family. It has been reported to be targeted to the mitochondrial outer membrane via its C-terminal transmembrane domain but the exact mechanisms of how Bok induces cell death remains unknown. As certain tissues of *bax*^{-/-}*bak*^{-/-} mice develop quite normally up to late stage of embryogenesis, it is conceivable that another Bax-like protein, for example Bok, may have critical functions in those tissues.

Our experiments show that inducible Bok overexpression triggers cell death in various cell lines (e.g. Ba/F3) in a caspase dependent manner involving cytochrome c release. Furthermore we show that full length Bok fused to EGFP or Flag localizes mainly to the ER/nuclear outer membrane at early stages of expression (12h-14h), while at later time points, or after addition of apoptosis inducers (etoposide, staurosporine, UV-irradiation), Bok changes its subcellular localization to vesicle-like structures not colocalizing with the ER anymore. Deletion of the Bok C-terminal anchor domain abrogates organelle targeting but, surprisingly, maintains its ability to induce cell death.

Elucidate the Beneficial Effect of Natural Factors in Induction of Apoptosis in Human Cancer Cells

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The increasing incidence of human cancer has led to the performance of intense investigations searching for efficient anti-tumorigenic compounds against this disease. Inducing apoptosis is an efficient method for preventing malignant transformation, where dysregulation of apoptosis cause significant human disease and promote cancer development. Focusing on identifying apoptotic factors inducing apoptosis in human cancer and understanding their molecular mechanisms may lead to the development of new strategies for the prevention and treatment of human cancer.

Epidemiological studies have demonstrated a decreased incidence of cancer in humans consuming large amounts of cruciferous vegetables. These vegetables contain glucobrassicin, which hydrolysis by myrosinase to indole-3-carbinol that converted in a low pH to 3,3'-diindolylmethane. The effect of the indole-derivatives on cell proliferation of human cancer cells was tested in *in vitro* and *in vivo* biological systems.

It was previously shown by us that these compounds have inhibitory effects on the viability and proliferation of human breast, prostate and colorectal cancer cells. These studies demonstrated that the indolic compounds exert their effects in the cancer cells through the induction of an apoptotic cell response. Moreover, we have shown that the indolic compounds suppress the growth of human cancer cells in a dose dependent manner by inducing apoptosis through p53, bcl-2 family proteins and fasL - independent pathway. DIM induced apoptosis in hormonal dependent as well as in hormonal - independent human cancer cells. Apoptosis was induced through the mitochondrial pathway, which involves the translocation of cytochrome C from the mitochondria to the cytosol and the activation of initiator caspase 9 and the effectors caspases, 3 and 6 leading to poly ADP-ribose polymerase (PARP) cleavage. The potential therapeutic effects of DIM in an *in vivo* model were tested. We found that DIM (5 and 10 mg/kg, 3 times a week) caused a significant deceleration in the volumes and weights of tumours which were induced in C57BL/6 mice, by transplanting the TRAMP-C2 prostate cancer cell line subcutaneously. Moreover, histopathological studies indicated that DIM induces apoptosis in the tumour cells. The treatment of DIM was not accompanied by liver and kidney toxicity as it was detected by biochemical tests. Interestingly, pre-treatment of animals with pre-apoptotic factors for five weeks before transplanting the TRAMP-C2 cells, significantly reduced tumor development as compared to controls. Tumors were developed in 80% of control and 20% of treated animals. The tumors developed in treated animals were significantly ($p < 0.001$) smaller than that developed in controls. Thus, it appears that DIM may offer an effective and non-toxic therapeutic mean against tumour growth in rodents, and may serve as a potential natural anti-tumorigenic compound in humans.

Effects of zinc on autophagy during development of *Drosophila melanogaster*

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Programmed cell death (PCD) at the end of the larval development of *Drosophila melanogaster*, triggered by ecdysone, seems to involve two processes, autophagy and apoptosis. We used haemocytes, insect's blood cells, of the third instar larvae as a model to study effects of zinc on physiologically induced autophagic cell death. In the earlier study it has been suggested that Zn^{2+} regulates PCD of haemocytes enhancing degradation of mitochondria and activation of caspases. The aim of this study was to examine expression of two genes in *Drosophila*, homologs of mammalian autophagy genes; *atg4* and *atg7*, and formation of autophagosomes in haemocytes treated with two concentrations of zinc ions: 0.35 mM, 1.7 mM. The obtained results showed that the expression of *atg7*-homolog is increased in zinc-treated cells in dose-dependent manner in comparing with the untreated cells. Surprisingly, the opposite effect was observed in case of *atg4*-homolog expression. In addition we found that the number and size of autophagosomes was significantly increased in Zn^{2+} -treated haemocytes measured using monodansylcadaverine and LysoTracker fluorescence. These results suggest that zinc enhances processes of ecdysone-induced autophagy in the fruit fly's haemocytes by modulating expression of genes involved in autophagy and autophagosome formation.

Anti-apoptotic role of HIF-1 and AP-1 in paclitaxel exposed breast cancer cells under hypoxia

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Hypoxia is a hallmark of solid tumors and is associated with metastases, therapeutic resistance and poor patient survival. In this study, we showed that hypoxia protected MDA-MB-231 breast cancer cells against paclitaxel- but not epirubicin-induced apoptosis. The possible implication of HIF-1 and AP-1 in the hypoxia-induced anti-apoptotic pathway was investigated by the use of specific siRNA. Specific inhibition of the expression of these two transcription factors was shown to increase apoptosis induced by chemotherapeutic agents under hypoxia indicating an involvement of HIF-1 and AP-1 in the anti-apoptotic effect of hypoxia. After HIF-1 specific inhibition and using TaqMan Human Apoptosis Array, 8 potential HIF-1 target genes were identified which could take part in this protection. Furthermore, Mcl-1 was shown to be a potential AP-1 target gene which could also participate to the hypoxia-induced chemoresistance. Altogether, these data highlight two mechanisms by which hypoxia could mediate its protective role via the activation of two transcription factors and, consecutively, changes in gene expression encoding different anti- and pro-apoptotic proteins.

NO. 21

Localization of Caspases in Human Cells Using Labeled Designed Ankyrin Repeat Proteins (DARPin) and Fluorescence Microscopy

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We have selected a large number of Designed Ankyrin Repeat Proteins (DARPin) [1] as binders against human caspases. Caspases, a family of cysteine proteases, play important roles in apoptosis and inflammation [2] and are therefore essential targets in structural and biochemical investigations.

The selected DARPin showed highly specific binding with affinities in the low nanomolar range; characterized by size exclusion chromatography (SEC), competition ELISA, isothermal titration calorimetry (ITC) and surface plasmon resonance. Furthermore, crystal structures of caspase-3 and caspase-8 in complex with specific DARPin were solved and showed in comparison two different epitopes.

The specific DARPin can be used as molecular markers of caspases by fluorescent labeling. For that reason, DARPin-fluorophore coupling is performed by maleimide chemistry to cysteine residues localized either at the N- or the C-terminus.

Fluorescent or luminescent DARPin will be then used for localization of caspases in human cells using fluorescence microscopy with the aim to gain a deeper insight into the molecular pathways of apoptosis inside apoptotic cells.

[1] Binz et al. High-affinity binders selected from designed ankyrin repeat protein libraries. Nat Biotechnol (2004) vol. 22 (5) pp. 575-82

[2] Riedl and Shi. Molecular mechanisms of caspase regulation during apoptosis. Nat Rev Mol Cell Bio (2004) vol. 5 (11) pp. 897-907

NO. 22

Coronopilin induces tumor cell-selective apoptosis and cell cycle arrest

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Sesquiterpene lactones (SLs), a large and diverse group of natural products, have been shown to possess a wide spectrum of biological activities. Here we demonstrated the anti-leukemic potential of coronopilin (CP), a pseudoguaianolide SL from *Ambrosia arborescens* Mill. Interestingly, CP inhibited Jurkat and U937 growth, but displayed low toxicity against PBMC, taken as normal counterpart. The most prominent response in Jurkat was the activation of apoptotic cell death, as assessed by cell morphology, phosphatidylserine exposure and DNA fragmentation analysis. Apoptosis was typically caspase-dependent, as confirmed by inhibition experiments with ZVAD and by WB analysis (caspase 3 activation and cleavage of its substrate PARP). Mitochondrial depolarization and cyt *c* release were not prevented by ZVAD, just indicating that caspase pathway is activated downstream mitochondria. In U937, CP did not induce rapid apoptosis, instead drug exposure leads to an accumulation in G2/M phase of the cell cycle. In particular, the activation of Cdk1 and the increased cyclin B1 levels confirmed the G2/M arrest, as Cdk1-cyclin B complex is required for entry into mitosis. Accordingly, Giemsa dye staining revealed a marked increase of the mitotic index. Altogether our results suggest that CP induced U937 death by mitotic catastrophe.

By which signaling pathways does gliotoxin, the major virulence factor of the mold *Aspergillus fumigatus*, induce apoptosis?

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Abstract

Gliotoxin (GT), a potent virulence factor of the pathogenic mold *Aspergillus fumigatus* is the major cause for Invasive Aspergillosis (IA), an opportunistic pulmonary disease in immunocompromised patients. We showed in our group that GT induces apoptosis of mouse embryo fibroblasts and human lung epithelial cells in a detachment-dependent manner, a process called anoikis. In addition, GT majorly kills cells through the intrinsic, mitochondrial pathway, implicating the Bcl-2 family member Bak (see poster by Andreas Geißler).

Here we investigated in human lung epithelial cells the signaling pathways that are involved in GT mediated apoptosis. We identified five protein kinase signaling cascades that are affected after cellular treatment with GT: (i) PI3 kinase/AKT, (ii) MEK/ERK, (iii) focal adhesion kinase (FAK), (iv) p38MAPK and (v) JNK. In addition we found that both GT treatment and anoikis by detachment (trypsinization and preventing reattachment by polyhema coated plates) induced the pro-apoptotic protein Bad. Using the specific MEK inhibitor U0126, we showed that Bad induction was MEK/ERK dependent. However, neither Bad downregulation by shRNA nor inhibition of the MEK/ERK pathway by U0126 had any effect on GT-induced cell death. These data indicate that although GT can activate the MEK/ERK pathway leading to Bad induction, this event is dispensable for GT-induced apoptosis. We are currently investigating the role of the PI3K/AKT, p38MAPK and JNK pathways as upstream mediators of GT-induced mitochondrial membrane permeabilization.

The fourth isoform of the adenine nucleotide translocator inhibits mitochondrial apoptosis in cancer cells

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The adenine nucleotide translocator (ANT) is a protein involved in many models of mitochondrial apoptosis and located in the inner membrane of mitochondria, where it catalyzes the exchange of ADP and ATP. The human ANT sub-family is composed of four isoforms, whose expression is highly regulated. Previous studies have demonstrated that ANT1 and 3 appear to be pro-apoptotic, whereas ANT2 prevents mitochondrial apoptosis. However, the role of the recently identified isoform ANT4 in the apoptotic pathway has not yet been elucidated. Here, we investigated the effects of stable overexpression of ANT4 in human cancer cells, using ANT3 isoform as a pro-apoptotic control. By contrast with ANT3, we demonstrate for the first time that ANT4 enhanced cell growth without impacting mitochondrial morphology or respiration. Similarly to ANT3, ANT4 differentially regulated the intracellular levels of hydrogen peroxide without affecting superoxide anion levels. Finally, unlike the apoptosis sensitizing activity of ANT3, stable ANT4 overexpression protected cancer cells from lonidamine- and staurosporine-induced apoptosis independently of Bcl-2 expression. Altogether, these results demonstrate a cytoprotective activity of ANT4 and provide new insights on ANT sub-family, with ANT1 and 3 isoforms functioning as pro-apoptotic factors while ANT2 and 4 isoforms make cells resistant to death inducing stimuli.

A novel TNFR1-triggered apoptosis pathway mediated by p38 MAPK, class IA PI3Ks, and ROS in neutrophils

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The most common form of neutrophil death is apoptosis. Here, we report surprising differences in the molecular mechanisms used for caspase activation between FAS/CD95- and TNF receptor 1 (TNFR1)-stimulated neutrophils. Whereas FAS-induced apoptosis was followed by caspase-8 activation and required Bid to initiate the mitochondrial amplification loop, TNF-alpha-induced apoptosis strikingly involved class IA phosphoinositide 3-kinases (PI3Ks), which were constitutively bound to TNFR1 and activated by mitogen-activated protein kinase p38. TNF-alpha-induced PI3K activation resulted in the generation of reactive oxygen species, which activated caspase-3, a mechanism that did not operate in neutrophils without active NADPH oxidase. Taken together, in neutrophils, pro-apoptotic pathways following TNFR1 stimulation are initiated by p38 and PI3K, but not caspase-8, a finding that should be considered in anti-inflammatory drug development strategies.

NO. 26

GLIOPTOSIS – APOPTOSIS INDUCED BY THE A.f. VIRULENCE FACTOR GLIOTOXIN

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The ubiquitous mold *Aspergillus fumigatus* (*A.f.*) and other related fungi are the causative pathogen for the severe pulmonary disease, termed invasive aspergillosis (IA) in immunosuppressed individuals. Among other secondary metabolites, *A.f.* produces gliotoxin (GT), an epipolythiodioxopiperazine, known to exhibit a broad range of immunosuppressive and cytotoxic properties. The recent finding that an *A.f.* mutant deficient in GT is less virulent than wt *A.f.*, when administered intranasally to mice, underscores the cardinal contribution of GT to the pathogenic potential of *A.f.* in this host species. Since GT has also been detected in specimens taken from *A.f.*-infected patients, the combined studies suggest that GT is also a virulence factor in human IA.

Recent work by Pardo *et al.* showed that the pro-apoptotic Bcl-2 family member protein Bak plays a fundamental role in the induction of apoptosis in mouse embryonic fibroblasts after GT treatment. In agreement with these findings Bak^{-/-} mice were less effectively killed by *A.f.* infection than wt mice.

To uncover the exact molecular mechanism of GT action and to investigate if GT induced apoptosis is crucial for human IA, we started to use human lung epithelial cells as a model system. By downregulating Bax and/or Bak by shRNA techniques, we found that as with MEFs, GT mainly uses Bak to induce apoptosis. Surprisingly, and in contrast to other apoptotic stimuli, Bax/Bak double knock-out cells still detached with GT, indicating that GT targets adherence molecules on the cell surface and induces detachment (anoikis) in a Bax/Bak independent manner. In addition, we used isolated mitochondria from these human epithelial cells to further characterize the mode of action of GT on mitochondria. We found that GT does not directly activate Bak and does not seem to use mitochondrial or cytosolic factors to do so. These results suggest that upstream signaling pathways acting between the plasma and mitochondrial membrane are crucial mediators of the action of GT. We could identify five protein kinase signaling cascades that are altered in response to cellular treatment by GT: (i) PI3 kinase/AKT, MEK/ERK, focal adhesion kinase (FAK), p38MAPK and JNK. We are in the process of further investigating the role of these pathways for GT-induced mitochondrial membrane permeabilization and apoptosis.

NO. 27

Regulation of FasL and TNF α -induced cell death in murine neutrophils

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We are interested in the regulation of apoptosis by Bcl-2 family members, which regulate the mitochondrial (stress-induced) apoptotic pathway, and the crosstalk between the death receptor-induced and the mitochondrial apoptotic pathway. For apoptosis induced by the death receptor Fas (CD95) it has been shown that this crosslink is required in some (called type II cells) but not in others (type I cells). The crosslink is mediated by the BH3-only protein Bid, which is cleaved and activated by caspase-8, the initiator caspase downstream of Fas. To verify whether a Fas ligand (FasL) sensitive cell type behaves like type I or type II one has to interfere with the initiation phase of the mitochondrial pathway. This can be achieved by either overexpressing anti-apoptotic Bcl-2 family members (such as Bcl-x_L or Mcl-1) or by deleting the BH3 only protein Bid. By such approaches it has been shown that hepatocytes are type II like cells, as Bid-deficient hepatocytes were refractory to FasL-induced killing.

Here, we investigate whether murine neutrophils, which are sensitive to Fas-induced killing, behave like type I or type II cells. In a first approach we are comparing FasL-induced killing in primary mature neutrophils (Gr1⁺) isolated from wildtype and *bid*^{-/-} mice. Our data suggests that FasL-induced death is indeed delayed in *bid*^{-/-} neutrophils at early time points. Surprisingly, and in contrast to hepatocytes, addition of the pan-caspase inhibitor Q-VD-oph results in a very moderate protection only. Therefore we also investigated caspase independent events as death receptors, especially TNF-R1, have been described also to induce necroptosis (programmed necrosis) and non-caspase like proteases, such as cathepsin D, have been linked to some forms of neutrophil apoptosis. Preliminary data indicate that mouse granulocytes can in fact be better protected from cell death when Q-VD-oph is added in combination with a RIP1 kinase inhibitor Necrostatin-1 (which blocks necroptosis). Similar experiments using TNF α , a death ligand that activates the death receptor TNF-R1 and can induce apoptosis as well as necroptosis, indicate that in mouse neutrophils TNF α -induced death is fully blocked by addition of Q-VD-oph.

In a second approach, we are using a system that allows *in vitro* differentiation of Hoxb8-immortalized myeloid progenitor cells into mature neutrophils. Using this approach, progenitor cell lines can be generated from any desired genetically modified mouse strain and mature neutrophils can be obtained in sheer unlimited numbers, which is a major drawback of experiments with primary mouse neutrophils.

NO. 28

Activation of the transcription factor FOXO3/FKHRL1 by doxorubicin and etoposide induces reactive oxygen species production and programmed cell death in neuroblastoma cells.

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Forkhead boxO (FOXO) transcription factors are regulators of cell death, cell cycle progression and contribute to longevity. FOXO3/FKHRL1 was shown to protect against reactive oxygen species (ROS) by regulating detoxifying enzymes. Etoposide and doxorubicin treatment activates FOXO3, induces ROS and elevates the expression of Noxa and Bim in neuroblastoma cells. Conditional activation of FOXO3 induced two sequential waves of ROS the first one being associated with elevation of Bim and Noxa. Knockdown of Bim or retroviral overexpression of Bcl-xL both prevented ROS production and delayed apoptosis which implies that FOXO3-induced ROS in neuronal cells is downstream of Bcl2 proteins. The decline after the first ROS wave correlated with increased expression of the peroxiredoxin Sestrin3. Knockdown of Sestrin3 prevented ROS decline and led to continuously increasing ROS levels and accelerated cell death. The combined data suggest that programmed cell death by FOXO3 involves ROS production downstream of Bcl2 rheostat and that FOXO3 in parallel activates ROS-protection by Sestrin3. Prolonged FOXO3 activation however overcomes Sestrin3 protection, induces a secondary ROS burst and leads to cell death.

NO. 29

YB1 is a repressor of apoptosis and hypertrophy in cardiomyocytes of adult rat

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Apoptosis and hypertrophy both contribute to heart failure progression and are controlled at a transcriptional level. SMADs and AP-1 were identified as pro-hypertrophic and apoptotic transcription factors. The transcription factor YB-1 is known to interact with SMADs and

AP-1 and thereby may influence apoptosis and hypertrophy in cardiomyocytes.

To support this hypothesis, we generated an adenovirus (AdYB-1) for overexpression of YB-1.

TGF β ₁ induced apoptosis ($13.7 \pm 1.6\%$, $n=7$, $p<0.05$) was prevented by overexpression of YB-1 ($6.6 \pm 1.1\%$, n.s. vs. unstimulated controls). Moreover, phenylephrine (PE) induced hypertrophy, determined by an increase in cell size to $671.8 \pm 15.4\mu\text{m}$ ($n=14$, $p<0.05$) and enhancement of the rate of protein synthesis to $138.0 \pm 16.6\%$ ($n=8$, $p<0.05$), was blocked in YB-1 overexpressing cardiomyocytes ($629.7 \pm 14.7\mu\text{m}$ and $87 \pm 12.3\%$, n.s. vs. unstimulated controls). However, enhancement of hypertrophic growth by GDF-15 was not blocked by AdYB-1: Cell size and rate of protein synthesis still increased in GDF-15 stimulated, YB-1 overexpressing cardiomyocytes.

Conclusion: We identified YB1 as a repressor of apoptosis and α -adrenergic hypertrophy in cardiomyocytes. YB-1 therefore provides new perspectives for therapeutic approaches against heart failure progression.

NO. 30

Transcriptional regulation of Autophagy related gene 5 (Atg5) gene expression

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Many cellular stress factors, such as, starvation, oxidative stress, or accumulation of protein aggregates, are capable to induce autophagy, a process, in which parts of the cytoplasm or organelles are sequestered by a double-membraned autophagosome and subsequently degraded by the lysosomal enzymes. Low level of autophagy can rescue the cells by reestablish homeostasis, whereas massive autophagy triggers type II cell death, the so-called autophagic cell death. As a key molecule of autophagy, Atg5 contributes to the double-membraned vesicle expansion and completion by forming the complex with Atg12 and Atg16. Furthermore, Atg5 can also be proteolytically activated to become a pro-apoptotic molecule that translocates to mitochondria and triggers apoptosis. Because of the multiple functions of Atg5, it is quite important to know how its expression is regulated.

Based on the results of luciferase assay, we could identify members of p53 gene family, especially p73 can activate Atg5 gene promoter. Chromatin Immunoprecipitation(ChIP) assay showed the binding of p73 on one putative p73 binding site of Atg5 promoter. Using inducible Saos2 cells which are p53 deficient, we found that Atg5 expression is induced at both mRNA and protein level after induction of p73. In summary, our data suggest that p73 may be a potential transcriptional activator of Atg5.

NO. 31

Identifying ER stress induced apoptotic factors with “click-chemistry” based mass spectrometry

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Apoptosis induced by endoplasmic reticulum (ER) stress has been associated with an increasing number of pathologies like neurodegenerative disorders and diabetes over the last two decades. Nevertheless, not much is known about the signaling pathways and proteins implicated in this type of cell stress. It has been shown that apoptosis induced by ER stress proceeds via both caspase-dependent and -independent processes in multiple mammalian cell lines. The caspase-independent step involves a component that acts upstream of mitochondrial membrane permeabilization and which can be blocked by pan-serine protease inhibitors such as AEBSF. In order to unravel this component (or components) we synthesized an alkyne version of AEBSF. This probe was used to “click” a biotin tag onto the modified inhibitor after its cellular uptake and covalent binding to the target protein(s) in order to perform pull down experiments and subsequent mass-spectrometry analysis. This method is termed “click chemistry”, a technique, which allows clicking two compounds irreversibly together in a copper-catalysed azide-alkyne reaction *in vitro*. Using this method we obtained some promising candidates, which we are currently validating. This will allow us to better understand the signaling pathway from ER stress to mitochondria-mediated apoptosis.

NO. 32

Mitochondria-shaping proteins are required for cardiomyocyte differentiation of mouse embryonic stem cell

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Mitochondria play essential and versatile roles in cardiomyocyte pathophysiology: they are the main source of ATP; they control apoptosis, and Ca²⁺ signaling,

impacting on almost any process, like outcome of ischemia/reperfusion and electrical synchronization during EC coupling. Mounting evidence is supporting a role for mitochondrial shape in determining organellar function. Mitochondrial morphology is controlled by a growing family of “mitochondria-shaping” proteins that includes the pro-fusion large dynamin-like GTPases optic atrophy 1 (OPA1) and mitofusin (MFN) 1 and 2 and the pro-fission dynamin-related protein 1 and its mitochondrial receptor FIS1. In addition, these mitochondria-shaping proteins not only control mitochondrial morphology, but also have crucial roles in key cellular processes, such as apoptosis, tethering of organelles and Ca^{2+} homeostasis. However, if mitochondria-shaping proteins regulate differentiation into functional and specific cell lineages is largely unknown, in particular in the cardiomyocyte. To understand the role of mitochondria-shaping proteins in cardiomyocyte differentiation, we used mouse embryonic stem (ES) cells lines (*Opa1*^{gt} and *Mfn2*^{gt}), which are heterozygous for a gene trap in *Opa1* or *Mfn2* gene respectively. Hanging-drop differentiation showed that OPA1 and MFN2 are required for proper differentiation into beating embryoid bodies. The differentiation defect was corrected by the calcineurin inhibitor FK506, as well as dominant negative form of calcineurin. These findings show that OPA1 and MFN2 are required for differentiation into cardiomyocytes, and suggest that mitochondrial shape regulates differentiation by impinging on a pathway involving the phosphatase calcineurin.

NO. 33

Sensitization of TRAIL-induced apoptosis by β -Ionone

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β -Ionone (ION), an end-ring analogue of β -carotenoid, has been known to inhibit tumor cell growth and induce apoptosis in various types of cancer cells. Nevertheless, its apoptosis-related molecular mechanisms remain unclear. Here, we first investigated the molecular mechanisms by which ION sensitizes cancer cells to the therapeutic potential of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). Notably, treatment with subtoxic concentrations of ION and TRAIL effectively inhibited cell viability in the hepatocellular carcinoma cell line Hep3B and other cancer cell lines such as colon carcinoma cell line HCT116 and leukemia cell line U937. Combined treatment with ION and TRAIL was also more effective in inducing DR5 expression, caspase activities, and apoptosis than treatment with either agent alone. ION-mediated sensitization to TRAIL was efficiently reduced by treatment with a chimeric blocking antibody or siRNA specific for DR5. EMSA and a chromatin immunoprecipitation assay confirmed that ION treatment upregulates the binding of transcription factor Sp1 to its putative site within the DR5 promoter region, suggesting that Sp1 is an ION-responsive transcription factor. In addition, ION significantly increased hepatocellular carcinoma cell sensitivity to TRAIL by abrogating TRAIL-induced NF- κ B activation and decreasing the expression of antiapoptotic proteins such as XIAP and IAP-1/2. Taken together, these data suggest that ION is a useful agent for TRAIL-based cancer treatments.

NO. 34

Sulforaphane increases gemcitabine mediated cytotoxicity towards pancreatic cancer stem-like cells

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Abstract

Despite intense efforts to develop treatments against pancreatic cancer, agents that cure this malignant disease are still not available. Cancer stem cells may be responsible for high resistance and early metastasis of pancreatic cancer. Considerable attention has focused on targeting of pancreatic cancer stem cells by broccoli compound sulforaphane. Since increasing evidence suggests that chemotherapeutic treatment results in an enrichment of cancer stem cells, we investigated whether sulforaphane can increase drug-mediated cytotoxicity in a pancreatic cancer cell line highly enriched in cancer stem cell characteristics. While gemcitabine and sulforaphane were effective in inducing apoptosis and preventing viability on their own, combination of an individual drug with sulforaphane was most effective. These data were confirmed for short and long term combination treatment. Most importantly, combined treatment with sulforaphane and gemcitabine inhibited self-renewal and aldehyde dehydrogenase 1 activity stronger than each single substance, indicating that cancer stem cell characteristics are affected. *In vivo*, combination treatment totally abolished tumor growth of cancer stem cell xenografts. No pronounced side effects were observed in mice. Our data suggest that sulforaphane increases the effectiveness of various cytotoxic drugs against cancer stem cells without side effects in mice.

NO. 35

Exploring the role of Mfn2 in tethering organelles

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Juxtaposition between endoplasmic reticulum (ER) and mitochondria provides the physical basis for intercommunication during Ca²⁺ signalling. The molecular mechanisms underlying this process are largely unknown, although our laboratory recently discovered that lack of mitofusin-2 (Mfn2) a dynamin-related protein mutated in Charcot-Marie-Tooth type IIa (CMT2a) leads to ER fragmentation and to a reduction in the ER-mitochondria interactions, impacting on Ca²⁺ transfer between

the two. The molecular mechanism by which Mfn2 regulates ER morphology and tethering, as well as the partners by which it accomplishes its tethering function are unknown. We will present data on an in vitro system to recapitulate ER fusion from vesicles isolated from Mfn2^{-/-} cells and on the characterization of Mfn2 containing complexes at the ER and at the ER-mitochondria interface.

NO. 36

Identification of the cell death pathway induced by hydrolyzed pectin in HepG2 and A549 cells

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Several studies have shown that modified forms of citrus pectin induce tumoral cell death. However, the nature of the active fragments and the mechanisms by which they act remain unclear. By heat hydrolysis we produced a mixture of pectin fragments (HFPC), which were tested on two cancer cell lines (HepG2 and A549 cells). HFPC induced HepG2 and A549 cell death, as measured by LDH release and MTT staining, while unmodified pectin did not have any cytotoxic effect. Western blotting of the active form of caspase 3, an apoptosis effector, and of the cleaved PARP protein, an apoptosis marker, were assessed, indicating an increase of the abundance of these proteins in

HepG2 and A549 cell incubated with HFCP. However, caspase 3 activity was not increase, even if its active form is more abundant. Even more, observation of the nucleus did not show the characteristic vacuolisation that occur during apoptosis. western blotting of the LC3-II protein, an autophagy marker, was assessed, indicating an increase of the abundance of this protein in HepG2 and A549 cells incubated with HFCP. Once identified, the HFCP active fraction could be used in complement to conventional chemotherapeutics in cancer treatment.

NO. 37

Dietary sphingomyelin-triggered apoptosis in intestinal epithelial cells is mediated by cathepsin D- and Bid-activation

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Sphingomyelin (SM), a lipid component of the plasma membrane, is prevalent in animal products. Metabolism of SM in the bowel generates ceramide which acts as lipid messenger and influences numerous cellular functions including apoptosis of intestinal epithelial cells (IEC). Recent data suggest that the underlying mechanism is triggered by cathepsin D (CTSD) activation and subsequent cleavage of Bid to tBid.

We aimed to investigate whether dietary SM provokes apoptosis during acute dextran sulfate sodium- (DSS-) induced colitis.

Acute colitis was induced to female C57-BL/6J mice by 2 % DSS over 7 days. Mice received 4 mg SM a day, resuspended in water and applied by oral gavage. IEC were isolated *ex vivo*.

Dietary SM was accumulated in IEC and metabolized to ceramide. CTSD activity was increased upon SM treatment both in mice without and with acute colitis. tBid/Bid ratio was significantly augmented in DSS colitis as well as due to dietary SM in healthy control mice. During colitis activation of caspase-3 and TUNEL analysis revealed significantly increased apoptosis due to dietary SM.

Our findings provide evidence that ceramide-triggered activation of CTSD results in the induction of apoptosis signaling by cleavage of pro-apoptotic Bid and subsequent initiation of the caspase cascade.

NO. 38

The role of MCL1 phosphorylation for *in vivo* lymphocyte development

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MCL1 is required for survival of T and B lymphocytes and has also been shown to maintain hematopoietic stem cell survival. We previously showed that MCL1 is phosphorylated by GSK3, resulting in loss of MCL1 mediated protection from apoptosis. The GSK3 phosphorylation mutant, MCL1-S159A, exhibited a delay in apoptosis upon growth factor withdrawal.

In this study, we compared the protective activity of MCL1-WT versus phosphorylation deficient MCL1-S159A *in vivo*, employing adoptive transfer of bone

marrow (BM) cells retrovirally transduced with MCL1-WT or MCL1-S159A in an IRES-GFP cassette.

Equal numbers of BM cells expressing MCL1-WT or MCL1-S159A were transferred into irradiated recipient mice. Peripheral blood, BM, thymus, spleen and lymph node cells were analysed after 4 and 6 weeks, respectively.

In peripheral blood, the proportion of neutrophil granulocytes, lymphocytes, monocytes, eosinophil granulocytes and basophil granulocytes did not differ in mice expressing MCL1-WT compared to those expressing MCL1-S159A. However, the total amount of white blood cells (WBC) was elevated in mice expressing MCL1-S159A. The distribution of thymocyte subpopulations remained unchanged upon expression of MCL1-WT vs. MCL1-S159A, as well as the T and B cell populations in spleen and lymph nodes.

NO. 39

Unexpected subcellular localization of the tumor suppressor BARD1: mitochondria vs. endoplasmic reticulum

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Mitochondrial morphology is tightly determined by a dynamic equilibrium between fission and fusion events. These dynamic processes are controlled by a growing number of proteins. Up to now, the mechanisms that regulate proteins impinging on mitochondrial morphology are only partially understood. Recent evidence demonstrated that proteins that regulate morphology could be modulated by post-translational modification, including phosphorylation, ubiquitination and SUMOylation. The BRCA1-associated RING domain protein (BARD1) is a putative tumor

suppressor gene, which is mutated in a subset of breast and ovarian cancers. BARD1 interacts with BRCA1 through its respective RING domains and functions as a heterodimer in nuclear DNA repair, dependent on BRCA1-BARD1 ubiquitin E3 ligase activity. Interestingly, recent evidence also indicates mitochondrial localization of BARD1 where it exhibits a BRCA1-independent apoptotic activity, which correlates with Bax activation and oligomerization. However, the underlying mechanism(s) of BARD1-induced apoptotic activity in the context of mitochondrial biology/morphology remains elusive. We therefore decided to investigate the subcellular localization of BARD1 and the role of this protein in the regulation of apoptosis and mitochondrial morphology. Here we will present our preliminary data on the unexpected retrieval of BARD1 in mitochondria-associated membranes as well as in the endoplasmic reticulum. BARD1 is accordingly a weak inducer of apoptosis and its function at the ER deserves further investigation.

NO. 40

Down-regulation of autophagy gene 5 (Atg5) in malignant melanoma contributes to melanoma tumorigenesis

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Cutaneous melanoma is a common malignancy of melanocytes. In its early stage malignant melanoma can be cured by surgical resection, but it becomes extremely difficult to treat once it progresses to the metastatic stage due to its high resistance to current chemotherapeutic drugs. Autophagy is a highly conserved cellular self-eating process, in which proteins and organelles are sequestered and subsequently degraded in a double membrane structure called autophagosome. Autophagy related genes (Atgs) are the main players during this process. Autophagy has been shown to be involved in the pathogenesis of numerous diseases, including neural degenerative

diseases, infections and cancer. We are interested in the role of Atg5 in melanoma. We found that Atg5 expression is down-regulated in malignant melanoma comparing with benign melanocytic nevus patients. Furthermore, down-regulation of Atg5 has also been shown in melanoma cell lines. Over-expression of Atg5 increases the susceptibility of melanoma cells towards chemotherapeutic drug induced cell death. With our data, we hypothesize that down-regulation of Atg5 may contribute to melanoma tumorigenesis and drug resistance.

NO. 41

Myelotoxicity of thienopyridines

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Clopidogrel and Ticlopidine are thienopyridine prodrugs that need enzymatic activation by cytochrome P450 (CYP) 3A and 2B6 in order to exert their anti-platelet effects. Activation of these drugs is associated with the generation of a free mercapto group, which may be responsible not only for the therapeutic effect, but also for their toxicity. The most important toxic effects include myelotoxicity (neutropenia and agranulocytosis). Since the active metabolites formed in the liver may be too reactive to be transported into the myelon, points to chance of potential activation of thienopyridines in the myelon. We investigated the toxic mechanisms using the HL-60 promyelocytic leukemia cells, which have an intense oxidative activity. Cytotoxicity experiments showed a dose and time dependent increase of toxicity with the compounds tested. We used a colony forming unit (CFU) assay to further investigate the myelotoxicity of thienopyridines. Preincubation of the thienopyridines with

microsomes showed a reduced number of colony formation. We plan to further develop these techniques, and decipher the mechanism behind thienopyridine-induced myelotoxicity.

NO. 42

Possible role of Atg5 in anti-cancer drug induced mitotic catastrophe

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Many chemotherapeutic drugs such as etoposide, taxol, nocodazole and cisplatin at sub-lethal doses induce an autophagic response in a variety of different cells in culture, concomitantly with an increase in cell size and an arrest of cell division at the G2/M phase of cell cycle. Autophagy-related gene 5 (Atg5), which encodes a product forming a conjugate with Atg12 required for the formation of autophagosomes, is upregulated by etoposide or cisplatin treatment and is associated with the G2/M arrest which culminates in severe mitotic defects, polyploidy or aneuploidy and mitotic catastrophe. Surprisingly, ectopic enforced Atg5 overexpression using a Lentiviral transduction system by itself causes the same phenotype as etoposide, ie. aberrant mitoses, abnormal spindle formation, incomplete chromosomal segregation and mitotic catastrophe. We show that enforced expression of Atg5, in addition to promoting autophagic activity, increases cell size and arrests cell division at G2/M

phase of cell cycle concomitantly with upregulation of p53, p21 and G2/M check-point kinases (Chk2). Furthermore, the arrest of cell cycle at the G2/M phase and mitotic catastrophe caused by Atg5 overexpression is independent of p53 and p73 function, and is also independent of the autophagic response itself, since other inducers of the autophagy such as starvation, rapamycin treatment or ectopic Beclin-1 (Atg6) expression do not cause such arrest. On the other hand, silencing the Atg5 gene with short hairpin RNA (Sh-RNA) resulted in apoptosis. The effect of Atg5 overexpression can perhaps be explained by the fact that it shuttles from the cytoplasm to the nucleus upon enforced expression as well as drug treated cells and physically interacts with survivin, accumulating in the nuclear compartment. A mutant of Atg5 lacking the putative Nuclear Export Signal (NES) was constructed, which following expression, induced autophagy, but failed to cause G2/M arrest or aberrant mitoses. Taken together, our results show that Atg5 is required for cell cycle progression and is essential for both cell proliferation and survival. Therefore, Atg5 may prove to be molecular target for treatment of human malignancies.

NO. 43

Photokilling of cancer cells by ethylene glycol porphyrin derivatives involves endoplasmic reticulum stress response

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Photodynamic therapy (PDT) is a treatment modality for cancer. It combines selective accumulation of chemical compounds, called photosensitizers (PS), and light to irreversibly damage cancer cells via oxidative stress. In our study we tested porphyrin derivatives with the ethylene glycol (EG) chain linked to the phenyl ring at meta or para position. Such subtle changes in the structure of porphyrins resulted in their different intracellular localization, so that porphyrin with EG chain at para position (p-TPP(EG)4) was localized mainly in lysosomes, whereas porphyrin with EG chain at meta position (m-TPP(EG)4) in the endoplasmic reticulum (ER). m-TPP(EG)4 displayed superior PDT efficacy leading to permanent ablation of human mammary carcinoma (MDA-MB-231) in immunodeficient mice (Kralova et al., *J.Med. Chem.* 2008, 51, 5964–5973). The importance of the p38 MAP kinase signalling mechanism for the induction of apoptosis in various cell lines was demonstrated for p-TPP(EG) 4, while the mechanism for m-TPP(EG)4-mediated apoptosis remains to be elucidated. Because of prevalent localization of m-TPP(EG)4 in ER, which is the main cellular store of Ca²⁺, we evaluated the impact of Ca²⁺ homeostasis on the cell death pathway. Cells pre-loaded with membrane-permeable intracellular calcium chelator BAPTA-AM displayed a reduced level of m-TPP(EG)4-mediated apoptosis.

The effect on cytosolic Ca²⁺ levels was monitored by loading cells with fluorescent Ca²⁺ indicator Fluo-4-AM and then measured by FACS. A major rise of (Ca²⁺)_{cyt} was observed within one minute after the cells were exposed to the laser beam. These experiments indicate that the disturbance of Ca²⁺ homeostasis substantially contributes to m-TPP(EG)4-mediated apoptosis. Results of further investigation of the exact mechanism and implication of ER stress sensors are presented.

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NO. 44

Opposite roles of distinct caspase-10 isoforms in death receptor apoptotic signalling.

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Resistance to TRAIL-induced apoptosis due to caspase-8 silencing is common in aggressive neuroblastoma (NB). Both caspases-8 and -10 are often co-silenced in childhood tumour. Here we analysed the particular contribution of the four distinct caspase-10 isoforms in the death receptor-mediated apoptosis, and their ability to substitute for caspase-8.

Over-expression of caspase-10-A or caspase-10-D isoforms strongly increased TRAIL and FAS-L sensitivity of caspase-8 expressing NB and colon carcinoma cells, whereas over-expression of caspase-10-B or caspase-10-G has no effect or was weakly anti-apoptotic. Surprisingly, a complete opposite effect of the distinct caspase-10 isoforms was observed in Jurkat cells, where caspase-10-A and caspase-10-D displayed an anti-apoptotic role in the extrinsic apoptotic pathway, while caspase-10-B was weakly pro-apoptotic.

Silencing of caspase-8 in TRAIL-sensitive NB cells resulted in their complete resistance to TRAIL. This indicates that caspase-10 at endogenous expression level was unable to substitute for caspase-8. In contrast, over-expression of caspase-10-A and -D in caspase-8 silenced NB cells restored TRAIL-mediated apoptosis.

These data highlight a differential cell type-related pro- or anti-apoptotic role for the distinct caspase-10 isoforms in the death receptor signalling. Moreover they suggest that, at endogenous expression level, caspase-10 may modulate the extent of the apoptotic response, while when over-expressed caspase-10-A and -D isoforms can substitute for caspase-8 in downstream activation of apoptosis in NB cells.

NO. 45

Role of autophagy in the hypoxia-induced resistance against paclitaxel-induced apoptosis in MDA-MB-231 breast cancer cells.

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Hypoxia is a microenvironment that is often associated with tumor resistance and therapy failure. Multiple mechanisms are responsible for this adaptive response observed in cancer cells when exposed to hypoxia. This work aims to understand what are the mechanisms activated under hypoxia that induce a resistance to chemotherapy-induced apoptosis.

In order to investigate the effect of hypoxia on paclitaxel-induced apoptosis, MDA-MB-231 breast cancer cells were incubated under normoxia or hypoxia with or without paclitaxel at 50 nM and caspase 3 activity was assessed. We showed that paclitaxel (50 nM) triggers apoptosis since an increase in caspase 3 activity was observed. Hypoxia prevents this activation, indicating chemoresistance. We also showed that paclitaxel induces autophagy since LC3II accumulation and an

increased colocalization of the autophagosomes and lysosomes were observed. In order to investigate if autophagy is modulated by hypoxia and whether it is involved in the protection observed under hypoxia, Atg5 and Atg7 siRNA were used. After exposure to paclitaxel LC3II abundance was increased in control cells. However, no variation in LC3 II accumulation via the LC3I/LC3II ratio was observed in paclitaxel incubated cells, after transfection with Atg5 siRNA suggesting that the autophagy induced by paclitaxel is activated independently of Atg5 in normoxia. Under hypoxia, the invalidation of Atg5 induced LC3II accumulation indicating that under hypoxia, Atg5 contributes to autophagy and that its inhibition blocked the autophagic flow. Finally, we showed that paclitaxel induced eif2a phosphorylation, probably via the activation of the unfolded protein response (UPR). This hypothesis needs to be further investigated since the UPR is known to influence not only adaptation and survival during ER stress but also cell death through regulation of different effector pathways such as apoptosis or autophagy.

NO. 46

Comparative effects of new generation oxazaphosphorines on cancer cell death

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Programmed cell death - inducing potential of various anticancer agents has recently become a topic of interest. New generation oxazaphosphorines have attracted much attention in a broad spectrum of cancer therapy. However, scarce information on cancer cell death - induction by these agents is available. The present study was undertaken to gain a deeper insight into the anticancer action of three new generation oxazaphosphorines (NIOMECH, Bielefeld). The effects of D-17272 (mafosfamide cyclohexylamine salt), D-18864 (4-hydro-peroxy-cyclophosphamide) and D-19575 (glufosfamide, beta-D-glucosyl-isophosphoramidate mustard) on triggering programmed death in leukemic cells, were determined. The research was conducted using flow cytometry, spectrophotometry, and light microscopy methods. Temporary functional and morphological alterations occurring in dying cells, were compared. The exposure of leukemic cells to the oxazaphosphorine action resulted in

the plasma membrane impairment and phosphatidylserine externalization, mitochondrial dysfunction and DNA degradation. The three oxazaphosphorines induced apoptosis and also non-apoptotic forms of programmed cell death, in a dose and time dependent manner. The different anticancer activity of D-17272, D-18864 and D-19575, was observed. A better understanding of the mechanisms of programmed death triggered in cancer cells by the oxazaphosphorine agents is relevant to developing novel therapeutic strategies.

NO. 47

Lipid rafts and apoptosis of COLO 205 human colorectal adenocarcinoma cells

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Some kinases (e.g. PDK1, PDK2/TORC2, AKT) don't interact (phosphorylation) unless they are assembled at LR. LR facilitate recruitment and activation of PKB/Akt (phosphorylated at Ser473 residue), upon phosphatidylinositol-3-kinase (PI3-K)-dependent phosphatidylinositol-3,4,5-triphosphate modification. This reaction is fundamental for cell survival in many cell types. TNF-R1 receptor may oligomerize (trimer) on LR in response to ceramides freed from sphingomyelin by sphingomyelinases (SMA-ses). Keeping in mind small diameter of LR it is highly unlikely, that signal is evoked in single raft. Thus, it is apparent that signaling cascades have to be ignited by the clustering of several rafts. This phenomenon occurs accidentally, but it could be considerably facilitated by some enzymes (PI3-K for IGF-1R or SMAse for TNF-R1). By changes in raft composition one can modulate the activity of signaling cascades. We decided to examine two critically important signaling pathways, namely PI3-K/Akt/GSK-3 β and TNF- α /TNF-R1 in COLO 205

cells. Several factors have been tested (including anti-cancer drugs) in order to find out whether they act through LR and how do they affect above-mentioned signaling cascades. To provide evidence at ultrastructural level scanning (SEM) and transmission (TEM) electron microscopy were used. As far as we know, we showed for the first time that TNF-R1 receptors are clustered in COLO 205 cells without cognate ligand. We also provide compelling evidence that acid sphingomyelinase (aSMAse) inhibitor imipramine (IMP) blocks the TNF-R1 surface appearance, and that apoptosis stimulation is associated with proteomic rearrangement in pro and anti-apoptotic proteins.

NO. 48

Elucidation of the Physiological Role of the Bcl-2 Pro-Survival Homologue A1

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The anti-apoptotic protein A1/Bfl-1 reportedly plays a role in lymphocyte and myeloid cell development and maturation. The physiological role of A1, however, is still unclear because conventional knockout techniques cannot be applied to generate a suitable mouse model.

In order to overcome this problem we have used an alternative strategy based on RNA interference (RNAi). We chose to generate an inducible as well as a tissue-specific transgenic mouse model to knock-down A1. Therefore, we designed an expression construct encoding a shRNA targeting A1 mRNA in the context of the miR30 micro RNA. In one model, this miR30-A1 sequence was embedded in the 3'UTR of a cDNA encoding the fluorescent marker gene *Venus* transcribed from a modified version of the *Vav*-gene promoter containing lac-repressor (lacI) binding sites (lacO), which is specific for the hematopoietic system and can be regulated by IPTG in the context of lacI. In a second approach the miR30-A1 sequence is

expressed in the context of a Tet-*CMV*^{min} promoter followed by *EGFP* cDNA sequence driven by the *ubiquitin* promoter. These *Tet-miR30-A1* mice were crossed with *VavP-tTA* mice in order to drive the expression of the mir30-A1 in all hematopoietic cells. In addition, we generated Hoxb8-myeloid progenitor cell lines that can be differentiated into neutrophils or macrophages *in vitro* from *Tet-miR30-A1* mice to establish an *in vitro* system allowing manipulation of A1.

First results suggest that A1 may be important for thymocytes survival during negative selection and for B cell maturation. Furthermore, the differentiation potential into the granulocytic lineage also seems dependent on A1 availability.

NO. 49

Potential involvement of the myotonic dystrophy protein kinase in different mitochondrial cell death pathways

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Myotonic dystrophy1 (DM1) is a multi-system, autosomal dominant disorder that results from a CTG repeat expansion located in the 3' untranslated region of the gene, which encodes the DMPK Ser/Thr kinase. Mouse models have demonstrated that decreased levels of DMPK contribute to the development of DM1 muscular dystrophy. Despite its pathological relevance and ubiquitous expression, the biological functions of the protein are still poorly understood. Among six major DMPK isoforms, the human isoforms A and C have been shown to localize to the outer mitochondrial membrane by their hydrophobic C-terminal tails. Here we investigate the effects of the expression of human DMPK isoform A in the SAOS-2 osteosarcoma cell model which lacks the endogenous kinase. Our results indicate that DMPK expression alters susceptibility of SAOS-2 cells to death stimuli such as detachment of hexokinase II from mitochondria, oxidative stress and nutrient depletion. In all these conditions the expression of DMPK displays a protective effect. Hexokinase detachment and oxidative stress caused by diamide are well-known

inducers of the mitochondrial permeability transition pore (PTP) whose dysregulation is involved both in cancer and degenerative disorders. The possibility of an unprecedented regulatory mechanism that functionally links mitochondria-anchored DMPK isoform A to the PTP could therefore be relevant for the pathogenesis of DM1 and could possibly enhance the tumorigenic process through regulation of cell death/survival mechanisms.

NO. 50

Impaired intrinsic and extrinsic apoptosis in neutrophils after major trauma

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Deregulated neutrophil apoptosis after major trauma is related to overshooting immune responses, systemic inflammatory response syndrome (SIRS) and multiple organ failure (MOF). Here, we demonstrate that neutrophils from severely injured patients display impaired activation of the intrinsic apoptosis pathway, as evidenced by limited staurosporine (STO)-induced cell death. Pre-incubation of neutrophils from healthy donors or differentiated HL60 cells with patient serum fully protected cells from STO-induced cell death. This apoptosis resistance was found to depend on serum-derived GM-CSF, which in turns leads to the up-regulation of the anti-apoptotic factor Mcl-1. However, recombinant GM-CSF alone did not protect cells from STO-induced apoptosis. Apoptosis resistance could be overcome by ex vivo stimulation of neutrophil Fas with agonistic anti-Fas IgM antibodies.

Interestingly, we found increased serum concentrations of soluble Fas (sFas) early after multiple trauma, which is known to antagonize in vivo Fas/FasL

interaction. We postulate that the anti-apoptotic activity of sFas in combination with the impaired intrinsic apoptosis pathway in neutrophils after trauma might lead to ongoing inflammatory injury and its sequelae. Indeed, serum sFas levels showed a positive correlation with neutrophil elastase (PMNE) and patients' prognosis. Taken together, neutrophil Fas as well as sFas might represent therapeutic targets to limit posttraumatic hyperinflammation.

NO. 51

Investigating the role of the PIDDosome in *B* cell lymphomagenesis

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The "PIDDosome", a protein complex containing the death domain containing proteins PIDD and RAIDD is proposed to act as an activation-platform for caspase-2. Caspase-2 has been proposed to facilitate apoptosis and exhibits tumor suppressor potential. In order to investigate the physiological role of the PIDDosome in B-cell lymphomagenesis, *caspase-2* and *pidd*-deficient mice were crossed with *Eμ-myc* transgenic mice that develop pre-B or IgM⁺ B-cell lymphomas within their first six months.

Consistent with published results, we observed a significantly accelerated lymphoma onset in mice lacking caspase-2 when compared to wt, but no such effect was noted in mice lacking PIDD.

Notably, loss of *caspase-2* favored the development of more mature IgM⁺ B-cell over pre-B-cell tumors. It is well known that considerable number of *Eμ-myc* driven lymphomas show inactivation of the p53 signaling-axis, and our analysis revealed that the rate of p53 inactivation in *caspase-2* deficient tumors was significantly reduced.

Analysis of tumor cell apoptosis revealed that neither *caspase-2* nor *pidd* deficient

cells show a survival advantage, when treated with different anti-cancer drugs. Taken together, we could demonstrate that caspase-2 plays a potent role in tumor suppression but this tumor suppressive effect does not require the PIDDosome as an activation-platform for caspase-2.

NO. 52

Bcl-x_{AK}, a novel splice product of the Bcl-x gene, is critically controlled by pro- and anti-apoptotic Bcl-2 proteins, despite its lack of the BH3 domain

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Bcl-2 proteins are critical regulators of apoptosis via mitochondrial cell death pathways. Most antiapoptotic Bcl-2-related proteins share four Bcl-2 homology domains (BH 1-4), whereas the proapoptotic factors of the Bcl-2 family enclose BH1, -2 and -3 (Bax, Bak) or only BH3 (BH3-only proteins). The Bcl-x gene gives rise to alternative splice products, as antiapoptotic Bcl-x_L and proapoptotic Bcl-x_S. Recently, we have described a novel proapoptotic Bcl-x splice product (Bcl-x_{AK}, atypical killer), which carries BH2 and BH4 but lacks BH1 and BH3. This is the first proapoptotic Bcl-2-related protein without BH3.

For investigating the mechanism of the Bcl-x_{AK}-mediated apoptosis, we constructed an adenovirus for tetracycline-regulatable (Tet-Off) expression of Bcl-x_{AK}. Adenovirus-mediated expression of Bcl-x_{AK} resulted in efficient induction of apoptosis (DNA fragmentation) in melanoma cell lines at 48 h after transduction. In contrast, adenovirus-encoded BH3-only proteins (Bik/Nbk) triggered apoptosis already at 24 h. Apoptosis induction by adenovirus-mediated high Bcl-x_{AK} expression was associated

with caspase activation (caspase-3, -8 and -9) as well as disruption of the mitochondrial membrane potential ($\Delta\Psi_m$) in melanoma cells. Apoptosis induction by Bcl-X_{AK} appeared as dependent on Bax and Bak, as determined by Bax/Bak knockdown and re-constitution. Furthermore, Bcl-X_{AK}-induced apoptosis was completely blocked by overexpression of antiapoptotic Bcl-2 proteins as Bcl-2 or Bcl-X_L.

These findings imply new mechanisms for the mutual regulation of Bcl-2 proteins in the control of apoptosis, independent of the BH3 domain. New pathways may provide additional strategies for overcoming apoptosis resistance of human melanoma cells.

NO. 53

USP18 modulates the responsiveness to Interferon-alpha (IFN- α) induced apoptosis in glioblastoma cells: identification of the critical players.

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Downregulation of USP18, a type I IFN-induced protein that deconjugates the ubiquitin-like modifier ISG15 from target proteins, augments drugs-induced apoptosis. This increased apoptotic susceptibility depends on the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and the interferon pathway (Potu et al Cancer Res. 2010, 70:655). USP18 is an important negative regulator of the IFN pathway that protects cells by IFN induced apoptosis by preventing TRAIL up regulation. Glioblastoma multiforme is among the most lethal tumors with a median survival of about 1 year from the time of diagnosis. Glioblastoma cells are highly resistant to apoptosis as induced by chemotherapy. We have decided to investigate the role of USP18 in regulating resistance to IFN-induced apoptosis in different glioblastoma cell lines. We have observed that in T98G but not in U87MG cells the downregulation of USP18 potently promotes IFN-induced apoptosis. This apoptotic response is elicited by a dramatic up-regulation of TRAIL. In U87MG cells silencing of USP18 is not enough to boost consistently TRAIL expression although several IRFs are up-regulated. When the TRAIL promoter was transfected in U87MG and T98G cells transcription of the reporter gene in response to IFN was similarly observed. To evaluate differences in the TRAIL promoters of T98G and U87MG cells we cloned both promoters and evaluated their transcriptional activity when transfected in glioblastoma cell lines. Both promoters equally elicited transcription of a reporter gene in response to IFN. Studies are in progress to unveil the methylation status of the TRAIL promoter in the two cell lines. The responses to IFN in terms of apoptosis, TRAIL up-regulation and the contribution of USP18 are also evaluated in primary glioblastoma cells obtained from different patients.

In addition to defect in the TRAIL up-regulation in response to IFN, U87MG cells are also resistant to recombinant TRAIL-induced apoptosis. Gene array data, confirmed by western blot analysis, revealed that U87MG cells, compared to T98G cells, have several apoptosis related gene differentially expressed, in particular FLIP (high), BID (low) and IAPs (high). Finally, to efficiently kill U87MG cells in addition to USP18 silencing and IFN treatment, cells were challenged with several drugs in order to find a compound capable of synergizing with IFN to induce apoptosis. Preliminary data show that ER stress inducing drugs are the most promising candidates in synergizing with IFN.

NO. 54

The role of MAPK pathway in controlling mitochondrial pathway

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Mitochondrial morphology depends on the balance between fusion and fission, regulated by a number of 'mitochondria shaping proteins'. Changes in mitochondrial shape are associated with the progression of the apoptotic cascade, and cell proliferation, suggesting that fine mechanisms control mitochondrial morphology. However, very little is known about the signalling pathways that regulate mitochondria shaping proteins. MAP cascade of pleiotropic kinases is involved in the regulation of cell death. They phosphorylate and activate ERK1 and 2, which have been reported to be localized also at the mitochondrial level and to protect from apoptosis.

We have found that a dominant negative form of MAP kinase kinase (MAPKK, also know as Mek1) causes elongation of mitochondria in mouse embryonic fibroblast (MEF). A genetic analysis proved that this depended on the mitochondria-shaping proteins mitofusin 1 but not on mitofusin 2. Further investigation showed that ablation of Mek1 delayed apoptosis in wild type (wt) and Mfn2 knockout (Mfn2^{-/-}) cells but not in Mfn1 knockout (Mfn1^{-/-}) cells indicating that MekDN requires Mfn1 to delay apoptosis in MEFs. Proteomic studies showed that Mfn1 can physically interact with Erk. Our data point to a novel role for Mek and Erk in the modulation of mitochondrial shape.

NO. 55

Sensitization of melanoma cells for TRAIL by the clotrimazole analog TRAM-34 correlates with enhanced death receptor expression and caspase activation

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Melanoma is a highly metastatic tumor, which responds only poorly to chemotherapy. Potassium channels are suspected as involved in tumor progression and may thus serve as new targets. TRAM-34 is a selective inhibitor of the calcium-dependent potassium channel KCNN4. TRAIL is a well-known death ligand, which triggers apoptosis in a variety of cancer cells. Prevalent as well as inducible TRAIL resistance however limits its efficient use in cancer therapy. TRAIL resistance in melanoma cells has been recently shown by us as associated with downregulation of its agonistic receptors TRAIL-R1/DR4 and TRAIL-R2/DR5.

For the present study, we used TRAIL-sensitive melanoma cell lines (A-375, Mel-HO), permanently resistant (MeWo, Mel-2a, SK-Mel 28) as well as cell lines with acquired TRAIL resistance upon continuous cultivation and selection with TRAIL (A-375-TS, Mel-HO-TS). All cell lines responded with enhanced apoptosis to combined treatments with TRAIL and TRAM-34. Unravelling the signalling pathways in A-375-TS revealed early and strong enhancement of the main effector caspases-3 even before apoptosis was detectable. Enhanced surface expression of DR4 and DR5 as well as processing of initiator caspases-8 was indicative for activation of extrinsic

apoptosis pathways. Furthermore, TRAM-34 treatment resulted in activation of the transcription factor p53, known also to transactivate TRAIL receptors.

The data show that downregulation of TRAIL receptors is a most critical issue in resistance to TRAIL. Furthermore, KCNN4 appear as involved. Thus, selective inhibitors as TRAM-34 may be helpful in overcoming apoptosis resistance and, in combination therapies with TRAIL, may be suitable for targeting the highly therapy-resistant malignant melanoma.

NO. 56

Proteomic approaches to identify novel binding partners of the survival factor Bcl-xL

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Overexpression of the Bcl-xL protein protects a variety of cell types from apoptosis by sequestering initiators (BH3-only proteins) as well as effectors (Bax, Bak) for outer mitochondrial membrane perforation and subsequent cytochrome c release and caspase activation. Endogenous Bcl-xL is crucial for the homeostasis of the immune system as well as for embryonic development and the formation and maintenance of the neuronal network. However, despite intense research, it has remained elusive which proteins/factors regulate endogenous Bcl-xL under surviving and apoptotic conditions. We therefore established proteomic techniques to identify novel binding partners of Bcl-xL in the cytosol and on mitochondria of healthy and apoptotic monocytes and fibroblasts. Bcl-xL protein complexes of various cell lines were purified by gel filtration analysis and analyzed via SDS-polyacrylamide gel electrophoresis. Furthermore, Bcl-xL immunoprecipitation and co-immunoprecipitations were performed. Preliminary data indicate that Bcl-xL is present in protein complexes in the range between 60 – 150 kD, both in the cytosol as well as on mitochondria. This pattern is independent of the cell type used and is

clearly different from the elution pattern of recombinant Bcl-xL which is only found in lower molecular weight fractions. Since known Bcl-xL binding partners such as Bax, Bak or BH3-only proteins exhibit molecular masses between 20 – 30 kD and the possibility of Bcl-xL multimers could be excluded, these data indicate that ca. 28 kD Bcl-xL is likely to be bound to other, yet unidentified proteins. To identify these interaction partners currently mass spectrometry analyses are performed employing immunoprecipitations of endogenous as well as exogenous Bcl-xL.

NO. 57

Apoptotic death induced by the alkylating chemotherapeutics temozolomide and nimustine in glioma cell lines: influence of DNA repair and p53 triggered signalling

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Chemotherapeutics such as temozolomide (TMZ) and nimustine (ACNU), methylating and chloroethylating agents respectively, are widely used in the treatment of brain tumours. Here, we show that malignant glioma cells undergo apoptosis following treatment with these agents. Clonogenic cell death and apoptosis following TMZ is greatly stimulated by p53, while the inverse was observed for ACNU. This p53 dependence was verified by chemical inhibition of the transcriptional activity of p53 and siRNA knock-down of p53. Transfection experiments with O⁶-methylguanine-DNA methyltransferase (MGMT) and depletion of MGMT by O⁶-benzylguanine showed that, in gliomas, the apoptotic signal originate from the TMZ-induced O⁶-methylguanine and the ACNU-induced O⁶-chloroethylguanine, and that repair of these DNA lesions by MGMT prevent apoptosis. Both TMZ and ACNU caused the formation of DNA double-strand breaks (DSBs) in a replication dependent manner, which are considered to be the ultimate apoptosis-trigger for these agents. For TMZ, similar levels of DSBs were formed in p53 wild-type and mutated glioma cells, but triggered apoptosis more effectively in p53 wild-type cells. For ACNU, a much higher frequency of DSBs were observed in p53 mutated cells compared to wild-type cells. Functional p53, therefore, seems to stimulate the repair of ACNU-induced cross-links and/or DSBs generated from O⁶-chloroethylguanine. Expression analysis revealed an up-regulation of *XPC* and *DDB2* mRNA in response to ACNU in p53 wild-type but not p53 mutant cells, indicating that p53 regulates a pathway that involves these DNA repair proteins. We further demonstrate that O⁶-methylguanine- and O⁶-chloroethylguanine-triggered apoptosis employs both Fas/CD95/Apo-1 receptor and mitochondrial apoptosis activation in p53 wild-type glioma cells, whereas in p53 mutated gliomas the same DNA lesions only trigger the mitochondrial apoptotic pathway. Collectively, the data demonstrate that cell death induced by TMZ and ACNU in gliomas is

due to apoptosis and that determinants of sensitivity of gliomas to TMZ and ACNU are MGMT, p53 and DNA repair. The data further show that p53 has opposing effects in gliomas treated with methylating or chloroethylating agents, for methylating agents it stimulates apoptosis and for chloroethylating agents it stimulates DNA repair and, therefore, the p53 status should be taken into account when deciding which therapeutic drug to use.

NO. 58

The telomerase subunit TERT silencing with siRNA induces apoptosis in breast cancer cells.

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Telomeres are guanine-rich repeated sequences located at the ends of chromosomes. Loss of telomeric repeats after each cell division may function as a biological clock limiting the cell proliferation ability (Hayflick limit). Telomerase, an RNA-dependent DNA polymerase synthesizes telomeric DNA and enables cancer cells an unlimited proliferative potential. Human telomerase, composed of reverse transcriptase - TERT, RNA component-TR (functioning as a template for the telomeric DNA addition) and associated proteins is present in most malignant cells but undetectable in most normal cells. Thus, the enzyme and its altered activity, characteristic for cancer cells, is an attractive molecular target for anticancer therapy. We analyzed the gene expression profile (real-time PCR), performed telomerase activity assays (TRAP) and flow cytometry analysis.

We showed that silencing TERT telomerase subunit provoked telomerase activity significant decrease (up to 25%) and apoptosis increase in MCF-7 cells (over 13% relative to 3.6% in control cells). This effect was accompanied by antiapoptotic (BCL2, BCL2L2, BCL2L10, MCL1, TRAF2, TRAF6) decrease and proapoptotic (3,4,6,9 caspases precursors, BAD1, FADD, HSP90B1) genes expression increase.

Thus, we conclude that targeting telomerase subunits coding genes might be efficient anticancer tool due to apoptosis induction.

NO. 59

Ionizing radiation inhibits protein translation independent of Akt and mTOR activation

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The Akt signaling pathway is frequently deregulated in tumor cells and contributes to resistance against common anti-neoplastic therapies. The protein kinase Akt controls protein translation through mammalian target of rapamycin (mTOR) which, in turn, phosphorylates the initiation factor 4E binding protein (4E-BP1). In its hyperphosphorylated state, 4E-BP1 releases the eukaryotic initiation factor 4E (eIF-4E) to start cap-dependent translation. Inhibition of protein translation can result in decay of instable proteins, like the anti-apoptotic Mcl-1, and affect the vitality of the cell. However, the impact of anti-neoplastic therapies on protein translation is not well understood. In Jurkat T lymphoma cells, the Akt/mTOR pathway is constitutively activated. 3h after treatment with 50 μ M LY294002, which inhibits the pathway upstream of Akt, the translational inhibitor 4E-BP1 become dephosphorylated and Mcl-1 levels declined. Adequate Mcl-1 protein expression is essential for Jurkat cell survival, since downregulation of Mcl-1 by siRNA was sufficient to induce apoptosis. Surprisingly, ionizing radiation did not affect phospho-Akt and phospho-mTOR levels but induced a dephosphorylation of 4E-BP1. The inhibition of protein translation correlated with a drop of Mcl-1 levels and apoptosis induction.

Taken together, our data show that ionizing radiation bypasses Akt and mTOR to inhibit protein translation.

NO. 60

Regulation of mRNA splicing via PI3K-PKB-FOXO3 signalling in human neuroblastoma

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The transcription factor FOXO3 is a downstream target of protein kinase B (PKB) and controls metabolism, cell death and cell cycle progression of mammalian cells. In neuroblastoma (NB) FOXO3 is frequently inactive due to aberrant PKB signalling. Since FOXO3 activation induces apoptosis in NB cells we performed a proteomics screen for FOXO3-induced proteins. By this screen we identified several FOXO3-targets involved in mRNA processing, among them splicing factor 1 (SF1). We observed that FOXO3 increases SF1 protein levels without effecting SF1 mRNA expression and that the induction of SF1 precedes the onset of programmed cell death in human NB cell lines. Since alternative splicing is a hallmark of cancer development and may also contribute to death sensitivity we investigated if SF1 induction by FOXO3 affects mRNA splicing. For this purpose we established splice-variant-specific RT-PCRs for ER- β mRNA which is alternatively spliced in response to SF1 activation in colon carcinoma. FOXO3 activation induces SF1-specific alternative splice variants of ER- β suggesting that FOXO3 influences mRNA splicing and thereby links survival signalling pathways to global gene regulation in NB cells.

NO. 61

Understanding the switch from type II to type I FasL-induced apoptotic signaling in primary mouse hepatocytes

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The TNF-like receptor Fas/CD95 plays an essential role in the maintenance of the liver. Upon binding Fas ligand (FasL), Fas can trigger both the so-called type I (or direct) and type II (or mitochondrial) apoptosis pathways, depending on the cellular context. *In vivo*, mouse hepatocytes undergo apoptosis via the so-called type II pathway, *i.e.*, implicating the formation of tBid and its translocation to mitochondria, activation of Bax/Bak on the outer mitochondrial membrane and the subsequent release of cytochrome c into the cytosol to allow apoptosome formation and caspase-3 activation. Surprisingly, freshly isolated mouse cells cultivated on a monolayer of collagen I, do not require Bid for apoptosis signaling, bypassing mitochondria to directly activate caspase-3. Conversely, hepatocytes kept in suspension retain the type II signaling pathway. We compared FasL-induced type I/II signaling between primary hepatocytes cultured in suspension, on stiff collagen monolayer and in a collagen sandwich gel, in order to better understand the molecular mechanism of the signaling switch. We found that primary hepatocytes cultured in a collagen sandwich still exhibited the type II to I switch. However, we observed lower levels of FLIP_L in suspension cells and a higher caspase-3 activity and processing and faster apoptosis kinetics in the collagen sandwich as compared to monolayer culturing. We are currently investigating integrin signaling triggered by collagen I, which may cross-talk to Fas-induced signaling, probably promoting the type II to type I switch.

NO. 62

Characterisation of the anti-metastatic effect of Interferon- γ in Ewing`s Sarcoma

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Ewing`s Sarcoma (ES) is the second most common bone tumor in childhood and adolescence. Prognosis is particularly poor in patients with metastatic and recurrent disease, with an overall survival of around 20%. Recently, lack of caspase-8 expression has been shown to be linked to the formation of metastasis in neuroblastoma. In previous studies we were able to show that: 1. in a mouse xenograft model of ES development of metastasis is suppressed by application of interferon- γ and 2. Interferon- γ promotes the expression of caspase-8 in caspase-8-deficient ES cell lines.

In order to study the influence of interferon- γ on formation of metastasis in ES, we have investigated the migration in transwell- and scratch-assays. These experiments revealed that interferon- γ inhibits the migration in ES cell lines. To date it is unclear, which proteins are involved in the process of metastasis in ES. We currently aim to identify these proteins. Because caspase-8 is regulated by interferon- γ , caspase-8 is one of the first candidate proteins to prove.

NO. 63

Investigation of Hexokinase II as a target of GSK3 β phosphorylation and regulation

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Hexokinase II (Hxk II) is a central enzyme in glucose metabolism and overexpressed in many cancer cells. It contributes to the characteristic glycolytic phenotype of malignant cells, known as the Warburg effect. The majority of the metabolic enzyme was found to be bound to the outer mitochondrial membrane. In addition to its impact on the metabolism of the cell, mitochondrial Hxk II was shown to have antiapoptotic properties by interfering with cytochrome c release. The regulation of Hxk II localization was suggested to be mediated by the PI3K/AKT pathway. It has been demonstrated that Akt inhibits the detachment of Hxk II from the mitochondria, the exact mechanism however remains unknown.

In this study we are investigating glycogen synthase kinase 3 β (GSK3 β) as the missing link between AKT and Hxk II. GSK3 β represents a promising candidate in this process because it is negatively regulated via direct phosphorylation by AKT. In support of this idea the HxkII amino-acid sequence contains several conserved GSK3 β recognition sites.

We therefore tested if GSK3 β has the potential to modify Hxk II in-vitro and thereby influences the localization, activity or post-translational modification pattern of the enzyme.

NO. 64

Cancer cell death - induction by new derivatives of daunorubicin

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Chemical modifications of currently approved anthracycline antibiotics are an important way to improve their anticancer activity. In the search for new anthracyclines, daunorubicin (DRB) derivatives have been synthesized. In the present investigations, the effects of two DRB analogues containing an amidine group bonded to C-3' of daunosamine moiety with either morpholine (DRBM) or hexamethyleneimine (DRBH) ring, on triggering cancer cell death, were studied. The experiments were performed *in vitro* on two human leukemic cell lines ML-1 and MOLT-4. The research was conducted using microscopy, spectrophotometry, and cytometry methods. Temporary morphological and functional changes occurring in pathological hematopoietic cells after their exposure to the anthracycline antibiotics, were compared. The frequency of cells undergoing apoptosis and other forms of programmed death, the cell count and cell population distribution dependent on cell volume, the frequency of cells expressing DNA damage and the mitochondrial activity of leukemic cells, were determined. The effects of DRB, DRBM and DRBH on MOLT-4 and ML-1 cells were dependent on the agent tested and its dose, the time intervals after the anthracycline application, and the type of leukemic cells being stimulated to die. The different cancer cell response to the action of daunorubicin and its two derivatives, was shown.

NO. 65

Modulation of autophagy and cellular stress response pathways can influence survival of malignant melanoma cells

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Activation of the ERK MAPK pathway is critically important for melanoma development and survival, and small molecule inhibitors of the pathway have been studied as potential anti-melanoma drugs. In contrast, the roles of other MAPK pathways in melanoma biology still remain unclear.

We have tested in viability and proliferation assays the response of melanoma cell lines to SB202190, a small molecule inhibitor of the p38 MAPK. Simultaneously, cellular morphology was studied using light, electron and digital holographic microscopy.

Pharmacological inhibition of p38 had only small impact on the viability of malignant melanoma cells but it caused upregulation of autophagy-related subcellular structures. Autophagy has been reported to promote both cell survival and cell death. In our experiments, inhibition of autophagy using chemical inhibitors significantly decreased the viability of SB202190-treated cells, suggesting that autophagy might be required for melanoma cell survival upon p38 inhibition. Moreover, p38 inhibitor-treated cells became extremely sensitive to ER stress inducer thapsigargin, indicating that p38 signalling might be also important for the response of melanoma cells to ER stress.

Collectively, our results suggest that p38 signaling and autophagy play important roles in melanoma cell biology and together with the ER stress response pathway might constitute targets for the development of novel anti-melanoma therapeutic strategies.

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NO. 66

Effect of hypoxia and oxidized LDL on autophagic and apoptotic responses in THP-1 derived macrophages.

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Foam cells, which are present in the deepest layers of atherosclerotic plaque, can be submitted to hypoxia. Hypoxia is known to increase the lipid load and to modulate many pathways. We studied the effects of hypoxia on two aspects of human macrophage derived foam cells : lipid loading and death mechanisms. As experimental model, we used two types of oxidized LDL, copper sulfate oxidized LDL (Ox-LDL) and myeloperoxidase modified LDL (Mox-LDL) to make foam cell out of THP-1 derived macrophages. Both types of LDL induced a marked foam cell phenotype with increased expression of adipophilin. Hypoxia further enhanced the lipid load without increasing the amount of adipophilin in the presence of Ox-LDL. We then focused on the stress and death pathways induced in those heavily lipid loaded foam cells. No apoptosis was evidenced. However, we highlighted many markers of the UPR (eIF2 α phosphorylation, XBP-1 splicing, Grp 78 overexpression and CHOP overexpression and relocalisation) in cells incubated with Ox-LDL both under hypoxia and normoxia. When UPR was observed, the amount of LC3 II, a marker of the autophagy process, was also increased. Preliminary TEM results also show

features of autophagy in these conditions. The possible link between UPR and autophagy and the precise role of autophagy will be further studied.

NO. 67

Investigating the role of BH3-only proteins in BAFF-mediated B cell development

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B cells undergo many selection processes before becoming mature and immunocompetent. The TNF family ligand B cell-activating factor (BAFF), which binds three receptors: BCMA, TACI and BAFF-R, plays an important role in B cell development and survival. Its absence causes the loss of most mature B cells including transitional type 2 (T2), follicular (FO) and marginal zone (MZ) B cells. This deficit can be partially rescued by overexpression of Bcl2. The prosurvival function of Bcl2 is antagonized by BH3-only proteins such as Bim or Bmf. Since loss of these BH3-only proteins results in an accumulation of mature B cells we crossed *bim*^{-/-} and *bmf*^{-/-} animals with mice that overexpress a TACI-Ig fusion protein, in which BAFF is sequestered and functionally inhibited.

Preliminary results suggest, that the deletion of Bim or Bmf can restore in part the survival of T2, FO and MZ B cells in TACI Ig transgenic mice, which is even more pronounced when both BH3-only proteins were lacking.

We conclude that BAFF functions by modulating the expression and/or function of Bim and Bmf, the molecular basis remains to be investigated in full detail.

NO. 68

Processing of Metacaspase Into a Cytoplasmic Catalytic Domain Mediating Cell Death in *Leishmania major*

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Metacaspases are cysteine peptidases that could play a role similar to caspases in the cell death program of plants, fungi and protozoa. The human protozoan parasite *Leishmania major* expresses a single metacaspase (LmjMCA). In this study, we investigated the processing sites important for the maturation of LmjMCA catalytic domain, the cellular localization of LmjMCA polypeptides, and the functional role of the catalytic domain in the cell death pathway of *Leishmania* parasites. Although LmjMCA polypeptide precursor form harbors a functional mitochondrial localization signal (MLS), we determined that LmjMCA polypeptides are mainly localized in the cytoplasm due to an amino-acid sequence downstream of the MLS, which impaired its transport into the mitochondrion. In stress conditions, such as exposure to heat shock, hydrogen-peroxide (H₂O₂) or anti-*Leishmania* drugs, LmjMCA precursor forms were extensively processed into soluble forms containing the catalytic domain. We showed that this domain was sufficient to enhance sensitivity of parasites to H₂O₂ by disrupting the mitochondrion. These data provide experimental evidence of the importance of the activity of the catalytic domain in disrupting mitochondria and of LmjMCA processing into an active catalytic domain which could be relevant in the design of new drugs to fight leishmaniasis and likely other protozoan parasite diseases.

NO. 69

LOVASTATIN INDUCES APOPTOSIS OF CLL CELLS BY ACTIVATION OF CASPASES

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Chronic lymphocytic leukemia (CLL) is the most common type of adult leukemia in the Western world.

The aim of current research was to examine the proapoptotic potential of competitive HMG-CoA reductase inhibitor – lovastatin in mononuclear cells (MNCs) isolated from peripheral blood of untreated patients with CLL.

Flow cytometrical analyses revealed that this inhibitor, at concentrations which were harmless to normal MNCs, influenced on cell viability and triggered apoptosis of CLL cells *ex vivo*. Apoptosis course in model cells exposed to tested statin was confirmed by DNA fragmentation (apoptotic ladder, sub-G₁ cell population) and caspase-dependent cleavage of PARP-1 and lamin B. The expression levels of some cellular proteins related to life-or-death decision and cell cycle regulation were also assessed.

The obtained results indicate that lovastatin-induced apoptosis of CLL cells goes via intrinsic pathway with caspase-9 activation and rapid deprivation of Mcl-1 protein. The apoptosis was accompanied by a proteolysis of proapoptotic protein Bax to its 18 kDa cleavage product as well as by a degradation of cell cycle inhibitory protein – p27^{KIP1}.

In summary, our preliminary results imply that lovastatin induces apoptosis of CLL cells and should be considered as antileukemic drug administered individually or, perhaps, as an adjunct to conventional chemotherapy.

NO. 70

Inhibition of fatal renal cell apoptosis and prolonged survival in lupus-prone NZB/W F1 mice treated with the topoisomerase I inhibitor irinotecan

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Topoisomerase I are enzymes responsible for releasing torsional stress of DNA. They induce single-stranded DNA breaks enabling the unwinding of genomic DNA. Unwinding is followed by re-ligation of DNA which is also mediated by topoisomerase I. Inhibitors of topoisomerase I bind to the DNA-topoisomerase I complex thus preventing the re-ligation of DNA. In dividing cancer cells the complex of DNA, topoisomerase I and inhibitor can collide with replication forks resulting in the generation of double-stranded DNA breaks and apoptosis. This mechanism explains why inhibitors of topoisomerase I are widely used for chemotherapy of metastatic cancer. There is a different situation in non-dividing cells where inhibitors of topoisomerase I produce single-stranded DNA breaks which are not lethal but most likely change the structure of genomic DNA. In this context we recently found that the topoisomerase I inhibitor irinotecan reverses immune complex glomerulonephritis and prolongs survival in mice with systemic lupus erythematosus, an autoimmune disease which is defined by apoptosis-inducing anti-double stranded DNA antibodies. Furthermore, while the level of anti-double stranded DNA antibodies was not reduced in irinotecan-treated mice, irinotecan avoided the induction of fatal renal cell apoptosis which was determined by TUNEL and caspase-3 activity. Prevention of renal cell apoptosis was accompanied by an impressive reduction of subendothelial immune deposits being representative for severe stages of lupus-associated glomerulonephritis. In conclusion, our data show that the topoisomerase I inhibitor irinotecan can serve as an inhibitor of apoptosis under certain circumstances. It further suggests irinotecan as a new treatment option for systemic lupus erythematosus.

NO. 71

DMU-212 induced apoptosis in LOVO and DLD-1 cell lines

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The enzyme CYP1B1 is expressed at a high frequency in a range of human cancers including breast, colon, lung etc. This enzyme is not detectable in normal tissues, therefore the expression of CYP1B1 in tumour cells provides an interesting molecular target for the development of new anticancer drugs that could be selectively activated in tumour cells. It was reported before, that DMU-212 (3,4,4',5'-trans-tetrametoksystylbene) is metabolized by CYP1B1 to higher hydroxylated analogues. Hence, it may be hypothesized that products of DMU-212 hydroxylation by CYP1B1 may act as prooxidants and induce oxidative stress selectively in cancer cells. This suggestion has been tested on two colonorectal cell lines, LOVO and DLD-1. The calculated IC₅₀ values (MTT assay) for LOVO and DLD-1 were 10.4 µM and 0.9 µM, respectively. In both cell lines caspase-3/7 dependent apoptosis was induced. Incubation of both cell lines with DMU-212 resulted in an induction of oxidative stress which was shown using 2',7'-dichlorodihydro-fluorescein assay. It was found, however, that DMU-212 inhibited EROD (CYP1B1 marker) activity as well as decreased CYP1B1 expression in these cells, therefore it is more probably that DMU-212 is inactivated not by CYP1B1 but rather by CYP1A1/1A2 because their expression remained unchanged after incubation with DMU-212.

NO. 72

Autism Spectrum Disorder is related to endoplasmic reticulum stress induced by mutations in the synaptic cell adhesion molecule, CADM1

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Autism spectrum disorder (ASD) is a neurodevelopmental disorder with an unknown molecular pathogenesis. We previously found that patients with ASD carried one of two missense mutations, H246N or Y251S, in the gene encoding synaptic cell adhesion molecule-1 (CADM1). However, when the *Cadm1* gene was deleted in mice, they did not exhibit the core symptoms that patients with ASD exhibited. This suggested that loss of function alone may not give rise to the pathogenesis of ASD. *In vitro*, the mutated CADM1 exhibited slightly reduced homophilic interactions compared to wild type CADM1 but most of their interaction remains. The mutated CADM1 showed morphological abnormalities, including shorter dendrites, and impaired synaptogenesis in neurons. The mutated CADM1 accumulated in the ER and induced the up-regulation of C/EBP-homologous protein, and this could be inhibited with ER stress inhibitors, like 4-phenyl butyric acid and rapamycin. Modeling analysis suggested that the mutations were directly related to the reduced interactions and conformation alteration. Thus, CADM1 mutations induced both loss of CADM1 function and ER stress due to the unfolded protein response. ER stress may be involved as gain-of-function in the pathogenesis of ASD.

NO. 73

Targeting Phosphoinositide 3-Kinases Impairs Cell Cycle Progression and Survival in Small Cell Lung Cancer

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The phosphoinositide 3-kinase (PI3K) pathway, fundamental for cell proliferation, survival, and motility, is known to be frequently altered and activated in neoplasia, including carcinomas of the lung. Based on the high frequency of alterations, targeting components of the PI3K signalling pathway is considered to be a promising therapeutic approach in cancer treatment. In this study we investigated the potential of targeting PI3K signalling in small cell lung cancer (SCLC), which is the most aggressive of all lung cancer types and almost entirely related to smoking.

Over-expression of the PI3K isoforms p110 α and p110 β was shown by immunohistochemistry in primary SCLC tissue samples. Targeting the PI3K p110 α or p110 β with specific pharmacological inhibitors resulted in strongly affected cell viability of SCLC cells. Inhibition of p110 α resulted in G1 cell cycle arrest, increased apoptosis and was also able to sensitize SCLC cells to commonly used chemotherapeutic agents, such as etoposide and carboplatin. Expression and phosphorylation state of PI3Ks and signalling molecules were studied by Western blot and Taqman analysis. We could observe decreased phosphorylation levels in PI3K pathway components, such as Akt, ribosomal S6 protein, and 4E-BP1, and reduced expression of cell cycle-related molecules, like p21, cyclin D₁, and cyclin E. Together, our data suggest involvement of PI3K isoforms p110 α and p110 β in SCLC cell survival processes and our studies will lead to a better understanding of the biological function of PI3K isoforms in controlling cell responses, such as proliferation, apoptosis, and metastasis in SCLC.

NO. 74

Doxorubicin cancer therapy induces autophagy in adult cardiomyocytes

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Objective: Doxorubicin (Doxo) is a robust chemotherapy drug used in the treatment of various cancers. Its usefulness in the clinic has been hindered by its side effects, most notably that of dilated cardiomyopathy and congestive heart failure due to myocardial cell death. The role of autophagy in doxorubicin-induced cardiotoxicity is not fully understood. Our hypothesis is that Doxo induces autophagy, as a maladaptive response, leading to cell death.

Methods: Chronic effects of Doxo were studied in isolated adult rat cardiomyocytes cultured for a total of 12 days in medium containing 20% FCS and exposed to the cancer therapy for 48 hours. Measurement of mitochondrial membrane potential (MTT), release of lactate-dehydrogenase (LDH) and DNA degradation were used for testing apoptosis and necrosis. Autophagic activity was monitored by Western blotting for the autophagosome marker LC-3 I/II and accumulation of poly-ubiquitinated proteins and Cathepsin-D by immunofluorescence microscopy.

Results: LC-3 I/II protein was found to be increased in a dose-dependent manner by Doxo. Accumulation of polyubiquitin-positive aggregates, Cathepsin-D-positive vesicles and myofibrillar disarray were observed at 1 μ M Doxo. Regulation of autophagy by Doxo was further confirmed using modulators 3-MA and chloroquine. Typical markers for apoptosis (TUNEL) and necrosis (LDH-release, MTT) showed a significant increase of cell death only at supraclinical Doxo concentrations of >20 μ M for 48 hours.

Conclusions: We conclude that low doses of Doxo cause autophagy and the accumulation of oxidatively damaged macromolecules in the absence of DNA-degradation in adult ventricular cardiomyocytes. Higher doses of Doxo further increase autophagy stimulation leading to cell death. Manipulating autophagic activity in the myocardium challenged by cytotoxic therapies may emerge as meaningful intervention in order to enhance cardiomyocyte survival.

NO. 75

Genetic dissection of the relationship between cytochrome *c* release and mitochondrial fragmentation during apoptosis

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Background

The release of cytochrome *c* from mitochondria and the downstream activation of effector caspases is the point of no return during apoptosis induced by intrinsic stimuli. Permeabilization of the outer mitochondrial membrane is accompanied by remodelling of the cristae and fragmentation of the mitochondrial network. The temporal relationship between these events and their contribution towards cytochrome *c* release is a matter of debate. In particular, it is unclear if fragmentation precedes or follows release of cytochrome *c* and activation of caspases.

Results

In order to address this question, we are combining the use of genetic models of blockage of outer membrane permeabilization (*Bax*^{-/-}/*Bak*^{-/-}), caspase activation (*Apaf-1*^{-/-}) and mitochondrial fragmentation (*Drp1*^{-/-}), with simultaneous assessment of morphology of the mitochondrial network and of cytochrome *c* release. In *Apaf-1*^{-/-} cells cytochrome *c* is released with the same kinetics as in their wt counterparts. In *Drp1*^{-/-} cells however we see no release of cytochrome *c* within the observed timeframe and observe delayed fragmentation of mitochondria.

Conclusions

We do not find evidence for the involvement of Caspases in mitochondrial fragmentation or cytochrome *c* release during apoptosis. Furthermore, fragmentation of mitochondria can be genetically separated from permeabilization of the outer mitochondrial membrane and activation of downstream caspases.

NO. 76

Role of the Bcl-2 homolog Bim in T cell-mediated liver immunopathology

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Liver disease induced by the cytotoxic immune response directed against infections with non-cytopathic viruses (e.g. HBV, HCV) still represents one of the main cause of mortality all over the world. The pro-apoptotic BCL-2 family member Bim has been shown to play a central role in hepatocyte apoptosis, as well as in the control of immune homeostasis. Nevertheless the role of Bim in the development of liver damage during viral infections is far from being understood. Thus the aim of the present study is to understand the role of Bim in both, liver tissue damage and apoptosis of T cells during viral infections of the liver. For this purpose we are using the model system of T cell-mediated hepatitis in mice infected with high doses of Lymphocytic Choriomeningitis Virus (LCMV-WE).

The analysis of liver damage in LCMV-WE infected mice by measurement of alanine aminotransferase serum levels clearly demonstrated that Bim^{-/-} mice are partially protected from development of T cell mediated liver disease. Confirming these results we observed reduced caspase-3 activity in the livers of the infected Bim^{-/-} mice compared with WT animals. To clearly define if Bim plays a central role in hepatocyte apoptosis or is more implicated in the persistence of activated T cells and as a consequence liver destruction, we performed experiments with bone marrow chimeras. Therefore bone marrow from WT or Bim^{-/-} mice was transferred in lethally irradiated WT (WT[→]WT; Bim^{-/-}[→]WT) or Bim^{-/-} (WT[→]Bim^{-/-}) mice. Eleven days after infection WT[→]Bim^{-/-} mice exhibited reduced liver damage and caspase-3 activity compared to WT[→]WT mice. As expected both types of chimeras showed equal numbers of virus specific CD8⁺ T cells as well as comparable virus titers in their livers. On the other hand WT[→]WT and Bim^{-/-}[→]WT chimeras did not display any differences in induction of liver damage although Bim^{-/-}[→]WT chimeras generated a stronger LCMV specific cytotoxic T cell response.

Taken together these results suggest that Bim expressed in the hepatocytes represents an important apoptosis mediator during T cell induced liver damage in LCMV-WE infected mice while the absence of Bim in the lymphoid compartment does not play a role in this model of acute liver damage.

NO. 77

Inflammation-associated autophagy-related programmed necrotic neutrophil death characterized by organelle fusion events

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The most common form of neutrophil death, under both physiologic and inflammatory conditions, is apoptosis. Here, we report a novel form of programmed necrotic cell death, associated with cytoplasmic organelle fusion events, that occurs in neutrophils exposed to inflammatory cytokines upon ligation of CD44. Strikingly, this type of neutrophil death requires both class I and III phosphatidylinositol 3'-kinase (PI3K) activation, signalling events usually required for survival pathways and autophagy induction, respectively. In the death pathway reported here, PI3K is required for the generation of reactive oxygen species (ROS), which somehow trigger the generation of large cytoplasmic vacuoles, likely generated by the fusion of CD44-containing endosomes with autophagosomes and secondary, but not primary, granules. Neutrophils demonstrating vacuolization undergo rapid cell death that depends on receptor-interacting protein 1 (RIP1) kinase activity and papain family protease(s), but not caspases, that are most likely activated and released, respectively, during or as a consequence of organelle fusion. Vacuolized neutrophils are present in infectious and autoimmune diseases under in vivo conditions. Moreover, isolated neutrophils from such patients are highly sensitive toward CD44-mediated PI3K activation, ROS production, and cell death, suggesting that the newly described autophagy-related form of programmed neutrophil necrosis plays an important role in inflammatory responses.

NO. 78

The role of pro-apoptotic BH3-only proteins during engraftment and reconstitution of human haematopoietic stem cells

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During stem cell therapy the transplanted haematopoietic stem and progenitor cells (HSPC) sustain deprivation of survival signals (i.e. cytokines or adhesion molecules) normally provided by the stem cell niche. Differentiation as well as cell death occurring prior to successful engraftment hamper and limit this therapy. Transient inhibition of apoptosis in HSPC thus may lead to an increased efficacy of stem cell therapy.

It has been shown, that murine bone marrow cells overexpressing the anti-apoptotic protein Bcl-2 or lacking the pro-apoptotic BH3-only proteins Bim or Bmf are more competitive during reconstitution of lethally irradiated recipient mice and displace wild type lymphopoiesis completely in competitive reconstitution experiments.

To test whether inhibition of apoptosis is also able to improve the efficacy of human HSPC during stem cell therapy we are currently working with fresh, cord blood derived CD34+ cells enriched for HSPC. In order to mimic lack of survival signals occurring during transplantation we subjected the cells to cytokine withdrawal. Under this treatment we could observe activation of Bim and Bmf followed by apoptosis. Thus we are trying to inhibit these two BH3-only proteins by RNAi and thereby render human CD34+ cells more resistant against cellular stress. Whether this confers advantages during stem cell therapy will be answered using a xenograft mouse model for human haematopoiesis.

NO. 79

Lack of the BH3-only proteins Bim, Bmf and Puma in haematopoietic stem and progenitor cells facilitates early reconstitution and long term haematopoiesis

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During haematopoietic stem cell transplantation the transplanted haematopoietic stem and progenitor cells (HSPC) suffer a transient deprivation of survival signals (i.e. cytokines or adhesion molecules) normally provided by the stem cell niche. Therefore, cell loss may occur prior to successful engraftment and thus restrict haematopoietic reconstitution. Apoptosis induced in response to lack of cytokines or contact to the extracellular matrix is regulated by members of the Bcl-2 family. Using different mouse models lacking the BH3-only pro-apoptotic Bcl-2 family members Bim, Bmf or Puma, all implicated in leukocyte homeostasis, we aimed to delineate which one of these Bcl-2 family proteins is critically involved in limiting successful reconstitution of the haematopoietic system.

Our results demonstrate that HSPC lacking Bim show accelerated reconstitution of lethally irradiated recipient mice. Moreover, competitive reconstitution experiments reveal that wild type haematopoiesis is completely displaced in wt:bim^{-/-} and strongly suppressed in wt:bmf^{-/-} and wt:puma^{-/-} bone marrow chimeras. The effects of Bim deficiency are comparable to those induced by overexpression of Bcl-2 and can neither be exceeded by additional loss of Puma nor loss of Bmf, identifying Bim as the major BH3-only protein during haematopoiesis. Since both lymphopoiesis and myelopoiesis are similarly affected, a direct role of Bim on early progenitor or even stem cells can be assumed.

In summary, inhibition of apoptosis in HSPC by transiently interfering with Bim-function may be a promising strategy to increase the efficacy of HSCT and reduce transplantation-related morbidity.

**CONTROL OF INFECTIONS BY INFLAMMASOMES: ROLE OF NITRIC OXIDE
AND PIROPTOSYS**

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Keywords: Flagellin; iNOS; inflammasomes; caspase-1; pyroptosis

Innate immune recognition of flagellin is shared by transmembrane TLR5 and cytosolic NAIP5/NLRC4. TLR5 activates inflammatory genes through MYD88 pathway. NLRC4 and NAIP5 assemble multiprotein complexes called inflammasomes, leading to caspase-1 activation, IL-1 β /IL-18 secretion and infected cell death. Although both TLR5 and NAIP5/NLRC4 pathways cooperate to clear infections, little is known about the relative anti-pathogen effector mechanisms operating through each of them. Here we show that the cytosolic flagellin (FLA-BSDot) was able to activate iNOS, an enzyme previously associated with TLR5 pathway. Using *Nlrc4*- or *Naip5*-deficient macrophages, we found that both receptors are involved in iNOS activation by FLA-BSDot. Moreover, distinct from extracellular flagellin (FLA-BS), iNOS activation by intracellular flagellin is completely abrogated in the absence of caspase-1. Interestingly, IL-1 β and IL-18 do not seem to be important for FLA-BSDot-mediated iNOS production. Together, our data defined an additional anti-pathogen effector mechanism operated through NAIP5 and NLRC4 inflammasomes and illustrated a novel signaling transduction pathway that activates iNOS. Finally, this pathway contributes with inflammasome-induced pyroptosis to the control of *Salmonella typhimurium* and *Legionella pneumophilla* infections.

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