10th
TNF Superfamily Conference

Beau-Rivage Palace (Ouchy)
Lausanne 2004

Organizing Committee

Jürg Tschopp
Jeffrey Browning
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Pascal Schneider
Carl Ware

10th TNF Superfamily Conference
Lausanne 2004

Conference & Gala Dinner: Room Sandoz
Exhibition, Coffe & Lunch: Room Rotonde
Posters: Arcades and Library

Wednesday, September 29

1:00-8:00 pm Registration

6:30pm WELCOME ADDRESS
Jürg Tschopp (Lausanne, Switzerland)
WELCOME IN LAUSANNE
Doris Cohen-Dumani (Lausanne, Switzerland)

6:40 pm KEYNOTE LECTURE
Michael Karin (La Jolla, USA) L01
The IKK complex in inflammation, cancer and cell survival

7:30 pm WELCOME RECEPTION (Olympic Museum)

Thursday, September 30

8:15-10:00 am DEATH RECEPTORS

8:15-8:45 **David Wallach** (Rehovot, Israel) L02
Signaling by TNF/NGF receptor family: new functions for old players

8:45-9:15 **Richard Siegel** (Bethesda, USA) L03
Regulation of death receptor signaling by surface receptor oligomers and horizontal adapter protein interactions

9:15-9:30 **Henning Walczak** (Heidelberg, Germany) L04
TGF-β-induced apoptosis is mediated by TRAIL

9:30-9:45 **Frédéric Rieux-Laucat** (Paris, France) L05
Autoimmune lymphoproliferative syndrome (ALPS-III) caused by somatic mutations of TNFRSF6 (Fas)

9:45-10:00 **Hiroyasu Nakano** (Tokyo, Japan) L06
NF-κB inhibits TNFα-induced accumulation of reactive oxygen species (ROS) that mediate prolonged JNK activation and necrotic cell death

10:00 *Coffee*

10:30-12:15 am INFLAMMATION & IMMUNITY I

10:30-11:00 **Fabienne Mackay** (Sydney, Australia) L07
The role of BAFF in autoimmunity: Is it just a B cell story?

11:00-11:30 **Vishva Dixit** (San Francisco, USA) L08
De-ubiquitination and ubiquitin ligase domains of A20 downregulate NF-κB signaling

11:30-11:45 **Teresa Cachero** (Cambridge, USA) L09
BAFF lacking a histidine tag can form virus-like clusters in solution

11:45-12:00 **Pascal Schneider** (Lausanne, Switzerland) L10
Identification of a third "receptor" for APRIL

12:00-12:15 **Stefan Schütze** (Kiel, Germany) L11
Compartmentalization of TNF-Receptor 1 Signaling: Internalized TNF-Receptosomes as Death Signaling Vesicles

12:15-12:40 pm TUTORIAL
Marco Gut (Applied Biosystems)
Gene expression profiling: Moving forward in candidate gene screening and molecular signatures

12:30 *Lunch*

1:30-3:15 pm INFLAMMATION & SIGNALLING

1:30-2:00 **George Kollias** (Athens, Greece) L12
Functional variations and differential thresholds of p55TNFR function in physiology and disease

2:00-2:30 **Anders Nykjaer** (Aarhus, Denmark) L13
Sortilin, a novel p75NTR co-receptor essential for proNGF-induced neuronal apoptosis

2:30-2:45 **Remco van Horssen** (Rotterdam, The Netherlands) L14
EMAP-II sensitizes endothelial cells towards TNF-induced apoptosis by facilitating TNF-R1 apoptotic signaling via TRADD-mobilisation

2:45-3:00 **Rudi Beyaert** (Ghent, Belgium) L15
Protective effect of NF-κB inhibition by adenoviral gene expression of ABINs in mouse models of inflammatory disease

3:00-3:15 **Isabelle Cremer** (Paris, France) L16
CD40 activation of primary B cells induces TRAF4 protein expression

3:15 *Coffee*

3:45-5:15 pm DEVELOPMENT & TISSUE REMODELING I

3:45-4:15 **Marja Mikkola** (Helsinki, Finland) L17
The role of Ectodysplasin A1 in placodal cell fate

4:15-4:45 **Bill Dougall** (Seattle, USA) L18
RANKL blockade as a novel therapy for human bone disorders: Biological rationale and clinical experience

4:45-5:00 **Nadia Corazza** (Bern, Switzerland) L19
The role of TRAIL (TNF-Related Apoptosis-Inducing Ligand) in thymocyte apoptosis

5:00-5:15 **Timothy Zheng** (Cambridge, USA) L20
Regulation of muscle satellite cell activation by Tweak/Fn14 pathway

5:15 *Coffee*

5:45-7:15 pm DEVELOPMENT & TISSUE REMODELING II

5:45-6:15 **Nancy Ruddle** (New Haven, USA) L21
Lymphoid organogenesis and neo-organogenesis: cytokines, chemokines and adhesion molecules

6:15-6:45 **Reina Mebius** (Amsterdam, The Netherlands) L22
Molecular and cellular aspects of secondary and teritary lymphoid structure formation

6:45-7:00 **Sergei Nedospasov** (Frederick, USA) L23
Novel knockin and transgenic models to study physiological functions of TNF and lymphotoxin

7:00-7:15 **Gerard Eberl** (New York, USA) L24
Lymphoid tissue inducer cells in the fetus and the adult gut

8:00 pm *Swiss Dinner (Optional)*

Friday, October 1

8:15-10:00 am INFLAMMATION & IMMUNITY II

8:15-8:45 **Eckhard Podack** (Miami, USA) L25
DR3 Signaling in the Lung Promotes IL-13 Production and Asthma

8:45-9:15 **Masayuki Miura** (Tokyo, Japan) L26
Genetic pathway of TNF/TNFR signaling in Drosophila

9:15-9:30 **François Leulier** (Gif-sur-Yvette, France) L27
Caspase regulation of NF-κB dependant innate immunity in Drosophila melanogaster

9:30-9:45 **Rama Khokha** (Toronto, Canada) L28
Abnormal TNF bioactivity in TIMP-3 null mice

9:45-10:00 **Roy Black** (Seattle, USA) L29
Shedding of TNF receptors by multiple metalloproteases and modulation of TNF-responsiveness

10:00 *Coffee*

10:30-12:15 am CO-STIMULATORY LIGANDS

10:30-11:00 **Michael Croft** (San Diego, USA) L30
Regulation of T cell Survival by OX40

11:00-11:30 **Sarah Hymowitz** (San Francisco, USA) L31
The crystal structure of the OX40-OX40L complex reveals an extensive interface between a small divergent ligand and a more conventional receptor

11:30-11:45 **Jan Paul Medema** (Leiden, The Netherlands) L32
The TNF family member APRIL promotes B-1 cell-associated neoplasm

11:45-12:00 **Tania Watts** (Toronto, Canada) L33
TRAF1 plays a positive role in T cell expansion in vivo

12:00-12:15 **Martijn Nolte** (Amsterdam, The Netherlands) L34
CD27 on hematopoietic stem and progenitor cells serves as a negative feedback mediator on leukocyte differentiation

12:15-12:40 am TUTORIAL
Keith Watling (Sigma/RBI)
Cell signaling

12:30 *Lunch*

1:30-3:15 pm HOST DEFENSE
1:30-2:00 **Carl Ware & Chris Benedict** (San Diego, USA) L35
A lymphotoxin-interferon axis essential for lymphocyte survival revealed during cytomegalovirus infection
2:00-2:30 **Tewis Bouwmeester** (Heidelberg, Germany) L36
Identification of novel regulators and pharmacological inhibitors of the pro-inflammatory TNFα/NF-κB pathway
2:30-2:45 **Klaus Pfeffer** (Düsseldorf, Germany) L37
Host defence mediated by the core members of the TNF superfamily and by interferons
2:45-3:00 **Wulf Schneider** (Regensburg, Germany) L38
Adenovirus 14.7K prevents TNF-induced apoptosis through inhibition of TNF-receptor 1 internalization and DISC formation
3:00-3:15 **Bertrand Huard** (Geneva, Switzerland) L39
Immunostimulation of spontaneous anti-tumor responses with a newly generated soluble form of CD40L

3:15 *Coffee*

3:45-6:00 pm POSTERS

The TNF superfamily
Poster presentation

7:00 pm *Aperitif / Concert / Gala Dinner*

Saturday, October 2

8:15-10:00 am CLINICAL APPLICATIONS: INFLAMMATION
8:15-8:45 **Marc Feldmann** (London, UK) L40
TNF blockade in arthritis: what have we learnt about inflammation in humans
8:45-9:15 **Kevin Tracey** (New York, USA) L41
The Inflammatory Reflex: Cholinergic Regulation of TNF and other Cytokines

9:15-9:30 **Eugene Zhukovsky** (Monrovia, USA) L42
Dominant-negative antagonists of TNF and BAFF: novel inhibitors for the treatment of inflammatory and autoimmune diseases

9:30-9:45 **Manolis Pasparakis (**Monterotondo, Italy) L43
In vivo analysis of IKK function in the central nervous system

9:45-10:00 **Rudolf Lucas** (Konstanz, Germany) L44
Dichotomal role of TNF in experimental pulmonary edema reabsorption

10:00 *Coffee*

10:30-12:30 am CLINICAL APPLICATIONS: CANCER

10:30-11:00 **Avi Ashkenazi** (San Francisco, USA) L45
Targeting Death Receptors in Cancer with Apo2L/TRAIL

11:00-11:30 **Dario Neri** (Zürich, Switzerland) L46
Antibody therapies targeting the stromal compartment

11:30-11:45 **Marc Dupuis** (Lausanne, Switzerland) L47
Preclinical evaluation of a highly active soluble FasL agonist: safety, pharmacokinetics, and efficacy

11:45-12:00 **Eberhard Stoeckle** (Bordeaux, France) L48
Limb salvage by isolated limb perfusion in patients with locally advanced soft tissue sarcoma

12:00-12:15 **Shie-Liang Hsieh** (Taipei, Taiwan) L49
Decoy receptor 3 (DcR3): a pleiotropic immunomodulator

12:15-12:30 **Timo L.M. ten Hagen** (Rotterdam, The Netherlands) L50
TNF: a key to solid tumor treatment?

1:00 pm End of the Conference

Michael Karin L01

Keynote Lecture

The control of TNF biological functions by the IKK and JNK effector pathways.

Karin M
University of California, San Diego, USA

TNF is a pleiotropic cytokine that can stimulate a plethora of biological responses including diagrammatically opposed effects on cell proliferation and programmed cell death (PCD). The functions of TNFα depend on its binding to two receptors: TNFR1 and TNFR2 and the activation of several effector pathways, including the IκB kinase (IKK) and Jun kinases (JNK). Whereas IKK activation prevents TNFα-induced PCD, sustained JNK activation enhances TNFα-induced PCD and in certain cell types is essential for TNFα-induced death. The anti-apoptotic function of IKK, which is a complex composed of two catalytic subunits (IKKα and IKKβ) and a regulatory subunit (IKKγ/NEMO), are mediated through activation of NF-κB transcription factors, which lead to induction of several key anti-apoptotic genes. These genes code for anti-apoptotic members of the Bcl2 family, caspase inhibitors and a decoy adaptor (cFLIP) that prevents caspase 8 activation in response to activation of TNFR1. Recent work has outlined an additional anti-apoptotic mechanism dependent on NF-κB activation based on inhibition of sustained JNK activation. We found that in the absence of IKKβ-dependent NF-κB activation, TNFα leads to sustained JNK activation through a mechanism that depends on production of reactive oxygen species (ROS) that lead to oxidation and inhibition of JNK phosphatases. Thus a major anti-apoptotic function of NF-κB in the context of TNFα signaling depends on its ability to suppress TNFα-induced ROS production. This function is specific because TNFα-induced ROS production is not required for stimulation of cell proliferation. I will present a number of biological examples based on in vivo studies that illustrate the important physiological function of the NF-κB-ROS-JNK triangle in inflammation and cancer.

David Wallach **L02**

Death Receptors

Signaling by the TNF/NGF receptor family: NEW FUNCTIONS FOR OLD PLAYERS

Wallach D, Ben-Moshe T, Kang T-B, Ramakrishnan P, Varfolomeev E and Wang W

Department of Biological Chemistry, The Weizmann Institute of Science, 76100 Rehovot, Israel

We tend to conceive of the cross-talk between cells as a language comparable to that of humans, i.e., composed of distinct terms with specific meanings. This has the unfortunate effect of leading to oversimplified notions of the molecular basis for cell–cell communication. Since the cytokines, once thought to each convey a single-functional message, have turned out to be pleiotropic and to act in a context-dependent manner, the quest for molecules with 'simple meanings' has shifted to the intracellular signaling proteins. Terms like 'death enzymes' or 'inflammatory signals' exemplify the inclination to conceive of signaling proteins in terms of single-functional messages. New findings in connection with two widely studied signaling enzymes that act downstream of receptors of the TNF/NGF family will be presented to demonstrate that the mode of function, also at this mechanistic level, is contextual. Caspase-8, an enzyme identified as the initiator of cell death in response to receptors of the TNF/NGF family, will be shown to serve a variety of non-apoptotic roles as well. NIK, a protein kinase known to mediate activation of the 'alternative' NF-κB pathway by the LTβR, CD40, and BlyS, will be shown also to mediate the functions of some other receptors of the TNF/NGF family and, in response to some of them, to activate the 'canonical' NF-κB pathway.

Richard Siegel **L03**

Death Receptors

Regulation of death receptor signaling by surface receptor oligomers and horizontal adapter protein interactions

Siegel R, Muppidi JR, Lenardo MJ
NIH, Bethesda, MD, USA

Fas (CD95), a member of the TNF receptor superfamily, signals for apoptosis via the ordered recruitment of the adapter and effector molecules FADD and caspase-8 to the intracellular death domain (DD) of the receptor. However, it not clear how recruitment of these proteins to the Fas death domain (DD) leads to activation of Caspase-8 in the receptor signaling complex. We have used high resolution confocal microscopy and live cell imaging to study the sequelae of early events in Fas signaling. These studies have revealed a new stage of Fas signaling in which receptor ligation leads to the formation of surface receptor oligomers that we term SPOTS (Signaling Protein Oligomerization Transduction Structures). Formation of SPOTS depends on the presence of an intact Fas death domain, FADD and caspase-8, but is independent of caspase activity. We have also found that FADD self-association through a conserved short peptide motif (RXDLL) in the death effector domain (DED) is required for death receptor signaling. Despite being able to bind both Fas and caspase-8 in isolation, FADD RDXLL motif mutants cannot reconstitute Fas mediated apoptosis in a FADD deficient cell line and fail to recruit caspase-8 into the receptor signaling complex. Abolishing self-association can transform FADD into a dominant-negative mutant that interferes with Fas-induced apoptosis and formation of microscopically visible receptor oligomers.

Henning Walczak **L04**

Death Receptors
Short oral communication

TGF-beta-induced apoptosis is mediated by TRAIL

Walczak H, Herzer K, Grosse-Wilde A, Ganten TM, Koppenhoefer D, Gersbach J, Krammer PH
German Cancer Research Center (DKFZ), Heidelberg, Germany

Transforming growth factor-beta (TGF-β) has been shown to induce apoptotic cell death in normal and transformed hepatocytes. However, the exact mechanism by which TGF-β induces cell death is unknown. We examined a potential role of various death receptor/ligand systems in TGF-β-induced apoptosis and have identified the tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) as the mediator of TGF-β-induced apoptosis in hepatoma cells. TGF-β-induced apoptosis is significantly impaired upon blockage of TRAIL. While TRAIL is upregulated in hepatoma cells upon treatment with TGF-β, TRAIL receptor levels remain unchanged. These results provide evidence that the TRAIL system is critically involved in TGF-β induced cell death.

Frédéric Rieux-Laucat **L05**

Death Receptors
Short oral communication

Autoimmune Lymphoproliferative Syndrome (ALPS-III) caused by somatic mutations of TNFRSF6 (fas)

Rieux-Laucat F, Holzelova E, Vonarbourg C, Stolzenberg MC, Selz F, Fischer A, Le Deist F
INSERM unité 429, Paris, France

Autoimmune lymphoproliferative syndrome (ALPS) is characterized by splenomegaly, lymphadenopathy, hyperglobulinemia (G and A) and accumulation of $TCRab^+$ $CD4^-CD8^-$ T cells (called Double Negative, or DN, T cells). ALPS is caused by a genetically determined impairment of lymphocyte apoptosis. Inherited mutations in the *fas* gene are responsible for many ALPS cases (ALPS-I). *Caspase 10* gene mutations are found in a few of the remaining cases (ALPS-II). In a third group of patients (ALPS-III), lymphocytes exhibit normal sensitivity to *in vitro* Fas-induced apoptosis and the underlying molecular cause remains unclear.

We performed analyses on FACS-sorted T cell subsets from six patients with ALPS-III. Surprisingly, heterozygous dominant *TNFRSF6* (*fas*) mutations were detected in the polyclonal DN T cells from all six patients. Moreover, in two cases, these mutations were found in a fraction of patients' peripheral $CD4^+$ and $CD8^+$ T cells as well as monocytes and $CD34^+$ hematopoietic precursors, but not in hair or mucosal epithelial cells. We identified *fas* mutations in freshly purified DN T cells but not from PHA-activated T cells. The normal *in vitro* Fas-induced apoptosis measured on activated T cells was thus consistent with the absence of *fas* mutations in such cells

In conclusion, we report here that some patients with ALPS-III have somatic *fas* mutations localized to hematopoietic lineage, in the absence of any malignancy. This is an example of a non-malignant, genetically acquired disease generated by a selective advantage (resistance to death) conferred by these newly described mutations. It may actually be a more frequent phenomenon than previously anticipated. The study also stresses the exquisite role of *fas* in peripheral T cell homeostasis.

Hiroyasu Nakano **L06**

Death Receptors
Short oral communication

NF-κB inhibits TNFα-induced accumulation of reactive oxygen species (ROS) that mediate prolonged JNK activation and necrotic cell death

Nakano H, Yagita H, Okumura K, Doi T
Juntendo University School of Medicine, Tokyo, Japan

NF-κB inhibits apoptosis by upregulating anti-apoptotic genes, such as c-FLIP, members of Bcl-2 and inhibitor of apoptosis (IAP) families. Recently, TNFα induces prolonged JNK activation in cells lacking RelA or IκB kinase β. This prolonged JNK activation promotes TNFα-induced apoptosis, indicating that one of the anti-apoptotic function of NF-κB is to downregulate JNK activation. However, the mechanism is not fully understood. We previously reported that murine embyonic fibroblast (MEF)s derived from TNF receptor-associated factor (TRAF)2 and TRAF5 double knockout (DKO) mice had a defect of NF-κB activation by TNFα. Here we show that delayed and prolonged JNK activation is induced in DKO MEFs despite the lack of early and transient JNK activation, indicating that TNFα-induced signaling pathways that regulate transient and prolonged activation of JNK are distinct, and the latter is TRAF-independent. Moreover, anti-oxidants dramatically reduce TNFα-induced prolonged JNK activation, but do not affect transient JNK activation in DKO and RelA KO MEFs. Importantly, TNFα promotes accumulation of ROS in DKO and RelA KO MEFs, but not wild-type MEFs. Combined these data together, prolonged JNK activation depends on ROS. Interestingly, dying cells show necrotic as well as apoptotic morphological changes as assessed by electron microscopy, and necrotic, but not apoptotic, cell death is substantially inhibited by antioxidant. Futhermore, microarray analysis shows that anti-oxidant enzymes are induced by TNFα in wild-type cells, but not DKO cells. Collectively, inhibition of NF-κB cascades induces ROS accumulation that subsequently mediates prolonged JNK activation and necrotic cell death. TRAF-mediated NF-κB activation normally suppresses TNFα-induced ROS accumulation by upregulating anti-oxidant enzymes.

Fabienne Mackay **L07**

Inflammation & Immunity

The role of BAFF in Autoimmunity: Is it just a B cell story?

Batten M, Fletcher C, Ng LG, Groom J, Wheway J, Laâbi Y, Xin X, Kalled SL, Browning JL, Schneider P, Tschopp J, Mackay CR and Mackay F

The Garvan Institute of Medical Research, Department of Arthritis and Inflammation, 384 Victoria Street, Darlinghurst, NSW 2010, Australia

The TNF-like ligand BAFF is a fundamental survival factor for B cells and essential during their maturation. However, overproduction of BAFF in transgenic (Tg) mice triggers autoimmune disorders similar to Systemic Lupus Erythematosus and Sjögren's syndrome, suggesting that BAFF affects one or more immune tolerance checkpoints. We showed that BAFF overexpression does not affect immune tolerance in the bone marrow but could prevent anergy and negative selection of B cells during maturation. Crossing BAFF Tg onto $TNF^{-/-}$ mice showed that germinal center (GC) formation was also not essential for disease in these mice, however autoimmune symptoms did correlate with the expansion of marginal zone (MZ) B cell population. MZ-like B cells were also abnormally detected outside the spleen, in lymph nodes and inflamed salivary glands of BAFF Tg mice. MZ B cells, however, did not participate in nephritis in BAFF Tg mice as BAFF Tg x $LT\beta^{-/-}$ mice which lack MZ B cells still developed lupus. In addition to B cells, BAFF promoted T cell activation possibly via increased cell survival mediated by BAFF-R expressed on effector T cells. Therefore, the role of BAFF in Autoimmunity appears to be the combined effect of inappropriate B cell survival and perturbed T cell activation.

Vishva Dixit **L08**

Inflammation & Immunity

De-ubiquitination and ubiquitin ligase domains of A20 downregulate NF-κB signaling

Wertz IE[1,4], O'Rourke KM[1], Zhou H[1], Eby M[2], Aravind L[5], Seshagiri S[2], Wu P[3], Wiesmann C[3], Baker R[6], Boone DL[7], Ma D[7], Koonin EV[5] & Dixit VM[1]

1Department of Molecular Oncology, 2Department of Molecular Biology and 3Department of Protein Engineering, Genentech Inc., South San Francisco, California 94080, USA. 4Department of Biological Chemistry, School of Medicine, University of California, Davis, California 95616, USA. 5Computational Biology Branch, NCBI, NLM, NIH, Bethesda, Maryland 20894, USA 6Division of Molecular Bioscience, John Curtin School of Medical Research, Australian National University, Canberra, Australian Capital Territory 2601, Australia 7Department of Medicine, University of California, San Francisco, San Francisco, California 94143, USA

NF-κB transcription factors mediate the effects of pro-inflammatory cytokines such as tumour necrosis factor-α and interleukin-1β. Failure to downregulate NF-κB transcriptional activity results in chronic inflammation and cell death, as observed in A20-deficient mice. A20 is a potent inhibitor of NF-κB signalling, but its mechanism of action is unknown. Here we show that A20 downregulates NF-κB signalling through the cooperative activity of its two ubiquitin-editing domains. The amino-terminal domain of A20, which is a de-ubiquitinating (DUB) enzyme of the OTU (ovarian tumour) family 3, removes lysine-63 (K63)-linked ubiquitin chains from receptor interacting protein (RIP), an essential mediator of the proximal TNF receptor 1 (TNFR1) signalling complex. The carboxy-terminal domain of A20, composed of seven C2/C2 zinc fingers 6, then functions as a ubiquitin ligase by polyubiquitinating RIP with K48-linked ubiquitin chains, thereby targeting RIP for proteasomal degradation. Here we define a novel ubiquitin ligase domain and identify two sequential mechanisms by which A20 downregulates NF-κB signalling. We also provide an example of a protein containing separate ubiquitin ligase and DUB domains, both of which participate in mediating a distinct regulatory effect.

Teresa G. Cachero **L09**

Inflammation & Immunity
Short oral communication

BAFF lacking a histidine tag can form virus-like clusters in solution

Cachero TG, Qian F, Krushinskie D, Eldredge J, Day E, Silvian L, Zeng C, Farrington G, Strauch K and Whitty A
Biogen Idec, Cambridge, MA 02142, USA

B cell activating factor, BAFF (BLyS, THANK, TALL-1, zTNF4, TNFSF13B), a member of the TNF ligand family, can signal through three different receptors, BCMA, TACI and BAFF-R. In the presence of IgM, BAFF co-stimulates the proliferation of B-cells. We have previously reported the crystal structure of BAFF extracellular domain, using a construct starting at residue Gln136 and with an N-terminal myc tag (Karpusas et al., JMB 2002). The structure of myc-Q136-BAFF, obtained at pH 4.5, showed two BAFF trimers per asymmetric unit. Interestingly, Liu et al. (Cell 2002) reported that in solution at pH 9.0 a his-tagged construct starting at residue Ala134 (His-A134-BAFF) formed a virus-like cluster containing 60 monomers. The formation of BAFF 60mer was pH dependent, and was abolished by deletion of the extended loop connecting b-strands D and E. Very recently, a report by Zhukovsky et al. (Nature 2004) suggested that BAFF, like other TNF family members, isa homotrimer, and that the 60mer formation reported by Liu et al. was an artifact of the histidine tag present in their construct. To further investigate the ability of BAFF to form a virus-like cluster in solution we engineered a construct with no N-terminal tag, A134-BAFF. The protein was expressed, purified, and characterized by analytical gel filtration, light scattering, crystallography, BIAcore, and in a cell-based functional assay (proliferation assay). We report here that A134-BAFF is able to form 60mers in solution (estimated molecular weight from lightscattering = 1055 kDa). Using analytical gel filtration we find that the 60mer formation is pH-dependent as was observed for their construct by Liu et al. Furthermore, we show by BIAcore that the affinity of A134-BAFF for BAFF-R and BCMA is identical to myc-Q136-BAFF, which we showed to be exclusively trimeric in solution, but A134-BAFF is more potent than myc-BAFF in inducing B cell proliferation *in vitro*. Taken together our results indicate that the observation of Liu et al that BAFF extracellular domain can form 60mers in solution was not an artifact of their his tagged construct, but rather is an intrinsic property of BAFF protein.

Pascal Schneider **L10**

Inflammation & Immunity
Short oral communication

Identification of a third "receptor" for APRIL

Zumsteg A, Ingold K, Rennert PD, Tschopp J, Schneider P
University of Lausanne, CH-1066 Epalinges, Switzerland

The TNF family ligands BAFF (BlyS/TALL1) and APRIL share the two receptors TACI and BCMA that are mainly expressed by B cells at various stages of differentiation. BAFF also binds BAFFR, a receptor that mediates the B cell and activated/memory T cell survival function of BAFF. Although APRIL does not bind to BAFFR, its ability to stain cells devoid of BCMA and TACI points to the existence of an additional APRIL-specific receptor unable to interact with BAFF.

Truncated recombinant forms of APRIL revealed two independent receptor-binding domains in APRIL. The TNF homology domain is required to bind BCMA and TACI, whereas a linear, positively charged amino acid sequence preceding the TNF homology domain is necessary but not sufficient for binding to APRIL-R. The corresponding region of BAFF is negatively charged and does not interact with APRIL-R. APRIL-R was identified as the negatively charged sulfated glycosaminoglycan side chains of proteoglycans. Although T cells and T cell lines did not bind APRIL to any significant extent, transfection of syndecans and glypicans in Jurkat T cells conferred high binding to APRIL that was entirely dependent on the basic sequence. Consistently, treatment of cells with a sulfation inhibitor or with heparin prevented APRIL binding. The glycosaminoglycan binding domain of APRIL may play a role in targeting APRIL to syndecan positive cells, in the interaction with the extracellular matrix or in preventing long range APRIL diffusion.

Stefan Schütze **L11**

Inflammation & Immunity
Short oral communication

Compartmentalization of TNF-Receptor 1 Signaling: Internalized TNF-Receptosomes as Death Signaling Vesicles

Schneider-Brachert W, Tchikov V, Neumeyer J, Jakob M, Winoto-Morbach S, Held-Feindt J, Merkel O, Kabelitz D, Schütze S
University Hospital of Schleswig-Holstein, Campus Kiel, D-24105 Kiel, Germany

The molecular regulation of the recruitment of initial signaling complexes at the TNF-R1 is poorly defined. We demonstrate here for the first time that within minutes internalized TNF-R1 (TNF-receptosomes) recruit TRADD, FADD, and caspase-8 to establish the "death-inducing signaling complex" (DISC). In addition, we identified the TNF-R1 internalization domain (TRID) containing the YXXW motif required for receptor endocytosis and provide evidence that TNF-R1 internalization, DISC formation and apoptosis are inseparable events. Analyzing cell lines expressing an internalization-deficient receptor (TNF-R1 ΔTRID and YXXW consensus-sequence point mutations) revealed that recruitment of RIP-1 and TRAF-2 to TNF-R1 occurred at the level of the plasma membrane. In contrast, aggregation of TRADD, FADD, and caspase-8 to establish the TNF-R1-associated DISC is critically dependent on receptor endocytosis. Furthermore, fusion of TNF-receptosomes with trans-golgi vesicles results in activation of acid sphingomyelinase and cathepsin D in multivesicular endosomes. Cleavage of Bid by Cathepsin D transmits endosomal death signals further downstream via caspase-9 and caspase-3 activation. Thus, TNF-receptosomes establish the different TNF signaling pathways by compartmentalization of plasma membrane-derived endocytic vesicles harboring the TNF-R1-associated DISC.

George Kollias **L12**

Inflammation & Signalling

Functional variations and differential thresholds of p55TNFR function in physiology and disease

Kollias G
Biomedical Sciences Research Center, Vari - Athens, Greece

We have shown previously that the immunosuppressive properties of TNF inautoimmune disease do not require the function of the p55TNFR, whereas the same receptor is necessary for most of the detrimental effects of TNF in inflammatory disease. Based on this finding we have put forward a hypothesis for an advantageous usage of 'anti-p55TNFR' treatments in human chronic inflammatory and autoimmune conditions. In this presentation we will be discussing the role and quantitative requirements for p55TNFR in several patho/physiological settings including lymphoid organ structure and function, host defence, chronic inflammation and autoimmunity. We show that while minimal levels of p55TNFR expression are sufficient to support its lymphoid developmental and host-defence properties, maximal levels are required for the detrimental pro-inflammatory properties of this receptor in chronic inflammatory disease. Thus, incomplete blockade of p55TNFR in TNF-driven disease may inhibit its deleterious proinflammatory activities without compromising its physiological properties.

Anders Nykjaer **L13**

Inflammation & Signalling

Sortilin, a novel p75NTR co-receptor essential for proNGF-induced neuronal apoptosis

Nykjaer A
Aarhus University, Aarhus, Danmark

Nerve growth factor (NGF) regulates neuronal development through survival and death signalling via two distinct receptors, TrkA and $p75^{NTR}$. Both NGF and its precursor proNGF are released by cells, but in contrast to NGF, proNGF selectively promotes $p75^{NTR}$-dependent apoptosis. We lately demonstrated that Sortilin, a receptor expressed in proNGF-responsive tissues, is indispensable for the pro-apoptotic effect. Sortilin binds preferentially to the pro-domain of proNGF whereas $p75^{NTR}$ selectively binds to the mature NGF domain. In cells, Sortilin coprecipitates with $p75^{NTR}$ but not with TrkA. This interaction is potentiated by proNGF that can be crosslinked to a complex of $p75^{NTR}$ and Sortilin on the cell surface, but not on cells expressing either $p75^{NTR}$, Sortilin or TrkA alone. In $p75^{NTR}$-expressing neurons, Sortilin antagonists protect against proNGF-induced cell death. Together, these results indicate that Sortilin acts as a coreceptor and molecular switch governing the $p75^{NTR}$ mediated pro-apoptotic signals induced by proNGF. Here, we have characterized mice with a targeted disruption of the Sortilin gene to test a role for the receptor in neurotrophin function *in vivo*. Sortilin-deficient mice are viable and fertile. However, deficiency for Sortilin results in protection of SCG neurons from proNGF-induced killing and in hypoalgesia due to a functional deficiency in sensory neurons. Thus, our findings confirm a role for Sortilin as an important regulator of neurotrophin action *in vivo*.

Remco van Horssen **L14**

Inflammation & Signalling
Short oral communication

EMAP-II sensitizes endothelial cells towards TNF-induced apoptosis by facilitating TNF-R1 apoptotic signaling via TRADD-mobilisation

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Background While the anti-tumor effects of Tumor Necrosis Factor-α (TNF) in an Isolated Limb Perfusion are mediated by the tumorvasculature, endothelial cells *in vitro* are insensitive to TNFα. Because the inflammatory cytokine Endothelial Monocyte Activating Polypeptide-II (EMAP-II) is capable of rendering a TNF-insensitive tumor TNF-sensitive *in vivo* we examined the effects and mechanism of EMAP-II action on the TNF activity on endothelial cells.
Methods We pre-treated Human Endothelial Umbilical Vein Cells (HUVEC) with EMAP-II (1 hr) before treatment with TNF (1 hr) and checked for apoptotic cell death using a YoPro-staining. TNF-R1 and TRADD expression and localisation after EMAP-II treatment was determined by immunofluorescence staining. For cellular of membrane expression we precipitated biotinylated cell-membranes and determined expression of TNF Receptor Associated Death Domain (TRADD) and Fass Associated Death Domain (FADD) (control) on the membrane.
Results A short pre-treatment of HUVEC with EMAP-II renders the cells sensitive towards TNF-induced apoptosis. Without the pre-treatment the cells are insensitive. Immunofluorescent staining of EMAP-II-treated HUVEC revealed a mobilisation of both TNF-R1 and TRADD out of the golgi-storage pool. A double staining for TNF-R1 and TRADD however revealed that they localize in different vesicles. When we treated HUVEC with EMAP-II we detected TRADD protein both within the cell and on the membrane as well, while FADD was only expressed within the cell.
Conclusion EMAP-II can make TNF-insensitive endothelial cells sensitive to effective TNF–induced apoptosis. The mechanism by which EMAP-II acts is both by a mobilisation of TNF-R1 as well as by inducing a translocation of TRADD from inside the cell to the cell-membrane.

Rudi Beyaert **L15**

Inflammation & Signalling
Short oral communication

Protective effect of NF-κB inhibition by adenoviral gene expression of ABINs in mouse models of inflammatory disease

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NF-κB is an essential transcription factor whose activity is modulated by a wide range of inducers, including cytokines and bacterial or viral products. Many of the NF-κB responsive genes play a key role in the regulation of inflammatory and immune responses, but also in the regulation of apoptosis and cell growth. Deregulation of NF-κB activity is often observed in several chronic inflammatory diseases such as rheumatoid arthritis, asthma and inflammatory bowel disease, as well as in acute diseases such as septic shock. Whereas members of the IκB family have been well studied as direct inhibitors of, a number of other proteins have been reported to negatively regulate NF dependent gene expression in response to TNF and IL-1. We are studying the in vitro and in vivo effects of the NF-κB inhibitory proteins ABIN-1, -2, and –3, which were originally identified as binding partners of the zinc finger protein A20. Although NF-κB is known to suppress TNF-mediated liver apoptosis, we unexpectedly found that in vivo adenoviral gene expression of ABIN-1 completely protects mice from TNF-induced acute liver failure and lethality. Protection was associated with a significant decrease in leukocyte infiltration and hepatocyte apoptosis. In contrast to ABIN-1, NF-κB inhibition by adenoviral delivery of an IκBα superrepressor did not have any protective effect against TNF-induced liver failure, indicating a different mechanism of action. Data on the therapeutic potential of adenoviral gene transfer of ABINs in a number of other mouse models of inflammatory disease will also be presented.

Isabelle Cremer **L16**

Inflammation & Signalling
Short oral communication

CD40 activation of primary B cells induces TRAF4 protein expression

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Members of the TNFR and IL-1R families are known to mediate important and diverse functions in immune system regulation. In particular, they represent key regulators in the balance between cell survival and cell death decisions in immune cells. The specific biological outcomes governed by these cell-surface receptors are dependent on various cytoplasmic signal transducing proteins including TRAF proteins. Although the primary structure of TRAF4 clearly places it in the TRAF family, the signaling pathway in which TRAF4 is involved remains elusive. TRAF4 is normally expressed throughout development in the thymus and can also be detected in the spleen, although at lower levels. Using multi-probe ribonuclease protection assay, TRAF4 mRNA was reported to be induced in both Daudi and tonsilar B cells following stimulation of CD40, a member of the TNFR family that plays a crucial role in T cell-dependent humoral immune responses. To further investigate a role of TRAF4 in immune cells, we have undertaken the analysis of TRAF4 protein expresssion under specific biological stimuli in human B cell lines and primary CD19+ B cells. Using an anti-TRAF4 monoclonal antibody, TRAF4 expression was detected in unstimulated B cell lines, including in Daudi, Raji and Ramos cells. In vitro stimulations by anti-CD40, PMA and LPS did not increase the level of TRAF4 protein expression. Interestingly, a specific induction of the TRAF4 protein following stimulation of primary B cells by anti-CD40 was observed in a time-dependent manner. Further evidence of a role of TRAF4 in B cell activation will be presented and discussed.

Marja Mikkola **L17**

Development & Tissue Remodeling I

The role of Ectodysplasin A1 in placodal cell fate

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Ectodysplasin-A1 (Eda-A1), a member of the TNF superfamily, together with its receptor Edar are necessary for normal development of ectodermal organs in humans and mice. We have previously shown that overexpression of Eda-A1 in transgenic mice under keratin14 promoter stimulates the formation and growth of several epithelial appendages. Organs developing as appendages of the ectoderm (such as teeth, hairs, mammary glands) are initiated from epithelial thickenings called placodes. Their formation is dependent on interactions between the ectoderm and the underlying mesenchyme, and several signalling molecules have been implicated as activators or inhibitors of placodal cell fate. In the present study, we have analysed the role of Eda-A1 in placode formation using in vivo and in vitro models in which both the timing and amount of Eda-A1 applied could be varied. The tooth and hair placodes of Eda-A1 transgenic embryos were enlarged, and extra placodes developed from the dental lamina and mammary line. Analysis of BrdU incorporation indicated that positive effect of Eda-A1 on placode expansion is not a result of increased cell proliferation. Exposure of embryonic skin to recombinant Eda-A1 protein in vitro stimulated the growth and fusion of hair placodes in a dose dependent manner. However, the timing of placode formation could not be accelerated suggesting that Eda-A1 is downstream of the primary inductive signal required for placode initiation. Taken together, our results suggest that Eda-A1-Edar signalling promotes placodal cell fate during early development of ectodermal organs

RANKL Blockade as a Novel Therapy For Human Bone Disorders: Biological Rationale and Clinical Experience

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RANKL (Receptor Activator of NF kappa B Ligand) is member of the TNF superfamily and an essential osteoclast differentiation, survival and activation factor. The action of RANKL requires signaling through its cognate receptor, the TNFR family member RANK. Mice containing homozygous deletions of either RANK or RANKL exhibit profound osteopetrosis and completely lack osteoclasts indicating that RANK and RANKL are essential signals regulating bone resorption. Osteoprotegerin (OPG) is a soluble decoy receptor for RANKL and functions as a physiological inhibitor of bone resorption. Knockout animal studies have also revealed the essential function for RANK and RANKL in two other tissues including the genesis of peripheral lymph nodes and the formation of lobulo-alveolar mammary structures that develop during pregnancy. Alterations of the RANKL/OPG ratio are critical in the pathogenesis of bone diseases that result from excessive bone resorption. Although many other cytokines have been shown to influence bone resorption, the primary in vivo effect of these cytokines appears to be indirect, by either increasing RANKL expression or decreasing OPG expression within the bone stroma. Evidence will be presented that the major cytokines and calciotropic factors that operate in pathologic bone resorption (e.g. PTHrP, IL-1, TNF) cannot increase osteoclastogenesis and subsequent bone resorption independently of a RANK or RANKL signal. RANKL blockade has been tested in animal models of pathologic bone turnover using recombinant forms of OPG or a soluble construct of the RANK extracellular domain fused to the immunoglobulin Fc (RANK:Fc). These reagents have been used to demonstrate the efficacy of RANKL blockade to reduce bone resorption in a number of animal models including hypercalcemia of malignancy, osteolytic and osteoblastic bone metastases, osteoporosis and inflammatory arthritis. A fully human monoclonal antibody to RANKL (AMG 162) has been developed and is being tested in a number of human bone diseases characterized by increased bone resorption. AMG 162 has been evaluated in two early clinical trials including women with breast cancer metastatic to the bone and post-menopausal women. The bone anti-resorptive activity, safety and tolerability of AMG 162 in these patients will be presented.

Nadia Corazza **L19**

Development & Tissue Remodeling I
Short oral communication

The role of TRAIL (TNF-Related Apoptosis-Inducing Ligand) in thymocyte apoptosis

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TRAIL represents a member of the Tumor Necrosis Factor (TNF) family with a potent apoptotis-inducing activity and it has particularly received strong scientific attention in tumor immune surveillance. TRAIL, however has also been implicated in various other aspects of immune cell regulation. Recently, Lamhamedi-Cherradi et al. have proposed TRAIL/TRAIL receptor-mediated cell death as an underlying mechanism of thymic negative selection.This finding is quite controversial and other investigators have not been able to demonstrate the implication of TRAIL in negative selection. Our own recent results may, however, shed a new light on the role of TRAIL in thymocyte apoptosis and may explain some of the controversies. Using TRAIL-deficient mice we observed reduced apoptosis of double-positive thymocytes upon T cell receptor activation in vivo and in vitro, thus confirming some of the results of Lamhamedi-Cherradi et al.. In addition, though, we have found also reduced thymocyte apoptosis upon in vivo and in vitro treatment with UV or γ-irradiation and glucocorticoids, but not with anti-Fas. It is well established that antigen-, glucocorticoid-, UV and γ-irradation-induced apoptosis in thymocytes proceeds predominantly via the activation of pro-apoptotic members of the Bcl-2 family associated with changes in the mitochondrial membrane permeability, causing the release of pro-apoptotic factors and subsequent caspase activation. We have analyzed the expression pattern of pro- and anti-apoptotic Bcl-2 members in thymocytes of wild type and TRAIL-deficient mice (i.e. Bim, Bmf, Bid, Bad, Bcl-w, Bcl-x and Bcl-2). No significant differences in protein expression were found in untreated thymocytes from TRAIL-deficient and control mice. However, upon TCR stimulation *in vitro* and *in vivo* wild type thymocyte showed a more pronounced increase in both, Bim mRNA expression and protein levels than TRAIL-deficient thymocytes. In conclusion, we demonstrated that TRAIL appears to have a significant apoptosis-modulating activity in thymocytes since TRAIL-deficient thymocytes show a marked decrease in apoptosis sensitivity to mitochondria-dependent pathways but not Fas-induced apoptosis.

Timothy S. Zheng **L20**

Development & Tissue Remodeling I
Short oral communication

Regulation of muscle satellite cell activation by Tweak/Fn14 pathway

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Many members of the tumor necrosis factor (TNF) superfamily regulate cellular outcomes of diverse cell types. Upon surveying target cell types of the TNF ligand Tweak, we observed that Tweak binds to all progenitor cells of the mesenchymal lineage, suggesting a possible role of this proinflammatory cytokine in modulating progenitor cell biology. Using the C2C12 myogenesis model of cell differentiation, we found that Tweak dramatically perturbed the cell cycle arrest of differentiating C2C12 myoblasts and potently inhibited their terminal differentiation. Similarly, Tweak also promoted the proliferation of isolated primary murine muscle satellite cells (MSCs) and inhibited their differentiation into myotubes. Importantly, primary MSCs isolated from mice deficient in the Tweak receptor Fn14 exhibited significantly reduced proliferative capacity and aberrant myotube formation. Following cardiotoxin injection, a known trigger for satellite cell-driven skeletal muscle regeneration, Fn14 knockout mice exhibited defective expansion of muscle satellite cells, altered inflammatory response and delayed muscle fibre regeneration compared to wild-type mice. These results indicate that the Tweak/Fn14 pathway is a novel regulator of muscle satellite cell activation and illustrate an important mechanism by which inflammatory cytokines could influence tissue repair and regeneration.

Lymphoid organogenesis and neo-organogenesis: cytokines, chemokines, and adhesion molecules

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Lymphoid organogenesis is an ordered process regulated by LT/TNF cytokines and chemokines in development. Neo-organogenesis is the process by which tertiary lymphoid organs (TLOs) arise in chronic inflammation in the course of autoimmune diseases or infection in environments characterized by chronic cytokine or chemokine over expression. Transgenic and knock-out mice are used here to determine how closely TLOs resemble lymphoid organs and to provide insight into the mechanism of these processes. Mice transgenic for a construct of the rat insulin promoter (RIP) driving expression of LT alpha and LT beta (RIPLT mice) exhibit lymphoid accumulations at the site of expression in the pancreas, kidney, and skin. These infiltrates have the characteristics of lymphoid organs with regard to cellular composition, chemokines, lymphatic vessels, and high endothelial venules (HEV). We demonstrate that the expression of PNAd and proteins that contribute to that HEV L-selectin ligand (GlyCAM-1, HEC-6ST) is controlled by LT, through the LTbetaR, predominately through the alternative NFB pathway, in both TLOs and lymph nodes. Most importantly, TLOs exhibit lymphoid organ function in their ability to present, process, and respond to exogenously administered antigen. This is demonstrated by the presence of antigen presenting cells (dendritic cells and follicular dendritic cells) that can present antigen to T cell lines, and the ability of antigen-specific T cells to proliferate in TLOs after administration of exogenous antigen. These studies indicate that TLOs exhibit all the properties of lymphoid organs and may contribute to protection in infectious diseases and to epitope spreading and clinical exacerbation in autoimmune diseases.

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Reina Mebius **L22**

Development & Tissue Remodeling II

Molecular and cellular aspects of secondary and tertiary lymphoid structure formation

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During embryonic development the formation of lymph nodes and Peyer's patches takes place. Essential for the organogenesis of these lymphoid organs is the interaction between lymphotoxin (LT) expressed by hematopoietic cells and the lymphotoxin receptor (LTβR) thought to be expressed on stromal cells. This interaction induces the expression of chemokines and adhesion molecules resulting in the rapid accumulation and retention of hematopoietic cells. In order to understand the details of this process we analyzed lymph node formation during embryonic life. At embryonic day 16.5 clusters of hematopoietic cells can be found of which the majority express IL7Rα, while a subset of these cells express also CD4, forming the CD45+CD4+CD3-LTαβ+ inducer cells believed to be responsible for the interaction with stromal organiser cells. Indeed, intermingled with these cells are VCAM-1+ICAM-1+ stromal cells. Upon analysis of peripheral lymph nodes versus mesenteric lymph nodes we observed a differential representation of VCAM-1+ICAM-1+LTβR+ subsets, leading to distinct stromal microenvironments within PLN and MLN anlagen. This could provide an explanation for the divergent developmental requirements observed for PLN vs. MLN. Furthermore, we show that upon intradermal injection of newborn lymph node derived cells, lymph node like structures as well as tertiary lymphoid structures can ectopically be induced. Formation depends on LT expressing donor cells, whereas further organization requires LT expressing recipient cells. These results indicate that the cellular and molecular requirements for the establishment of lymph nodes and tertiary structures are remarkably similar.

Sergei Nedospasov **L23**

Development & Tissue Remodeling II
Short oral communication

Novel knockin and transgenic models to study physiological functions of TNF and Lymphotoxin

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TNF and Lymphotoxin (LT) play critical roles in organogenesis and maintenance of lymphoid tissues, as well as in host defense. Since TNF and LTα can signal through the same receptors, and since their genes are very closely linked, a possibility was considered that TNF and LT knockout (KO) mice presented a complex phenotype, resulting from disruption of one gene and consequent deregulation of the other. It is known that insertion of neo selection cassette may cause artifacts in densely packed genomic loci. In particular, conventional LTαKO mice have been shown to produce suboptimal TNF levels, however this could be due to reciprocal regulation of TNF expression by LTα. Using LoxP-Cre technology, we generated neo-free LTα and TNF KO mice and compared them to their conventional KO counterparts. Strikingly, we found that neo-free LTα KO mice produce normal TNF levels in response to LPS, and behave differently from conventional LTα KO in several pathophysiological models. Together with our earlier data on neo-free TNF KO which lack Peyer's patches but show no other features of the LT deficiency, these findings strongly argue for the two cases of collateral damage when targeting murine TNF/LT locus. We also generated transgenic mice bearing a cosmid, encompassing complete human TNF/LT locus with all natural promoters and enhancer elements. Mice bearing this transgene on the triple TNF/LT KO background (Kuprash et. al. MCB, 22:8626) can be used in the future as a "humanized" model to study the effects of human TNF blockers. Mice with combination of human and murine TNF/LT genes possess an interesting phenotype which is transgene number-dependent, as will be discussed.

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Gerard Eberl **L24**

Development & Tissue Remodeling II
Short oral communication

Lymphoid tissue inducer cells in the fetus and the adult gut

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Lymphoid tissue inducer (LTi) cells are essential for the development of lymph nodes and Peyer's patches. In the fetus, LTi cells migrate to sites of lymphoid tissue development, and activate a leukocyte-recruitment program in specialized R-expressing mesenchymal cells. In the adult, smallbLT clusters of LTi-like cells are found within the intestinal lamina propria in so-called cryptopatches (CPs). It has been suggested that CP cells are precursors for the extrathymic generation of intraepithelial lymphocytes. We now show that CP cells are not lymphocyte precursors, but instead suggest that these LTi-like cells induce the formation of lymphoid tissue under inflammatory conditions. It therefore appears that the adult gut can recapitulate a fetal program of lymphoid tissue development. Our conclusions are derived from experiments using novel types of genetically engineered mouse models, and are based on our previous findings that the nuclear hormone receptor RORγt is specifically expressed in LTi cells and required for their development.

Eckhard R. Podack **L25**

DR3 Signaling in the Lung Promotes IL-13 Production and Asthma

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In order to study DR3 function we generated DR3 transgenic mice expressing DR3 on T cells under the CD2 promoter. DR3-tg CD4 T cells upon activation secrete large quantities of TH2 cytokines including IL-13 upon primary activation, and even larger amounts upon secondary activation. Since IL-13 is a signature cytokine for asthma DR3-tg B6 mice were compared to B6 w.t. mice in their susceptibility to asthma in the classic ovalbumin model. DR3-tg mice had dramatically exaggerated lung inflammation when compared to w.t. mice as measured by histopathology and analysis of bronchio-alveolar lavage. To determine whether blockade of DR3 ameliorated susceptibility to asthma we generated dominant negative transgenes (DN-DR tg) expressed under the same promoter. DN-DR3-tg mice were resistant to the induction of asthma and lung inflammation. Moreover DN DR3-tg cells could not be polarized in vitro to the TH2 phenotype in secondary activation, suggesting that DR3 signaling is required for asthma induction in vivo and Th2 polarization in vitro. To further test this hypothesis, we developed a blocking antibody to TL1A, the natural ligand for DR3. Treatment of ovalbumin sensitized mice with blocking anti TL1A prior to ovalbumin aerosol exposure abolished lung inflammation that was seen with control antibody.
Our data suggest that TL1A and DR3 interaction in bronchial lymphnodes of sensitized mice during secondary antigen exposure through the airways triggers CD4 cells to produce IL-13 and other signals that precipitate lung inflammation and airway hyper reactivity. Our data also indicate that blockade of DR3 signaling may be an effective way to treat asthma.

*This work contains part of the doctoral dissertation of Lei Fang.

Masayuki Miura **L26**

Genetic pathway of TNF/TNFR signaling in *Drosophila*

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We identified *eiger*, a TNF superfamily ligand in genetic screening of *Drosophila* cell death inducing genes. Genetic evidence showed that *eiger* induces cell death by activating the *Drosophila* JNK pathway. Although there are accumulating evidences that the JNK pathway plays an important role in TNF-induced cell death signaling in mammals genetic components that transduce the death signal to the proapoptotic molecules are poorly understood. To elucidate the signaling mechanisms of *eiger*-induced biological functions including cell death, we have conducted a dominant modifier screen to identify downstream molecules of Eiger signaling pathway using eye-specific *eiger* overexpressing flies (*GMR-Gal4*, *UAS-eiger[regg1]*) and a collection of deficiency-bearing flies. We identified Wengen, the first member of the *Drosophila* tumor necrosis factor receptor (TNFR) superfamily. Wengen is a type III membrane protein with conserved cysteine-rich residues (TNFR homology domain) in the extracellular domain, a hallmark of the TNFR superfamily. The small-eye phenotype caused by an eye-specific overexpression of *eiger*, was dramatically suppressed by downregulation of *wengen* using RNA interference. We also identified a *Drosophila cytochrome c* gene, *cyt-c-d*, as a suppressor of the eye ablation induced by *eiger*. We show genetically that the *eiger*-induced cell death signal is transduced to mitochondrial pathways, a *Drosophila* Bax-like protein, Drob-1 and Cyt c-d. Our observations provide the first genetic evidence that the mitochondrial pathway is involved in TNF-induced cell death *in Drosophila*.

François Leulier L27

Inflammation & Immunity II
Short oral communication

Caspase regulation of NF-κB dependant innate immunity in *Drosophila* melanogaster

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The *Drosophila* innate immune system discriminates between pathogens and responds by inducing the expression of specific antimicrobial peptide encoding genes through distinct signaling cascades. Both fungal and Gram-positive bacterial infections activate the NF-κB like transcription factors Dif and Dorsal via the Toll pathway. In fact orthologues of the Toll receptor in mammals, the members the Toll-Like Receptors family, also regulate immune responses. *Drosophila*'s anti-Gram-negative bacterial defence were less well defined but recent genetical studies have defined the Immune Deficiency (IMD) signalling pathway as the central regulator of defence against Gram-negative bacterial infection. Like the Toll pathway, the IMD pathway regulates antimicrobial gene expression via a third NF-κB like transcription factor, Relish. The IMD pathway shares structural and molecular parallels with the TNF-R1 pathway in mammals. Although a particular feature of this pathway is the absolute requirement for dFADD and Dredd, the *Drosophila* orthologues of FADD and caspase-8/10 respectively, for Relish activation. Here we present recent advances in understanding the molecular function of dFADD and Dredd in the direct activation of the NF-κB factor Relish.

Rama Khokha **L28**

Inflammation & Immunity II
Short oral communication

Abnormal TNF bioactivity in TIMP-3 null mice

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During homeostasis the inflammatory response is kept in check from aberrant activation. Upon tissue injury or infection the inflammatory response occurs rapidly and must also halt equally as rapidly to stop this reaction from inflicting self damage. Such a highly regulated process results from altering balances in proinflammatory signals and anti-inflammatory check points or breaks, and is orchestrated by many factors and cell types within the tissue microenvironment. TNF is one of the key inducers of inflammation. A metalloproteinase called ADAM-17 or TACE (TNF alpha converting enzyme) is the primary enzyme that cleaves and sheds membrane bound pro-TNF to its soluble form. To date, the only known physiological inhibitor of TACE is TIMP-3, a member of the tissue inhibitors of metalloproteinase family. We have discovered that TNF bioactivity is altered in mice with targeted deletion of TIMP-3. We will present our studies describing the role of TIMP-3 during liver tissue homeostasis, hepatic injury and cardiovascular disease. Overall, our results reveal the novel role of TIMP-3 as a negative regulator of TNF bioactivity and inflammation. TIMP-3 regulates the basal anti-inflammatory state as well as serves as a brake during active inflammation.

Roy Black **L29**

Inflammation & Immunity II
Short oral communication

Shedding of TNF receptors by multiple metalloproteases and modulation of TNF-responsiveness

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Soluble forms of TNFα and both of its receptors can be released from cells by proteolysis, a process termed "ectodomain shedding". Tumor necrosis factor-αconverting enzyme (TACE/ADAM-17) was originally identified as the metalloprotease responsible for releasing TNFα. TACE can also release soluble TNF receptors from cells, but whether TACE is the main enzyme responsible for generating soluble TNF receptors is unknown. We have examined the shedding of TNF receptors by monocytic cells derived from wild-type and TACE-deficient mice. Wild-type cells constitutively shed p55 and p75 TNF receptors, and the rates of release were enhanced by stimulation with either PMA or LPS. Unstimulated TACE-deficient cells shed p55-TNFR at about the same rate as wild-type cells did but released minimal p75-TNFR. Stimulation with PMA or LPS did not enhance shedding of p55- or p75-TNFRs by TACE-deficient cells. The constitutive release of p55-TNFR from TACE-deficient cells was inhibited by TIMP-1, TIMP-3, and hydroxamates, suggesting a role for ADAM-10 or a related metalloprotease as a constitutive p55-TNFR sheddase. We next examined the ability of induced TNFR shedding to modulate TNF and IL-1 responses in HUVEC cells. PMA treatment increased shedding of both TNF receptors and inhibited cell responses to TNF, but not IL-1. A metalloprotease inhibitor restored TNF-responsiveness, suggesting that the effect of PMA on TNF signaling was due to induced receptor shedding. Thus, multiple metalloproteases participate in constitutive and regulated TNF receptor shedding, and modulation of receptor shedding can control TNF responses.

Michael Croft **L30**

Co-Stimulatory Ligands

Regulation of T cell Survival by OX40

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A number of TNFR superfamily members are expressed on T cells and may be important for determining the magnitude of inflammation in many T cell driven immune diseases. We have focused on OX40 (CD134) and suggested it is critical to the expansion and survival of T cells. Unlike molecules such as CD28 and CD27 that are constitutively expressed on resting T cells, OX40 needs to be induced. As such it functions after the initial antigen recognition event. We have proposed that its primary role is to suppress T cell death. An overview of OX40 biology will be given, including its relevance to protective immunity and to autoimmunity. Data will be discussed regarding the role of OX40 in T cell memory, and how it regulates the number of T cells that persist and contribute to in vivo inflammation. Recent results will be presented suggesting that a PI-3-k/Akt pathway, common to CD28, is a major regulator of costimulation from OX40, and that downstream targets include both anti-apoptotic members of the Bcl-2 family and the inhibitor of apoptosis family member survivin.

Sarah Hymowitz **L31**

Co-Stimulatory Ligands

The crystal structure of the OX40-OX40L complex reveals an extensive interface between a small divergent ligand and a more conventional receptor

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OX40 is a T-cell co-stimulator, which is activated by interaction with OX40L. Blockade of this interaction has ameliorative effects in treating animal models of T-cell pathologies. In order to better understand the interaction between OX40 and OX40L, we have determined the crystal structure of the human OX40-OX40L complex at 2.3 A. This structure shows that OX40L has diverged from other TNF ligands in many ways. OX40L is an unusually small TNF ligand containing only 120 residues in the TNF domain. This domain is followed by an extension which is disulfide bonded back to the TNF domain. There is only a very short (6 residue) linker connecting the OX40L extracellular domain to the transmembrane domain suggesting that access to OX40L by OX40 may be restricted by the cell surface. Additionally, the arrangement of the OX40L protomers forming the functional trimer differs from that of other TNFSF by a ~15º rotation and by an opening of the trimer distal to the ligand terminii. The structure of OX40, by contrast, is more similar to that of other TNF receptors. OX40 makes more extensive contacts to OX40L than have been seen in other TNFSF-receptor complexes and uses residues from cysteine rich domains (CRDs) 1, 2 and 3 to form a binding interface. Site directed changes of the interfacial residues of OX40L suggests that this interface lacks a single "hot-spot" and instead that the binding energy is dispersed over at least two distinct areas. While both OX40L and OX40 are glycosylated, the interactions between receptor and ligand are independent of glycosylation. This structure also demonstrates the plasticity of TNFSF ligand-receptor interactions.

Jan Paul Medema **L32**

Co-Stimulatory Ligands
Short oral communication

The TNF family member APRIL promotes B-1 cell-associated neoplasm

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The TNF-like ligand APRIL (a proliferation-inducing ligand) is a secreted protein, named for its capacity to stimulate tumor cell proliferation *in vitro*. APRIL is expressed in various carcinoma tissues and a tumor-supporting role for APRIL has been suggested. Here, we describe that 9-12 month old APRIL transgenic mice develop lymphoid tumors that originate from the peritoneal B-1 B cell population. Initially, these cells expand in the peritoneum due to a survival advantage induced by APRIL. At later stages, hyperplasia in mesenteric lymph nodes and Peyer's patches, as well as disorganization of affected lymphoid tissues is observed. Mucosal and capsular infiltration is detectable, and eventually tumor cells infiltrate kidney and liver massively. Importantly, a large fraction of human B cell chronic lymphoid leukemia (B-CLL), for which murine B-1 B cell-derived tumors are a model, express APRIL mRNA, and B-CLL patients have significantly increased APRIL levels in serum. Our data therefore indicate that APRIL promotes the onset of B-1-associated neoplasms and that APRIL antagonism may provide a therapeutic strategy to treat B-CLL patients.

Tania Watts **L33**

Co-Stimulatory Ligands
Short oral communication

TRAF1 plays a positive role in T cell expansion in vivo

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TNF receptor family associated factor (TRAF) 2 acts downstream of several TNFR family members and links them to cell survival. TRAF1 appears to function as a modulator of TRAF2 function. To date, there have been conflicting results as to whether TRAF1 plays a positive or negative role in signaling by TNFR family members. To assess the role of TRAF1 during an immune response *in vivo*, we compared the immune response to influenza virus in WT versus TRAF1$^{-/-}$ mice. TRAF1$^{-/-}$ mice exhibit decreased expansion of D^b/NP366-374-specific CD8 T cells at all time points examined, during both primary and secondary responses to influenza virus. Adoptive transfer experments with TCR transgenic T cells revealed that the decrease in Ag-specific CD8 T cell numbers during an immune response *in vivo* can be attribtuted to TRAF1 in both T cells and non-T cells. To assess the function of TRAF1 in a specific signaling pathway in T cells, we analyzed responses to 4-1BB ligation of TRAF1 deficient and TRAF1 sufficient T cells. Purified T cells from TRAF1$^{-/-}$ mice showed reduced IL-2 production and reduced Bcl-X_L upregulation in response to anti-CD3 plus 4-1BB signaling whereas IL-2 production in response to anti-CD3 plus anti-CD28 was unchanged. TRAF1$^{-/-}$ T cells were also more prone to cell death in culture. Overall, the results show that TRAF1 in both T cells and non-T cells is a positive regulator of the T cell response.

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Martijn Nolte **L34**

Co-Stimulatory Ligands
Short oral communication

CD27 on hematopoietic stem and progenitor cells serves as a negative feedback mediator on leukocyte differentiation.

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Although the development of early hematopoietic stem cells to fully differentiated red and white blood cells has been well characterized, little is known about potential feedback mechanisms that influence this process. Here we describe that the TNF-R superfamily member CD27, which is expressed on a substantial population of stem/progenitor cells in bone marrow, serves as an important regulator of the hematopoiesis. Deletion of CD27 increased the colony forming potential of stem/progenitor cells, but also enhanced their reconstitution capacity in competitive transplantation experiments. On the contrary, stimulation of CD27+ progenitor cells with CD70, the unique ligand for CD27, inhibited leukocyte outgrowth both in vitro and in vivo and led to an accumulation of early progenitor cells. Since CD70 is only expressed on activated immune cells, we suggest that CD27 expression on stem/progenitor cells can serve as a negative feedback on the hematopoietic process during inflammatory responses.

Carl F. Ware L35

Host Defense

A lymphotoxin-interferon axis essential for lymphocyte survival revealed during cytomegalovirus infection.

Ware CF, Banks TA, Rickert S, Benedict, CA, Schneider K, de Trez C, Brinker K, Meier J, Ha W, Granger SW, Turovskaya O, Elewaut D, Otero D, Henry SC, Hamilton JD, Scheu S, Pfeffer K

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The importance of lymphotoxin (LT)-β receptor (LTβR) as a regulator of lymphoid organogenesis is well established, but its role as an effector system in host defense has yet to be fully defined. Here we report that mice deficient in LTβR signaling were highly susceptible to infection with mouse cytomegalovirus (MCMV) and at early times post-infection exhibited a catastrophic loss of T and B lymphocytes due to bystander cell death. Moreover, bone marrow chimeras revealed that LTβR expression on both stromal and hematopoietic cells is needed to prevent lymphocyte death. The induction of interferon-β (IFNβ) was also severely impaired in MCMV-infected $LT\alpha^{-/-}$ mice, but immunotherapy with an agonist LTβR antibody both restored IFNβ levels and prevented lymphocyte death. $IFN\alpha\beta$ $receptor^{-/-}$ mice demonstrated that they too exhibit profound lymphocyte death during MCMV infection, providing a genetic link between IFNβ induction and lymphocyte survival, implicating the LTαβ/IFN axis is important for host defense to MCMV.

Identification of novel regulators and pharmacological inhibitors of the pro-inflammatory TNFα/NF-κB pathway

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Tumor necrosis factor alpha (TNFα) is a potent cytokine involved in a number of physiological and pathological processes including inflammation, immuno-regulation, proliferation, apoptosis and anti-viral activities. There is unmet medical need to develop orally administered small molecule drugs against intracellular transduction components as alternative to protein therapeutics. We have mapped the protein interaction network around 32 known and candidate TNFα transduction components using an integrated approach comprising of: Tandem Affinity Purification (TAP), liquid-chromatography/tandem mass spectrometry (LC-MS/MS), bioinformatic pathway computation and selective biological assessment. We identified over 80 novel molecular associations, many of which are TNFα stimulus-dependent and represent unanticipated modulatory functions. The collective proteins form a single, coherent molecular network from membrane-associated receptor complexes to nuclear transcription complexes. To identify small molecule TNFα/NF-κB pathway antagonist we have conducted a phenotypic screen for compounds that block cytokine release in a monocytic cell line. We identified several novel, small molecule inhibitors of the critical signal transducers, p38 MAP kinase and IKKβ, which form the basis for developing clinical candidates that suppress TNFα/NF-κB signaling *in vivo*.

Klaus Pfeffer **L37**

Host Defense
Short oral communication

Host defence mediated by the core members of the TNF superfamily and by interferons

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Tumor necrosis factor receptor p55 (TNFRp55) has been shown to be essential for controlling the replication of *Listeria monocytogenes* and *Mycobacterium tuberculosis* in vivo. Interestingly, TNFRp55- deficient mice succumb to listeria infection despite activation of the classical antibacterial effector systems NADPH-oxidase and NOS2. New data indicate that lymphotoxin β receptor- (LTβR-) deficient mice are also highly susceptible to infections with listeria and mycobacteria. Surprisingly, LTβR-deficient mice produced similar levels of TNF and IFNγ in infected organs as compared to control mice. Flow cytometry revealed expression of LTβR on activated pulmonary and peritoneal macrophages. Ligands for the LTβR are LIGHT and $LT\alpha_1\beta_2$. Analysis of LIGHT-deficient mice revealed no increased susceptibility to mycobacteria infections whereas LTβ and LTα- deficient mice were susceptible, indicating a dominant role for $LT\alpha_1\beta_2$ heterotrimers via LTβR in innate host defence against intracellular pathogens. Taken together, both TNF/TNFRp55 and $LT\alpha_1\beta_2$/LTβR pathways are essential for the competence of macrophages to control and kill intracellular bacteria and, importantly, cannot substitute each other. A comparable phenotype has been observed for IFNγ/IFNγR.
In the search for novel antimicrobial effector mechanisms initiated by TNF, $LT\alpha_1\beta_2$, and IFNγ differential display PCR and Affymetrix expression profiling has been performed. Results relevant for host defence will be discussed.

Wulf Schneider **L38**

Host Defense
Short oral communication

Adenovirus 14.7K prevents TNF-induced apoptosis through inhibition of TNF-receptor 1 internalization and DISC formation

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During their coevolution with the immune system viruses have adopted sophisticated strategies to escape host defence mechanisms. The antiviral activity of TNF-induced apoptosis is mediated by the recruitment of TRADD, FADD, and caspase-8 to the death domain (DD) of tumour necrosis factor receptor 1 (TNF-R1) to establish the death inducing signalling complex (DISC). However, the functional contribution of receptor endocytosis to the activation of the pleiotropic TNF-signalling pathways, including apoptosis, is still poorly defined. Here we report that adenovirus 14.7K protein prevents TNF-induced apoptosis by inhibition of TNF-R1 endocytosis. Isolation of magnetically labelled TNF-R1 complexes by a novel immunomagnetic purification approach revealed that inhibition of TNF-R1 internalisation prevents recruitment of TRADD, FADD, and caspase-8. In contrast, 14.7K does not affect TNF-induced NF-κB activation via recruitment of RIP-1 and TRAF-2. Inhibition of endocytosis by 14.7K was mediated by an uncoordinated assembly of the endocytotic machinery at the site of the activated TNF-R1 missing essential components such as Rab5 and dynamin 2. Thus, this study describes a novel viral anti-apoptotic strategy to inhibit DISC formation by selective targeting of TNF-R1 endocytosis.

Bertrand Huard **L39**

Host Defense
Short oral communication

Immunostimulation of spontaneous anti-tumor responses with a newly generated soluble form of CD40-L

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CD40-L/CD40 is one of the main signaling pathways rendering antigen presenting cells competent in the induction of effector T cells. In this study, we tested the immunostimulatory effect of CD40 signaling in murine tumor models with a new form of soluble CD40-L. We grafted the extracellular domain of CD40-L onto a complement-like molecule resulting in the generation of an hexameric molecule (megaCD40-L). MegaCD40-L was found active in in vitro DC and B cell activation assays. In a murine tumor model, we found that a therapeutic treatment of animals bearing B16-OVA (B16 melanoma cells expressing the model tumor antigen ovalbumin) with megaCD40-L delays tumor growth and increases mice survival by about 10 days. The anti-tumor effector cells induced are likely to be CD8+ T cells, considering the previous studies reported with an agonistic mAb against CD40. Importantly, megaCD40-L induces a long-lasting immune protection against a second tumor challenge. This indicates that immunostimulation of naturally occurring anti-tumor responses with adjuvant-like molecules such as CD40-L may have therapeutic values in cancer.

Marc Feldmann **L40**

Clinical Applications: Inflammation

TNF blockade in arthritis: what have we learnt about inflammation in humans?

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The clinical effectiveness of blocking a single proinflammatory cytokine in a chronic disease, rheumatoid arthritis, in which multiple proinflammatory cytokines are expressed in large quantities was surprising to many. However it reproduced results using human rheumatoid synovial cultures, in which TNF blockade reduced IL-1, IL-6, GM-CSF, IL-8 for example. This highlights the relevance of using human disease tissue as a good model of a human disease.

Analysis of the mechanism of action of anti TNF revealed that essentially everything measurable was normalised, the cytokine profile (above), the haematological abnormalities (high platelets, high fibrinogen, low haemoglobin, the immunological abnormalities, low T cell response), the autoantibody response are reduced, the joint destruction is reduced probably due to the reduction in MMPs, but probably the major mechanism of benefit is by reduced cellularity and trafficking (reduced by > 50%). This probably explains the reason shy anti TNF is effective in many other diseases, including Crohn's disease, Juvenile RA, ankylosing spondylitis, psoriasis and psoriatic arthritis. But two other points of interest: protection of joints or healing of Crohn's fistulas can occur in the apparent absence of clinical benefit. So we do not know exactly how inflammation engages the tissue destructive pathways. Most importantly we now have insights into the mechanism of autoimmunity. Many patients are now on TNF blockade for 5 years. This indicates that in contrast to cancer, the molecular mechanisms do not evolve, or if they do, very slowly. This suggests that it may be possible to go from current disease control to eventual remission or cure. The latter has not been accomplished, but with early treatment with TNF blockade and methotrexate, up to 40% remissions were obtained in two trials. The future dilemma is how to build on that base without increasing the infectious risk of blocking an important host defence molecule.

Kevin Tracey **L41**

Clinical Applications: Inflammation

The Inflammatory Reflex: Cholinergic Regulation of TNF and other Cytokines

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The "cytokine theory of disease" implies that the overproduction of cytokines can cause the clinical manifestations of disease. TNF was the first cytokine to be successfully targeted in for the treatment of human disease, and monoclonal anti-TNF antibodies have now achieved widespread clinical use for rheumatoid arthritis and Crohn's disease. Since the earliest recognition that the overproduction of TNF can be injurious to the host, a major question in this field has been: How is TNF normally regulated, such that its over-expression is controlled in normal health? A series of recent, surprising, discoveries revealed an unexpected neural mechanism that inhibits the production of TNF, HMGB1, and other cytokines. The "cholinergic anti-inflammatory pathway" is the efferent arm of the "inflammatory reflex", a physiological pathway in which the nervous system detects the presence of inflammatory stimuli and cytokines. Afferent signals are transmitted to the brain via the vagus nerve, which activates a reflex response that increases ACTH release and activates of the synthesis and release of glucocorticoids, and also includes increases activity in the cholinergic nervous system. Acetylcholine released from vagus nerve endings in the vicinity of macrophages in the reticuloendothelial system interacts specifically with alpha7 nicotinic subunits on TNF producing cells. Receptor ligand interaction deactivates macrophages, and inhibits cytokine release. This neural control of cytokine production, provides an important alternative to the humoral regulation of cytokine responses, because the neural regulation is fast, can be integrated and locally controlled, and is not specifically dependent on slow concentration gradients. There are potentially significant therapeutic implications derived from this mechanism.

Clinical Applications: Inflammation
Short oral communication

Dominant-Negative Antagonists of TNF and BAFF - novel inhibitors for the treatment of inflammatory and autoimmune diseases

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The therapeutic role of inhibitors of the TNF superfamily of ligands (TNFSF) in human diseases is well established. To receptor-specifically block TNFSF signaling, we utilized Dominant Negative (DN) technology that is based on the ability of multimeric proteins to exchange subunits with variants differing from the wild type by a small number of amino acids. The DN-TNFSF variants lack the ability to agonize corresponding TNFSF receptors and functionally antagonize them by forming heterotrimers with the wild type, thus sequestering endogenous TNFSF members in non-signaling heterotrimers. We utilized Xencor's Protein Design Automation technology and models or crystal structures of the ligand/receptor complexes to create DN libraries for TNF and BAFF. We determined the size of BAFF constructs with various N-terminal sequences by size-exclusion chromatography, since there were conflicting reports regarding the oligomerization state of native BAFF (3-mer vs. 60-mer) and we expected only trimeric cytokine to efficiently exchange with DN-BAFF. This analysis showed that native BAFF is a trimer that displays full biological activity and higher order oligomers are an artifact of N-terminal His-tagging. We developed in vitro assays to assess TNF and BAFF variants' propensities to exchange, to bind cognate receptors of TNF or BAFF, and to agonize and antagonize the appropriate native cytokines in cell-based assays. We also tested the efficacy of DN antagonists in vivo. This strategy identified antagonists of TNF and BAFF with distinct selectivity profiles toward their respective receptors. Based on the in vitro cell-based assays and in vivo models, the inhibitory properties of DN-TNF and DN-BAFF are similar to those of either marketed (TNF) or the development-stage (BAFF) antagonists. Comparison of binding to BAFF receptors and B-cell proliferation activity by DN-BAFF confirms the role of BAFF-R as the BAFF B-cell proliferation signal transducer; binding to BCMA is inconsequential to B-cell proliferation. Several DN-TNF variants specifically inhibit soluble, but not membrane-bound TNF, and demonstrate marked reduction of side effects attributed to marketed TNF antagonists. In conclusion, our study suggests that the DN antagonist paradigm can produce novel therapeutic inhibitors of TNF and BAFF for treating inflammatory and autoimmune diseases, and that specific DN-TNF and DN-BAFF variants may be useful in the investigation of the multi-receptor (and multi-ligand) biology of these TNFSF cytokines.

Manolis Pasparakis **L43**

Clinical Applications: Inflammation
Short oral communication

In vivo analysis of IKK function in the central nervous system

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The transcription factor NF-κB controls the expression of many genes that are involved in cell survival, proliferation and apoptosis, and has a well recognised function in the regulation of immune and inflammatory responses. Recent evidence suggests that the NF-κB pathway plays a role in the central nervous system. Constitutive NF-κB activity is detected in neurons indicating that NF-κB may be involved in physiological neuronal functions such as learning and memory. Increased NF-κB activity is also found in CNS tissue from multiple sclerosis patients, suggesting it may play a role in the pathophysiology of this inflammatory demyelinating disease.

The IκB kinase (IKK), composed of the IKK1(IKKα) and IKK2(IKKβ) catalytic subunits and the NEMO/IKKγ regulatory subunit, is essential for NF-κB activation by most stimuli. To investigate the function of the NF-κB pathway in the central nervous system we are using CNS-specific targeting of IKK subunits. Mice with deletion of either IKK2(IKKβ) or NEMO/IKKγ in all cells of the CNS during early embryonic development develop normally suggesting that these IKK subunits do not play a critical role in neuronal development. Neurons lacking either IKK2 or NEMO do not show constitutive NF-κB activation in vivo demonstrating that this activity is mediated by the "classical" NF-κB pathway. We are currently analysing the behavioural responses of CNS-specific IKK2- or NEMO-deficient mice in order to evaluate whether the NF-κB pathway plays a role in learning and memory. To study the function of NF-κB activation in cells of the CNS in the pathogenesis of inflammatory demyelinating disease we are analysing the response of CNS-restricted IKK2- and NEMO-knockout mice to experimental autoimmune encephalomyelitis (EAE). The results of these experiments will be discussed.

Rudolf Lucas **L44**

Clinical Applications: Inflammation
Short oral communication

Endotoxin induced bronchoconstriction is TNF-dependent, while neutrophil recruitment depends on TLR4/MAPK p38 signalling

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Endotoxin derived from Gram-negative bacteria induces an acute respiratory distress syndrome (ARDS). Mitogen-activated protein kinase (MAPK) may play a critical role in acute inflammation. We show that the p38 MAPK inhibitor, NVP-AAZ102, prevents LPS induced ARDS, as TNF secretion, bronchoconstriction and recruitment of neutrophil in the lung and BAL fluid are dose-dependently inhibited. TNF inhibition explains individual effects of the p38 MAPK inhibitor, as bronchoconstriction is absent in TNF deficient mice, although neutrophil sequestration in the lung is normal. However, neutrophil recruitment in TNF deficient mice is abrogated by p38 MAPK inhibition. Therefore, bronchoconstriction is TNF-dependent, while neutrophil recruitment depends on the TLR4 signalling which is p38 MAPK mediated.

Targeting Death Receptors in Cancer with Apo2L/TRAIL

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Apo2L/TRAIL is a recently discovered member of the TNF superfamily that triggers apoptosis through engagement of two specific death receptors: DR4 and DR5. Upon engaging DR4 and/or DR5, Apo2L/TRAIL assembles a death-inducing signaling complex (DISC) that activates the apoptosis-initiating proteases caspase-8 and caspase-10 through the adaptor molecule Fas-associated death domain (FADD). Two selective decoy receptors, DcR1 and DcR2, as well as FLIP, an enzymatically inactive relative of caspase-8, inhibit apoptosis induction by Apo2L/TRAIL. As a soluble, zinc-coordinated trimer, Apo2L/TRAIL induces apoptosis in many kinds of tumor cells but not in most normal cell types. Unlike conventional cancer therapeutic agents, Apo2L/TRAIL activates cell death independently of the p53 tumor suppressor gene. In several cancer xenograft models, based upon established tumor cell lines or patient-derived tumors, Apo2L/TRAIL has demonstrated single agent anti-tumor efficacy as well as synergy with chemotherapy. Thus, Apo2L/TRAIL might be effective not only for the second-line treatment of tumors that have acquired resistance to conventional agents, but also for augmenting the efficacy of current first-line therapy in several types of cancer.

Dario Neri **L46**

Clinical Applications: Cancer

Antibody therapies targeting the stromal compartment

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One avenue towards the development of more selective, better anti-cancer drugs consists in the targeted delivery of bioactive molecules (drugs, cytokines, procoagulant factors, photosensitizers, radionuclides, etc.) to the tumor environment by means of binding molecules (e.g., human antibodies) specific for tumor-associated markers.
A good-quality marker for both tumoral and non-tumoral neovasculature is the extra-domain B (ED-B) of fibronectin, a sequence of 91 aminoacids that can be inserted into the fibronectin molecule by a mechanism of alternative splicing. In collaboration with Prof. Dr. Luciano Zardi (Istituto Nazionale per la Ricerca sul Cancro, Genova, Italy), we have generated several high-affinity antibody fragments specific for fibronectin containing the ED-B domain, and mapped their epitope onto the three-dimensional structure of the antigen. One antibody (the high-affinity human antibody L19) has been shown to efficiently target tumor neo-vasculature in animal models of angiogenesis-related diseases and in patients with cancer. A number of derivatives of the L19 antibody (fusions to cytokines, pro-coagulant factors, photosensitizers, drugs, radionuclides, etc.) have been studied in animal models. The results may be of therapeutic relevance, since the ED-B has identical sequence in mouse and man, is expressed in the majority of aggressive solid tumours, but is undetectable in normal vessels and tissues.

Marc Dupuis **L47**

Clinical Applications: Cancer
Short oral communication

Preclinical evaluation of a highly active soluble FasL agonist: safety, pharmacokinetics, and efficacy

Dupuis M, Etter AL, Germain S, Bassi I, Beltraminelli N, Sordat B, Delaloye JF, Rosat JP, Demotz S
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A highly active soluble agonist of FasL was engineered by fusion of its extracellular domain with the collagen stalk of ACRP30/adiponectin. The resulting product is a hexameric soluble fusion protein (MegaFasL), which triggers apoptosis in a wide variety of cell lines and can be produced in large quantities. These studies sought to determine the single dose safety and clearance of MegaFasL in rodents and non-human primates, and its efficacy to slow the progression of a mouse xenograft model of human ovarian cancer. For preliminary safety studies, MegaFasL was given as an i.v. bolus to mice and cynomolgus monkeys. No advert clinical symptoms were observed at up to 20 mg/kg, although hematology and blood chemistry data in monkeys showed that the limiting toxicity was due to a 9-fold decrease in platelet numbers, and to a 4 to 11-fold raise in alanine- and aspartate aminotransferases (ALAT and ASAT) activity at 6 hr after injection. These parameters were already returning to baseline levels at 48 hr, and reached the baseline levels at 14 days after injection. For clearance studies, MegaFasL was given as an i.v. or i.p. bolus to mice, and as an i.v. bolus to cynomolgus monkeys. In mice, MegaFasL was rapidly eliminated from the bloodstream with a half-life of 7 to 28 min after i.v. injection. In contrast, MegaFasL was gradually released in the serum after i.p. injection, reaching a peak at 60 min and decreasing to background levels at 120 min.

For efficacy studies, MegaFasL was tested on human ovarian cancer cells lines, fresh tumor samples *in vitro*, and in immunodeficient mice with established ovarian tumor xenografts. MegaFasL induced apoptosis in ovarian cancer cell lines and fresh tumor samples as measured by Annexin V and TUNEL staining, as well as in a cytotoxity assay. Pretreatment with the drug cisplatin significantly increased the sensitivity of ovarian cancer cells to MegaFasL. Administration of MegaFasL in mice bearing peritoneal orthotopic ovarian HOC79 xenografts produced a significant, dose-dependent prolongation of survival versus control animals.

In conclusion, these studies provide information that will be used to predict the safety and clearance of MegaFasL in humans, and support the hypothesis that the use of MegaFasL in combination with platinum salts may be an effective strategy in slowing the progression of human ovarian cancer.

Clinical Applications: Cancer
Short oral communication

Limb salvage with isolated limb perfusion in 100 adult patients with locally advanced soft tissue sarcoma: a randomized phase II study comparing four doses of TNFα

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Optimal dose of TNFα delivered by isolated limb perfusion (ILP) in patients with locally advanced soft tissue sarcoma is still unknown.
Method: randomised phase II trial comparing hyperthermic ILP (38-40°) with melphalan and one of the 4 assigned doses of TNFα: 0.5mg, 1mg, 2mg, 3mg upper/ 4 mg lower limb. Main objective: objective response on MRI. Secondary objectives: histological response, rate of amputation and toxicity. Resection of the remnant tumour was performed 2 to 3 months after ILP. The sample size was calculated assuming a linear increase of 10% in the objective response rates between each dose level group.

Results: 100 patients (25 per arm) were included, median tumour size lower/upper limb 100/70 (mm), grade I, II, III in 15, 39, 43 patients, 63 recurrences, 43 multiple tumours. Thirteen-percent patients had a systemic leakage (median 3%) with a cardiac toxicity in 6 patients correlated with high doses TNFα. Objective responses were: 68%, 56%, 72% and 64% in the 0.5mg, 1mg, 2mg and 3/4mg arms respectively (NS). Sixteen-percent patients were not operated, 71% had a conservative surgery, 13% were amputated with no group specifits. With a median follow-up of 24 months, the 2 years overall and disease free survival rates (95% CI) were 82% (73%-89%) and 49% (39%-59%) respectively.

Conclusion: At the range of TNFα doses tested, there was no dose effect detected for OR, but systemic toxicity was significantly related to high doses TNFα. Efficacy and safety of low dose TNFα could greatly facilitate ILP procedures in the next future.

Shie-Liang Hsieh **L49**

Clinical Applications: Cancer
Short oral communication

Decoy receptor 3 (DcR3): a pleiotropic immunomodulator

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Tumor cells have developed many strategies to escape host immunity attack and get extra nutrient via the suppression of immune responses and angiogenesis. DcR3 is a member of TNFR superfamily which is overexpressed in tumor cells as well as in the amnion and in the decidua capsularis. Moreover, DcR3 is also up-regulated in patients of autoimmune diseases.

Recently, we have demonstrated that DcR3-treated dendritic cells can bias T cell activation and differentiation into Th2 phenotypes, and injection of DcR3-treated dendritic cells into nonobesity diabetes (NOD) mice can inhibit the onset of diabetes. In addition, the ability of phagocytosis, production of free radicals and uptake of apoptotic cells are severely impaired in DcR3-treated macrophages. Strikingly, addition of DcR3 to HUVEC can induce angiogenesis, and transgenic mice overexpressing DcR3 under the control of insulin promoter completely inhibit the onset of diabetes. Recently, we further demonstrated that DcR3 also enhance the activation and differentiation of osteoclast, thus DcR3 might be one of the contributing factors to induce osteoporosis or bone erosion in cancer patients. Transgenic mice overexpressing DcR3 are used as a model system to further confirm our observation *in vitro*.

Timo L.M. ten Hagen **L50**

Clinical Applications: Cancer
Short oral communication

TNF: a key to solid tumor treatment?

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Targeting the tumor vasculature in the treatment of solid tumors is a highly favorable approach. Not only is the vasculature the first target encountered by systemically injected drugs, it is also very important for the survival of the tumor. Studies with anti-angiogenic agents demonstrated that inhibition of the tumor vascular growth can have a profound effect on tumor development. Moreover, destruction of the tumor associated vasculature (TAV), or manipulation of the endothelial lining of the TAV, to facilitate for instance augmented extravazation of chemotherapeutic agents, was shown to improve greatly the concomitant chemotherapy. We showed in an Isolated limb Perfusion (ILP) setting that tumor necrosis factor-α (TNF) strongly enhances accumulation of co-administered drug in tumor tissue, which was accompanied by a strongly enhanced tumor response. Secondly, also in the systemic treatment setting TNF at low dose acts on the tumor-associated vasculature inflicting increased permeability. Hallmark of the TNF approach is an increased accumulation of the co-administered drug in tumor tissue. TNF is an example of a drug that has rather specific activity on tumor endothelial cells, resulting in morphology changes, integrin expression alterations and apoptosis. As the TAV is recognized as a major candidate in tumor therapy it is becoming more and more important to understand the TNF inflicted anti-vascular effects better. Especially models, which allow real-time monitoring of tumor vascular events *in vivo*, are valuable in this type of research. One such model is intravital microscopy using the dorsal skin-fold window chamber on mice or rats. With this model we study the TNF-induced tumor vascular effects to understand the key functions of TNF in its generation of tumor permissiveness to chemotherapy.

Clinical Applications: Cancer **P01**

Intra-tumoral TNF and cytokine induction by DMXAA for cancer therapy

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Tumor necrosis factor (TNF) induces necrosis of solid tumors through its ability to destroy the tumor vasculature. The efficacy of TNF in cancer therapy however is severely limited by its systemic toxicity. As an alternative approach, we have been investigating small synthetic molecules that can induce TNF synthesis in situ in tumor tissues. An example of this approach, DMXAA, developed in this laboratory is currently under clinical evaluation as an anti-vascular agent for the treatment of cancer. While DMXAA induces significant intra-tumoral TNF synthesis in transplantable murine tumors, and in methylnitrosourea-induced rat tumors, only limited hemorrhagic necrosis and TNF production was observed in three tumor biopsies from patients in the clinical trial. The poor TNF response from clinical samples could not be explained entirely by a difference in dosage or pharmacokinetics, and suggested a difference in regulation of TNF production in humans compared with that in mice or rats. In an effort to understand its molecular mechanism of action, in vitro systems for the induction of TNF and other cytokines by DMXAA were investigated. The intra-tumoral TNF is produced mainly by the infiltrated host macrophage and lymphocyte populations, and we have found that in vitro splenocyte cultures best modeled in vivo intra-tumoral cytokine induction by DMXAA, both in terms of the spectrum of cytokines produced, and in their relative amounts. Gene array and protein array analyses showed up-regulation and production of IL-6, MIP-1a, MIP-2, and RANTES across a broad range of concentrations; IL-4, IL-9, and IFN-g at high concentrations (300 mg/ml), while TNF was produced only at low concentrations (10 mg/ml). The addition of deacylated-lipopolysaccharide, which alone does not induce cytokines although it still binds to the CD14 receptor, led to synergistic amounts of TNF produced, but had no effect on IFN-g production in response to DMXAA in splenocyte cultures. Collectively, the results suggest that DMXAA induces both lymphoid and macrophage derived cytokines, and preferential stimulation of different sets of cytokines can be achieved at different concentrations. TNF induction by DMXAA can be amplified with either agonists or protagonists of CD14/TLR4 signalling, suggesting strategies for further selective and preferential stimulation of TNF production in the clinical setting.

Clinical Applications: Cancer **P02**

Charge modulation in the tip region of TNF-alpha trimer influences its in vitro as well as in vivo biological activity

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In the past numerous attempts have been made to prepare human tumour necrosis factor alpha (TNF) analogues with the aim of increasing TNF specific anti-tumour activity and reducing the undesired systemic toxicity of this pharmaceutically interesting cytokine. The flexible N-terminus of TNF was recognised as a good location for the introduction of various modifications. Thorough analysis of the published data shows that introduction of more basic amino acid residues into the N-terminal region of the TNF molecule, resulting in analogues with increasing isoelectric point (pI), exibits a general trend towards increased in vitro cytotoxicity.
In our laboratory the tip region of the trimeric TNF molecule was studied and found equally useful for introducing modifications. Our previously published results showed that charge reversal in the tip region of human TNF analogues led to significantly increased cytotoxicity in L929 cells in vitro as well as to reduced systemic toxicity of TNF analogue LK-805 (E107K) in tumour-bearing mouse models (fibrosarcoma Sa-1). In this study we demonstrate that a good correlation between isoelectric point of the respective analogue and its in vitro cytotoxicity is found for the tip region. Actually, the correlation is much better for this part of the molecule, since no other influences are included, such as different lengths of the N-termini, which also have a certain impact on cytotoxicity.
Furthermore, modulation of pI by introduction of various mutations in the tip region influences in vitro cytotoxicity in both, mouse and human cell lines. Studying cytotoxicity of several new TNF analogues with gradually increasing pI values, the same behaviour was observed for two mouse cell lines, L929 and WEHI-164 clone 13, as well as for human cell line KYM-1D4. In general the tip region of TNF appears to be an attractive site for introduction of new structural variations that modulate biological activity. Implication for other ligands of the TNF family e.g., Lymphotoxin-alpha, FasL, TRAIL and others, is suggested.

Clinical Applications: Cancer **P03**

Engineered members of the TNF family: Novel fusion proteins with tumor localized activity

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Many cytokines or derivatives thereof provoke severe toxic side effects after systemic application. Facing these limitations we have developed cytokine fusion proteins that lack biological activity in their soluble form but regain cytokine function after targeting to tumor tissue. For this purpose we applied two distinct concepts: i) We designed FasL-based fusion proteins consisting of the extracellular domain of FasL fused to the C-terminus of a targeting module, represented either by a single chain antibody or an extracellular receptor domain. Similar to soluble homotrimeric natural FasL these fusion proteins are devoid of systemic toxicity in mice. This lack of biological activity is reversed after cell surface immobilization via the targeting module, mimicking membrane FasL and thus leading to cell death induction of the targeted and juxtaposed cells. ii) For TNF, which is bioactive in its soluble trimeric form via TNFR1 signaling, a prodrug construct was generated in which cytokine function is inhibited by addition of a TNF receptor fragment to the C-terminus of a scFv-TNF fusion protein. A protease sensitive linker between the TNF module and the receptor fragment allows specific cleavage by recombinant matrix metalloproteinase-2 (MMP-2), an enzyme known to be frequently overexpressed in tumors. Moreover, we present evidence that this MMP-2 sensitive TNF prodrug can be activated by endogenous proteases of tumor cells and, more important, that this process of prodrug conversion into a bioactive molecule is strictly dependent on specific targeting to the cell surface via the tumor associated antigen recognized by the scFv moiety of the prodrug.

Clinical Applications: Cancer **P04**

Modulation of tumor permissiveness for liposomal formulation by Tumor Necrosis Factor alpha

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Successful treatment of solid tumors requires that anticancer agents reach the tumor cells. Rapid metabolism and clearance of drugs, heterogeneous tumor perfusion and increased interstitial fluid pressure result in low drug concentrations at the tumor site. Liposomal encapsulation of anticancer drugs improves therapy because of prolonged circulation time and leakage through the tumor vasculature. In previous studies we have shown that addition of low-dose TNF to Doxil treatment resulted in pronounced tumor response and increased drug uptake. In the present study a B16BL6 mouse tumor model has been used to investigate the effect of TNF on biodistribution of 67Ga-labeled Stealth liposomes of different sizes. Additionally we examined tumor distribution of liposomal drugs real-time in B16BL6 melanoma implanted in the mouse dorsal skin-fold chamber.
After 12 and 24 hours a 6-fold higher tumor accumulation of 100 nm liposomes was found when TNF was co-administered, compared to liposomes alone. For the 400 nm the enhanced accumulation, caused by TNF, was 5- and 9-fold (12 and 24 hours, respectively) and for the 800 nm a 12-fold increase was shown after 24 hours, but no increase was found after 12 hours. Intravital microscopy experiments showed that RhoPE liposomes of 100 nm were mainly present inside bloodvessels, whereas addition of TNF caused an enhanced extravasation of liposomes into the tumor interstitium. Larger liposomes of 400 nm were mainly trapped inside vessels, and co-administration with TNF resulted in slightly enhanced extravasation of liposomes mostly in the periphery of the tumor. Doxil DiO liposomes were used to distinguish between localisation of liposomes and liposome-encapsulated drug. TNF induced an increased extravasation of Doxil DiO and liposome uptake in tumor cells and doxorubicin transport to the nucleus was observed.

In conclusion: we showed that TNF enhances accumulation of Stealth liposomes of 100, 400 and even 800 nm in tumor tissue. With intravital microscopy we saw that TNF caused an enhanced extravasation of liposomes into the tumor interstitium.

Clinical Applications: Cancer **P05**

Inhibition of cytokine-stimulated NF-kB activity by the tumor suppressor CYLD

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Familial cylindromas are benign adnexal skin tumors caused by germline mutations in the CYLD gene. Genetic evidence suggests that CYLD acts as tumor suppressor gene. CYLD is a protein of 953 amino acids harboring a functional deubiquitinating domain at the C-terminal end. A yeast two-hybrid screen using a HaCaT cDNA library identified TRIP (TRAF-interacting protein) as interactor with full-length CYLD. Mapping of the interacting domains revealed that the central domain of CYLD binds to the C-terminal end of TRIP. Far Western analysis and coimmunoprecipitations in mammalian cells confirmed these findings. Both TRIP and CYLD are inhibitors of NF-κB activation mediated by TNFa, IL-1b and the ectodysplasin receptor. The inhibition by CYLD requires the presence of the central domain interacting with TRIP and the deubiquitinase activity at the C-terminal end. Suppression of endogenous CYLD expression by shRNA technology diminished the inhibitory effect of TRIP on NF-κB activation indicating that inhibition by TRIP partially depends on CYLD. TRIP is a RING finger protein, a molecular domain previously shown in other proteins to exhibit E3 ubiquitin ligase activity. Overexpression of TRIP in 293T cells leads to polyubiquitination of TRIP, which is, however, not prevented by coexpressing CYLD. These findings indicate that constitutive NF-κB activation during the cell lineage differentiation of the pilosebaceous-apocrine unit leads to cylindroma tumor formation.

Clinical Applications: Cancer **P06**

Kupffer cell-expressed membrane-bound TNF mediates melphalan hepatotoxicity by means of activating both TNF receptors

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Isolated hepatic perfusion of non-resectable liver cancer using the combination of TNF and melphalan can be associated with a treatment-related hepatotoxicity. We investigated whether, apart from TNF, also melphalan is cytotoxic in primary murine liver cells *in vitro* and investigated mediators, mode of cell death and cell types involved. Melphalan induced a caspase-dependent apoptosis in hepatocytes, which was not seen in liver cell preparations depleted of Kupffer cells. Neutralization of TNF prevented melphalan-induced apoptosis and liver cells derived from mice genetically deficient in either TNF receptor 1 or 2, but not from *lpr* mice lacking a functional CD95 receptor, were completely resistant. Melphalan increased membrane-bound, but not secreted TNF in Kupffer cells and cell-cell contact between hepatocytes and Kupffer cells was required for apoptosis to occur. In accordance with the *in vitro* results, melphalan induced a significant hepatotoxicity in the isolated recirculating perfused mouse liver from wild type mice, but not from TNF receptor 1 or 2 knock-out mice. In conclusion, this study shows that melphalan elicits membrane TNF on Kupffer cells and thereby initiates apoptosis of hepatocytes *via* activation of both TNF receptor 1 and TNF receptor 2. The identification of this novel mechanism allows a causal understanding of melphalan-induced hepatotoxicity.

Clinical Applications: Inflammation **P07**

Circulating levels of tumor necrosis factor-alpha correlate positively with severity of hepatic encephalopathy in patients with chronic liver failure

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It has been demonstrated that serum levels of tumor necrosis factor-α (TNF) are significantly elevated in patients with acute and chronic liver diseases, where this elevation is independent of the etiology of the underlying disease. Serum levels of TNF were also shown to be significantly higher in patients with cirrhosis than in those without cirrhosis, reaching the highest levels in decompensated cirrhosis. It has also been shown that plasma levels of TNF correlate with severity of hepatic encephalopathy (HE) in patients with fulminant hepatic failure. However, the relationship between TNF and HE in patients with chronic lever failure has not previously been evaluated. The aim of this study is to determine the relationship between circulating levels of TNF and severity of HE in patients with liver cirrhosis, compensated and decompensated, due to various etiologies. Using a cmmercially available high-sensitivity enzyme-linked immunosorbent assay kit, serum levels of TNF were measured in 74 patients with liver cirrhosis due to various etiologies, in various clinical grades of HE (grades 0-4). The mean ± SEM values of serum levels of TNF at presentation before intervention in patients with grade 0 of HE (n = 23), grade 1 (n = 12), grade 2 (n = 14), grade 3 (n = 16), and grade 4 (n = 9) were 4.50 ± 0.46, 9.10 ± 1.0, 12.98 ± 1.22, 21.51± 2.63, and 58.26 ± 19.7 pg/mL, respectively. Serum levels of TNF were significantly higher in patients with HE than in those without HE ($p < 0.0001$). Furtheremore, a significant positive correlation was found between serum levels of TNF and severity of HE ($p < 0.0001$). No significant difference was found between infectious and non-infectious precipitating factors of HE regarding serum levels of TNF in patients with the same grade of HE. Furtheremore, for patients in the same grade of HE, comparison between the group of patients in their steady-state grade of HE and group of patients at presentation after acute deterioration due to precipitating factors, with respect to serum levels of TNF, revealed no significant difference between the two groups. In conclusion, the results of this study demonstrate, for the first time, a strong positive relationship between serum levels of TNF and severity of HE in patients with chronic liver failue, and may provide indirect evidence to suggest TNF as a possible mediator of HE in chronic liver failure.

Clinical Applications: Inflammation **P08**

Circulating levels of tumor necrosis factor-alpha correlate positively with severity of peripheral edema in patients with right-sided heart failure

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A variety of clinical and experimental studies have demonstrated that circulating levels of tumor necrosis factor-α (TNF) are elevated in humans and animals with advanced congestive heart failure (CHF). This cytokine has since been strongly implicated in the pathogenesis of this cardiac disease. However, the main origin of the increased circulating levels of TNF in CHF is still controversial and remains unknown. The majority of these studies focused mainly on left-sided heart failure, and only a few studies dealt with right-sided heart failure (RHF) in this regard. The aim of this study is to evaluate the relationship between circulating levels of TNF and severity of peripheral edema in patients with RHF related to ischemic heart disease (IHD) or idiopathic dilated cardiomyopathy (IDC), which has not been previously evaluated. Anticubital venous blood was collected at presentation from all control subjects and all patients for TNF assay. Using a commercially available high sensitivity ELISA kit, serum levels of TNF were measured in 15 healthy volunteers, and in 83 patients with RHF of various grades of peripheral edema. At presentation 13 patients were without peripheral edema (grade 0), 22 patients were with mild peripheral edema (grade 1), 23 patients were with moderate peripheral edema (grade 2), and 25 patients were with severe peripheral edema (grade 3). The mean ± SEM values of serum levels of TNF at presentation of the control group and of the above groups of various grades of edema were 2.98 ± 0.2 pg/mL, 4.22 ± 0.55 pg/mL, 4.67 ± 0.29 pg/mL, 7.66 ± 0.44 pg/mL, and 10.94 ± 0.67 pg/mL respectively. Serum levels of TNF were significantly higher in the group of patients with edema (7.89 ± 0.42 pg/mL) than in the group of patients without edema (4.22 ± 0.55 pg/mL) and than the control group (2.98 ± 0.2 pg/mL) ($p < 0.0001$). A significant positive correlation was found between serum levels of TNF and severity of peripheral edema ($r = 0.77$, $p < 0.0001$). In conclusion, circulating levels of TNF correlate positively with severity of peripheral edema in patients with RHF due to IHD or IDC. Since severity of peripheral edema may indirectly represent severity of bowel-wall edema in these patients, the results of this study may support the hypothesis that the origin of the high circulating levels of TNF in CHF could be the edematous bowel-wall.

Clinical Applications: Inflammation **P09**

Pleural fluid to serum ratio of tumor necrosis factor-alpha concentration is a good marker for discrimination between complicated and uncomplicated parapneumonic effusion

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We have recently demonstrated in a preliminary study that pleural fluid to serum ratio of tumor necrosis factor-α (TNF) concentration (TNFratio) is a good marker for discrimination between complicated parapneumonic effusion (CPPE) and uncomplicated parapneumonic effusion (UCPPE). The aim of this study is to investigate this issue in a significantly larger population, and to compare the efficacy of TNFratio with that of pleural fluid TNF concentration (TNFpf), glucose concentration, and lactate dehydrogenase (LDH) concentration in discrimination between CPPE and UCPPE. Using a commercially available high sensitivity ELAISA kit, levels of TNF were measured in serum and pleural fluid of 51 patients with UCPPE, and 30 patients with nonempyemic CPPE. The mean±SEM values of serum TNF (TNFserum), pleural fluid TNF (TNFpf), pleural fluid glucose, pleural fluid LDH, and TNFratio in the UCPPE group were 6.65±0.48 pg/mL, 10.85±0.74 pg/mL, 128±7 mg/dL, 439±68 U/L, and 1.70±0.07 respectively, and in the CPPE group were 7.59±0.87 pg/mL, 54.02±5.43 pg/mL, 51±9 mg/dL, 1758±279 U/L, and 9.08±1.42 respectively. While no significant difference was found between the two groups regarding levels of TNFserum ($p=0.31$), a highly significant difference between these two groups was found regarding levels of TNFpf, pleural fluid glucose, pleural fluid LDH, and TNFratio ($p<0.0001$ for each variable). TNFpf, at an optimal cut-off level of 25.0 pg/mL for discrimination between UCPPE and CPPE, achieved by the receiver operating characteristic analysis, has sinsitivity of 83.3%, specificity of 100%, accuracy of 93.8%, area under the curve (AUC) of 0.96, and $p<0.0001$. Pleural fluid glucose, at an optimal cut-off level of 74 mg/dL, has sensitivity of 83.3%, specificity of 94.1%, accuracy of 90.1% AUC of 0.91, and $p<0.0001$. Pleural fluid LDH, at an optimal cut-off level of 513 U/L, has sensitivity of 80%, specificity of 86.3%, accuracy of 83.9%, AUC of 0.89, and $p<0.0001$. TNFratio, at an optimal cut-off level of 3.0, has sensitivity of 96.7%, specificity of 98%, accuracy of 97.5%, AUC of 0.99, and $p<0.0001$. In conclusion, TNFratio is a good marker for discrimination between CPPE and UCPPE,and is better than the above other variables for this discrimination.

Clinical Applications: Inflammation **P10**

Relationship between tumor necrosis factor-α and severity of liver disease represented by the Child-Pugh score classes in patients with compensated or decompensated liver cirrhosis

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Blood levels of tumor necrosis factor-α (TNF) are high in patients with acute and chronic liver diseases, and this proinflammatory cytokine has been shown to be involved in the patogenesis of the liver damage in these patients, independent of the etiology of the underlying liver disease. It has also been demonstrated that blood levels of TNF are higher in patients with decompensated liver cirrhosis than in those with compensated liver cirrhosis. This study was undertaken to determine, for the first time, the relationship between circulating levels of TNF and severity of liver disease represented by the Child-Pugh score classes in patients with compensated or decompensated liver cirrhosis of various etiologies. Using a commercially available high-sensitivity enzyme-linked immunosorbent assay kit, serum levels of TNF were measured in 74 patients with liver cirrhosis, compensated or decompensated, of various etiologies (hepatitis B virus, hepatitis C virus, primary biliary, alcoholic, and cryptogenic). The mean ± SEM values of serum levels of TNF, and 95% confidence intervals (CIs) for means of group of patients with Child-Pugh score class A ($n = 10$), class B ($n = 29$), and class C ($n = 35$) were 4.22 ± 0.63 pg/mL (95% CI: 2.8-5.65), 7.67 ± 0.77 pg/mL (95% CI: 6.1-9.25), 28.51 ± 5.87 pg/mL (95% CI: 16.57-40.44) respectively. A significant difference was found between the various groups ($p < 0.0001$, ANOVA). Every two groups showed a significant difference. Also, a significant positive correlation was found between serum levels of TNF and severity of liver disease represented by the Child-Pugh score classes ($r = 0.8$, $p < 0.0001$). In conclusion, a strongly significant positive relationship is present between circulating levels of TNF and severity of liver disease in patients with liver cirrhosis.These results may indicate that TNF could be responsible, at least in part, of the progressive liver damage and the consequent liver failure in patients with liver cirrhosis.

Co-stimulatory Ligands **P11**

Dominant-Negative Antagonists of TNF and BAFF - novel inhibitors for the treatment of inflammatory and autoimmune diseases

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The therapeutic role of inhibitors of the TNF superfamily of ligands (TNFSF) in human diseases is well established. To receptor-specifically block TNFSF signaling, we utilized Dominant Negative (DN) technology that is based on the ability of multimeric proteins to exchange subunits with variants differing from the wild type by a small number of amino acids. The DN-TNFSF variants lack the ability to agonize corresponding TNFSF receptors and functionally antagonize them by forming heterotrimers with the wild type, thus sequestering endogenous TNFSF members in non-signaling heterotrimers. We utilized Xencor's Protein Design Automation technology and models or crystal structures of the ligand/receptor complexes to create DN libraries for TNF and BAFF. We determined the size of BAFF constructs with various N-terminal sequences by size-exclusion chromatography, since there were conflicting reports regarding the oligomerization state of native BAFF (3-mer vs. 60-mer) and we expected only trimeric cytokine to efficiently exchange with DN-BAFF. This analysis showed that native BAFF is a trimer that displays full biological activity and higher order oligomers are an artifact of N-terminal His-tagging. We developed in vitro assays to assess TNF and BAFF variants' propensities to exchange, to bind cognate receptors of TNF or BAFF, and to agonize and antagonize the appropriate native cytokines in cell-based assays. We also tested the efficacy of DN antagonists in vivo. This strategy identified antagonists of TNF and BAFF with distinct selectivity profiles toward their respective receptors. Based on the in vitro cell-based assays and in vivo models, the inhibitory properties of DN-TNF and DN-BAFF are similar to those of either marketed (TNF) or the development-stage (BAFF) antagonists. Comparison of binding to BAFF receptors and B-cell proliferation activity by DN-BAFF confirms the role of BAFF-R as the BAFF B-cell proliferation signal transducer; binding to BCMA is inconsequential to B-cell proliferation. Several DN-TNF variants specifically inhibit soluble, but not membrane-bound TNF, and demonstrate marked reduction of side effects attributed to marketed TNF antagonists. In conclusion, our study suggests that the DN antagonist paradigm can produce novel therapeutic inhibitors of TNF and BAFF for treating inflammatory and autoimmune diseases, and that specific DN-TNF and DN-BAFF variants may be useful in the investigation of the multi-receptor (and multi-ligand) biology of these TNFSF cytokines.

Co-stimulatory Ligands **P12**

A role for TL1A, the TNF-family ligand for the death receptor DR3, in APC:T cell interactions during T cell activation

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Accumulating evidence has implicated molecules in the death receptor pathway such as FADD, Caspase-8 and c-FLIP in T cell proliferation and survival. However, which if any TNF-family ligands and receptors mediate the pro-survival effects of caspases is not known. The TNF ligand-receptor pair TL1A-DR3 may be a candidate for this function, as DR3 is specifically expressed on activated lymphocytes and TL1A can function as a T cell costimulator. DR3 (TRAMP/LARD/WSL-1) is a death domain containing receptor that can activate caspases or NF-κB depending on the cellular context. Initial reports suggested that TL1A was expressed exclusively on endothelial cells. To determine whether TL1A may have a role in T cell costimulation, we have analyzed expression of TL1A mRNA in a variety of immune cell types before and after activation. TL1A mRNA is highly induced within 3 hours of T cell stimulation of unseparated human PBMC. However, activated or resting B or T cells do not express detectable TL1A. Instead, TL1A mRNA is induced in the adherent cell fraction. A small induction of TL1A mRNA is detected in T cells when stimulated in presence of APCs. Thus, T cell induction of TL1A mRNA in APC's may constitute a positive costimulatory feedback loop between T cells and APCs. At the protein level, a band corresponding to cleaved TL1A is detected in adherent cells, and intracellular TL1A can be detected by FACS on non-T cells. Surprisingly, addition of DR3-Fc, which should block TL1A-DR3 interactions, increased T cell cycling when T cells were activated in the presence of APC's. Taken together, these data suggest that APC's are the primary source of TL1A during in vitro stimulation of PBMC. Ongoing experiments will test the functional role of TL1A-DR3 interactions on T cell activation and long-term survival.

Co-stimulatory Ligands **P13**

Regulation of humoral responses by APRIL

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The TNF-like ligands APRIL and BLyS are close relatives and share the capacity to bind the receptors TACI and BCMA. BLyS has been shown to play an important role in B cell homeostasis and autoimmunity, but the biological role of APRIL remains less well defined. We used APRIL transgenic (Tg) mice to further analyse the effect of APRIL in humoral responses. Following immunisation with a T cell-independent type 1 antigen, APRIL Tg mice showed increased IgM and IgG titres throughout the immune response. Induction of a T cell-dependent humoral response by administration of vaccinia virus resulted in elevated IgM titres in the transgenic mice up to 100 days after immunisation; in contrast, IgG responses were downregulated more rapidly in APRIL Tg than in control mice. There are other members of the TNF ligand family that induce different signals. Fas ligand, for example, stimulates T cells during the early activation phase whereas it mediates apoptosis at later phases of T cell activation.
We are presently testing whether the observed distinct signaling of APRIL can be also observed during T cell-dependent humoral responses against non-vial antigens.

Death receptors **P14**

CD95 maintains effector T cell homeostasis in chronic immune activation

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Apoptosis is vital to maintain homeostasis of antigen-stimulated T cells. After cessation of antigen, cytokine withdrawal initiates an apoptotic pathway that depends on mitochrondrial release of cytochrome c, whereas repetitive antigenic stimulation induces expression of CD95-ligand and renders T cells susceptible for CD95-induced cell death. TRAF-binding members of the TNF-receptor family, such as OX40, 4-1BB and CD27, antagonize mitochondrion-dependent cell death by upregulating anti-apoptotic bcl-2 family molecules such as Bcl-xL and Bfl-1. We here show that triggering of CD27 sensitizes T cells for CD95-induced apoptosis. CD95-deficient (lpr/lpr) T cells massively expanded and differentiated into IFN-g-secreting effector cells in transgenic mice that chronically express the CD27-ligand, CD70. CD95-deficient/CD70-transgenic mice became moribund by 4 weeks of age with severe liver-pathology and bone marrow failure. Thus, CD95 is a critical regulator of effector T cell homeostasis in chronic immune activation.

Death receptors **P15**

Withdrawn

Death receptors **P16**

Soluble TNF coupled to micro-beads mimicks membrane-bound TNF a new tool for receptor stimulation

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The two known TNF-receptors, TNFR1 and TNFR2, have different requirements for optimum stimulation. TNFR1 is equally well triggered by soluble TNF (sTNF) and membrane-bound TNF (memTNF), whereas TNFR2 signalling mainly depends on a stimulation with memTNF. Although a memTNF-like stimulus can be produced by e.g. the combination of sTNF and a crosslinking, non-agonistic TNFR2-specific antibody, we were interested to develop a tool that would allow spatially restricted stimulation of TNF receptors. To achieve this, we generated a sTNF-derived mutein containing a Cysteine residue in its N-terminus. This mutein was covalently coupled to the surface of micro-particles which had been activated with a hetero-bifunctional cross linker. For applications in confocal live cell imaging, beads stained with Alexa-Fluor-568 were used. Mouse fibroblasts deficient of wildtype TNFR1 and -2, but expressing a TNFR2-Fas chimera were used as a test system. These cells are fully resistant to sTNF treatment, but rapidly develop apoptosis when stimulated with memTNF. Similar to memTNF, TNF-bearing micro-beads induced a strong apoptotic response in these cells. In TNF resistant cells, beads with a diameter of 1 μm or less were shown to be internalised after longer incubation times, presumably by phagocytosis. Mechanically induced contact of a selected TNF sensitive cell with a single 10 mm TNF-bead caused TNFR2-clustering and finally induced apoptosis. In HeLa cells, overexpressing TNFR2 and TRAF2-GFP, recruitment of TRAF2 to TNFR2 and colocalization of both molecules could be observed that was apparently independent of TNFR2 internalisation.

Death receptors **P17**

Cloning and characterization of a novel small size zinc finger-like protein Zfra that interacts with WOX1, JNK1 and NF-κB during stress response

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In this study, we isolated a cDNA, encoding a putative 31-amino-acid zinc finger-like protein that regulates apoptosis (named Zfra), from libraries of human and mouse. Northern blotting revealed the presence of a ~500 bp mRNA transcript in TNF-sensitive L929 fibroblasts, but not in TNF-resistant L929R cells. RT/PCR showed reduction of Zfra mRNA expression by TNF-α in a time-dependent manner. *In vitro* translation of the cDNA yielded a 4-kDa protein. Immunoprecipitation also showed presence of Zfra protein in L929, Molt4 T cells, and SK-N-SH neuroblastoma cells and rat brain extracts. Transient overexpression of Zfra induced apoptosis of L929, HEK-293 and ovarian ME180 cells, but not prostate DU145 and lung Mv1Lu cells. Upon stimulation of SK-N-SH and Molt-4 T cells TNF-α or UV light, Zfra rapidly became self-associated and bound with proapoptotic WOX1 (FOR2/WWOX), JNK1 and then NF-κB (p65) in less than 30 min, as determined by GST-Zfra pull down and immunoprecipitation assays. As determined by DNA fragmentation and cell cycle analyses, ectopic Zfra and WOX1 mediated apoptosis in both synergistic and antagonistic manners, whereas Zfra had no apparent effect on JNK1. Yeast two-hybrid mapping showed that Zfra interacted with the *N*-terminal first WW domain and the *C*-terminal short chain alcohol dehydrogenase/reductase (SDR) domain of WOX1, where Tyr33 phosphorylation in the WW domain was essential for this binding. Functionally, ectopic Zfra blocked UV-induced WOX1 nuclear translocation, whereas it enhanced phosphorylation and nuclear translocation of JNK1 and ERK. In conclusion, Zfra is a novel small size protein that participates in stress responses via interacting with WOX1, JNK1 and NF-κB.

Death receptors **P18**

Regulation of apoptosis and signal transduction by distinct CD40 domains.

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CD40 is frequently expressed in carcinomas where its stimulation results in induction of apoptosis when *de novo* protein synthesis is inhibited. The requirement of protein synthesis inhibition for efficient killing implies that CD40, like other TNF receptor family members transduces potent survival signals which suppress its pro-apoptotic effects. Indeed, we have recently shown that one mechanism by which CD40 promotes cell survival is via the regulation of the translational machinery in a PI3K and ERK MAPK dependent manner, and the subsequent increase in expression of the anti-apoptotic protein c-FLIP$_S$ (J. Biol. Chem. (2004) 279: 1010 – 1019). We have proceeded to identify the domains within the cytoplasmic tail of CD40 responsible for survival/pro-apoptotic effects. To this end, a panel of HeLa clones stably expressing CD40 receptors mutated within the TRAF binding domains were generated. Mutation of the TRAF2/TRAF3 binding sites significantly impaired CD40 ligand-induced activation of p38 MAPK whilst a mutation that abolished interactions with TRAF6 had minimal effect. Interestingly, ligation of CD40 mutated at the TRAF6 but not the TRAF2/TRAF3 binding domain induces apoptosis without the need for protein synthesis inhibition. This apoptotic effect was rapid, dependent on caspase-8 and caspase-3 but not caspase-9, and independent of p38 MAPK signal transduction. Importantly, ligation of CD40 mutated at both the TRAF6 and the TRAF2/TRAF3 binding domains prevented apoptosis implying that TRAF2 and/or TRAF3 are critical for this apoptotic response. Experiments using RNA interference support such a role. A better understanding of the mechanisms by which CD40 promotes survival or cell death will greatly facilitate the development of therapeutic agents that could be used in combination with CD40 ligand for cancer therapy.

Death receptors **P19**

Acid sphingomyelinase mediates DR5 clustering and is involved in DR5-induced apoptosis

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The Trail/DR5-system is known to trigger apoptosis in many tumor cells as well as in some normal cells, e.g. activated splenocytes. DR5-ligation induces the formation of a caspase 8-containing DISC that results in activation of caspase 3 and finally cell death. Here, we investigated the role of membrane lipids and alterations of the plasma membrane composition for DR5-triggered apoptosis. Our data demonstrate that stimulation via DR5 with 0.5 to 5 ng/ml Trail induces rapid activation of the acid sphingomyelinase and triggers a concomitant release of ceramide. Ceramide locates to the extracellular leaflet of the cell membrane upon DR5-stimulation and forms large ceramide-enriched membrane platforms. DR5 clusters upon stimulation in those platforms suggesting that ceramide-enriched membrane platforms serve to trap the receptor and to initiate a very high density of receptor molecules. Studies on acid sphingomyelinase deficient-peripheral lymphoblasts reveal that cells lacking the acid sphingomyelinase are resistant to Trail-induced cell death. The data indicate a novel player in Trail/DR5-induced apoptosis. They suggest that activation of the acid sphingomyelinase, the release of ceramide and the formation of ceramide-enriched membrane platforms serve to cluster DR5 that seems to be a pre-requisite for the induction of apoptosis.

Death receptors **P20**

Apo2L/TRAIL is a potent anti-cancer agent for the treatment of breast cancer growth in bone

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Breast cancer is the most common carcinoma that metastasises to bone. Bone metastases in breast cancer patients are associated with extensive bone destruction, leading to bone pain, hypercalcemia, pathological fractures, spinal cord compressions, and eventually death. To examine the *in vivo* anti-tumour effects of Apo2L/TRAIL, we established a mouse model in which MDA-MB-231 human breast cancer cells are transplanted orthotopically into the tibiae of athymic mice. These tumour cells grow reproducibly and produce osteolytic lesions in the area of injection. The animals transplanted with MDA-MB-231 breast cancer cells and left untreated developed large lesions that invaded the marrow cavity and eroded the cortical bone, as assessed by radiography, micro computed tomography (mCT) and histology. In contrast, animals treated i.p with 30 mg/kg/dose of Apo2L/TRAIL for five consecutive days followed by once weekly for four weeks, showed significant conservation of the tibiae with much reduced areas of osteolysis. The amount of bone loss in the tumour bearing tibiae of the untreated group was 40%, whereas in the Apo2L/TRAIL treated group, was only 5%. Histological examination of the Apo2L/TRAIL-treated animals, showed the presence of tumour cells in the marrow cavity, but the tumours were significantly smaller and were confined to the site of transplantation. The challenge for the future is to completely eradicate these tumours using combination of Apo2L/TRAIL with chemotherapy. Our study demonstrates for the first time that Apo2L/TRAIL protects the skeleton from the bone destruction induced by these tumours.

Death receptors **P21**

The histone deacetylase inhibitor, suberoylanilide hydroxamic acid (SAHA), overcomes resistance of human breast cancer cells to Apo2L/TRAIL

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The histone deacetylase inhibitor, suberoylanilide hydroxamic acid (SAHA), overcomes resistance of human breast cancer cells to Apo2L/TRAIL
Agents that combine with Apo2L/TRAIL to enhance apoptosis of breast cancer cells will increase the utility of this agent for breast cancer. We have found that the histone deacetylase inhibitor, suberoylanilide hydroxamic acid (SAHA), can sensitize breast cancer cells to Apo2L/TRAIL-induced apoptosis, at doses that individually have little effect on cell viability. Importantly, neither TRAIL alone, nor in combination with SAHA, affected the viability of normal cells. We selected Apo2L/TRAIL-resistant MDA-MB-231 breast cancer cells by long-term culture with Apo2L/TRAIL. These cells were re-sensitized to Apo2L/TRAIL-induced apoptosis by SAHA. Sensitization by SAHA was mediated by activation of caspases 8, 9 and 3, which was concomitant with BID and PARP cleavage. The expression of BAX increased significantly with SAHA treatment and high levels of BAX were maintained in the combined treatment with Apo2L/TRAIL. Treatment with SAHA increased cell surface expression of DR5 but not DR4, DcR2 or OPG. Interestingly, SAHA treatment resulted in a significantly greater increase in cell surface expression of DcR1. Taken together, our findings indicate that the use of these two agents in combination may be effective for the treatment of breast cancer.

Death receptors **P22**

Membrane FasL induces supramolecular Fas clusters of high stability

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Using fluorescent fusion proteins of Fas and FasL we show in this study that membrane FasL interacts with Fas to form supramolecular FasL-Fas clusters at contact sites between FasL and Fas expressing cells. These FasL-Fas clusters are of flexible shape but are nevertheless durable and very stable. Fluorescence loss in photobleaching experiments revealed that there is almost no release of Fas or FasL from the clusters. Importantly, Fas-FasL clusters are also formed when Fas-YFP was expressed in caspase-8 or FADD deficient cells or using a cytoplasmic deletion mutant of Fas. This suggests that the known Fas signaling pathways are not necessary for cluster formation. Moreover, deletion of the cytoplasmic domain of FasL and the presence of the raft destabilizing component β-cyclodextrin had also no effect on the stability of the Fas-FasL clusters. Together, these data suggest that the interaction of the extracellular domains of Fas and FasL alone is sufficient to drive the formation of supramolecular Fas-FasL complexes which are regarded as a structural prerequisite for Fas activation.

Death receptors **P23**

Hepatitis C Virus Core Protein modulates TRAIL-mediated Apoptosis by enhancing Bid cleavage and activation of Mitochondrial Apoptosis Signaling Pathway

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Hepatitis C virus (HCV) is a major human pathogen causing chronic liver disease, which leads to cirrhosis of liver and hepatocellular carcinoma (HCC). The HCV core protein, a viral nucleocapsid, has been shown to affect various intracellular events including cell proliferation, and apoptosis. However, the precise mechanisms of the effects are not fully understood. Here we show that HCV core protein sensitizes human HCC cell line, Huh7, conferred sensitivity to TRAIL- but not FasL-mediated apoptosis. The induction of TRAIL sensitivity by HCV core protein was not due to upregulation of surface TRAIL death receptors. Nevertheless, TRAIL-induced caspase-8 activation and Bid processing was enhanced by HCV core protein. HCV core protein also facilitates activation of mitochondrial transmembrane potential and cytochrome c release upon TRAIL engagement. Therefore, the HCV core protein–induced TRAIL-mediated apoptosis is dependent upon activation of caspase-8 downstream pathway to convey the death signal to mitochondria, leading to activation of mitochondrial signaling pathway and breaking the apoptosis resistance. These results combined indicate that the HCV core protein enhances TRAIL- but not FasL-mediated apoptotic cell death in Huh7 cells via a mechanism dependent on the activation of mitochondrial apoptosis signaling pathway. These results suggest that HCV core protein may have a role in immune-mediated liver cell injury by modulation of TRAIL-induced apoptosis.

Death receptors **P24**

NF-κB inhibition potentiates TRAIL mediated cell death.

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The TNF-related apoptosis-inducing ligand (TRAIL) induces apoptosis preferentially in tumor cell lines and not in normal cells. TRAIL binds the specific transmembrane receptors, activates caspases and induces NF-κB. However, the role of NF-κB in TRAIL-mediated apoptosis is still not well understood. We examined the role of NF-κB in TRAIL-induced apoptosis in different cell types using a highly specific small molecule IkappaB kinase complex inhibitor (I229). In all tested cellular models, e.g. THP-1, U937, HEK293, HEPG2, and HEP3B cells, TRAIL induces apoptosis moderately and inhibition of IkappaB kinase (IKK) complex dramatically potentiates TRAIL-induced apoptosis. The overexpression of IkBasd , non-degradable inhibitor of NF-κB, in HEK293 cells enhances TRAIL-induced apoptosis to the same extend as inhibition of NF-κB by I229. TRAIL activates caspase 3 and its inhibition with pan-Caspase inhibitor zVADfmk completely blocks TRAIL-mediated apoptosis. Several different genes were transiently overexpressed to test their potential protective effect against TRAIL-mediated apoptosis, including FADDdn, AKT, Bcl-x, FLIP. Only overexpression of FADDdn showed strong protective effects. Global gene expression profiling has been used to study mechanisms of TRAIL-signalling and role of NF-κB in anti-TRAIL protection. Our results suggest that NF-κB plays a central role in TRAIL-mediated signaling/apoptosis and blocking of TRAIL-mediated NF-κB activation leads to dramatic enhancement of the TRAIL-induced apoptosis. Primary human hepatocytes were found to be not sensitive to the apoptosis induction by TRAIL and IKK inhibitor. This suggests that a combined therapy with TRAIL and IKK-inhibition may be used as an efficient strategy to treat certain tumors.

Death receptors **P25**

TRAIL-mediated proliferation of tumor cells in the presence of cytotoxic drugs

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Recently, we described that the death inducing ligand TRAIL (TNF-related apoptosis inducing ligand) mediates survival and proliferation in tumor cells resistant against apoptosis induction by TRAIL. We now established a new FACscan-based technique to determine cell concentration. TRAIL induced proliferation was found in about 25 % of 30 TRAIL-resistant cell lines tested with a maximum of a two-fold increase in newly formed cells by addition of TRAIL. TRAIL mediated both apoptosis and proliferation simultaneously in the same cell line. TRAIL mediated proliferation in the presence of some, but not all cytotoxic drugs. All 9 cell lines tested with dysfunctional death inducing complex (dysfunctional FADD, loss of caspase-8 or overexpression of FLIP) showed proliferation after stimulation with TRAIL. We conclude that TRAIL-mediated proliferation of tumor cells is a relevant characteristic of TRAIL, which should be prevented during anti-tumor therapy.

Death receptors **P26**

Single chain TNF, a novel designed TNF mutein with enhanced stability

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Single chain TNF, a novel designed TNF mutein with enhanced stability. Bioactivity of the cytokine tumor necrosis factor (TNF) is linked to its homotrimeric structure, stabilized by hydrophobic interactions. To prevent dissociation of soluble TNF into monomers at low concentrations, we have designed a new variant of TNF, called single chain TNF (scTNF). In this molecule the three TNF monomers are connected by Glycin/Serine linkers to form a single polypeptide.
scTNF was produced in an *E. coli* expression system, purified to homogeneity and subsequently freed from lipopolysaccharides. Functional assays revealed similar apoptotic and gene inductory activities of scTNF when compared to soluble TNF using the Kym-1 cell line as well as stably transfected murine fibroblasts. Importantly, scTNF largely remains its bioactivity over a period of 3 weeks at a concentration of only 3 ng/ml, whereas soluble TNF loses its cytotoxic activity near to total. Similar results were obtained when scTNF was kept in the presence of non heat inactivated serum. Interestingly, determination of the binding affinity using radioiodinated TNF revealed a significantly higher affinity for scTNF binding to TNFR1 (Kd = 26+/-12 pM) as compared to wildtype TNF (Kd = 166+/-86 pM). In an in vivo mouse model of septic shock, where TNF was injected intravenously, scTNF shows a similar or even enhanced biological activity in comparison to wildtype TNF. These results are in line with the blood retention time of scTNF that is more than twice the value of wildtype TNF.
In summary we have created a new TNF mutant with significantly enhanced stability in vitro and in vivo retaining its full biological activity. Therefore, single chain TNF represents an interesting molecule for drug design.

Death receptors **P27**

TNF mediated neuroprotection against glutamate induced excitotoxicity is enhanced by NMDA receptor activation: Essential role of a TNF receptor 2 mediated, PI3 kinase dependent NF-κB pathway

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We have previously shown that the two TNF receptors (TNFR) exhibit antagonistic functions during neurodegenerative processes in vivo, with TNFR1 aggravating and TNFR2 reducing neuronal cell loss, respectively. In order to elucidate the neuroprotective signalling pathways of TNFR2, we investigated glutamate induced excitotoxicity in primary cortical neurons. TNF-expressing neurons from TNF transgenic mice were found to be strongly protected from glutamate-induced apoptosis. Neurons from wildtype and TNFR1$^{-/-}$ mice prestimulated with TNF or agonistic TNFR2 specific antibodies were also resistant to excitotoxicity, whereas TNFR2$^{-/-}$ neurons died upon glutamate and/or TNF exposures. Both PKB/Akt and NF-κB activation were apparent upon TNF treatment. Both, TNFR1 and TNFR2 induced the NF-κB pathway, yet with distinguishable kinetics and upstream activating components: TNFR1 induced only transient NF-κB activation, while TNFR2 facilitated long term, strictly PI3K-dependent NF-κB activation. Glutamate induced triggering of the ionotropic N-Methyl-D-Aspartate receptor was required for the enhanced and persistent, PI3K-dependent NF-κB activation by TNFR2, indicating a positive cooperation of TNF and neurotransmitter induced signal pathways. TNFR2 induced persistent NF-κB activity was essential for neuronal survival. Thus, the duration of NF-κB activation is a critical determinant for sensitivity towards excitotoxic stress and is dependent on a differential upstream signal pathway usage of the two TNFRs.

Death receptors **P28**

Enhanced apoptosis and tumor regression induced by a direct agonist antibody to TRAIL-R2

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Substantial evidence indicates that supraoligomerization of the death receptors for Fas ligand (FasL) and Tumor necrosis factor-related apoptosis-Inducing ligand (TRAIL) is necessary for efficient activation of the apoptotic pathway. Oligomerization of Fas or TRAIL receptors with bivalent IgG antibodies can mimic the natural ligands, but only after these antibodies are further oligomerized by the addition of secondary crosslinking reagents. We report here a novel fully human IgG antibody to TRAIL-R2, KMTR2, that directly activates tumor cell apoptosis in vitro without the requirement of crosslinking IgG. Size-exclusion chromatography demonstrated the apoptosis activity co-eluted with monomeric IgG and was effective independent of the presence secondary antibody or FcR-expressing effector cells. The KMTR2 antibody formed supracomplexes with soluble recombinant and membrane-anchored TRAIL-R2 and enhanced clustering of TRAIL-R2 on the surface of cell without crosslinking. The KMTR2 antibody was dramatically efficacious in reducing establish human xenograft tumors in vivo when compared to other anti-TRAIL-R2 antibodies of similar isotype and affinity suggesting the agonistic anti-tumor activity is independent of host effector function. These results indicate that this monoclonal agonist antibody can direct antibody-dependent oligomerization of TRAIL-R2 and initiates efficient apoptotic signaling and tumor regression.

Death receptors **P29**

The role of lipid rafts in the regulation of Fas ligand activity

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Lipid rafts are small cholesterol- and sphingolipid-rich domains of the cell membrane, and are known to be crucial in diverse signaling pathways, such as T cell receptor signaling or induction of cell death via TNF receptor 1. Upon now, nothing is known about the role of membrane lipid rafts in the regulation of Fas (CD95/APO1) ligand (FasL)-mediated cytotoxicity.
Since coordinated expression of FasL protein on the cell surface of cytotoxic T cells is important for its apoptosis-inducing activity, we have investigated the possibility whether cell surface FasL associates with lipid rafts and whether raft association is important for FasL activity. We here show evidence that FasL is recruited to lipid rafts and that this lipid rafts association is crucial for its cytotoxic activity. Disruption of lipid rafts in cytotoxic T cells and FasL transfectants leads to a reduced induction of cell death in the target cells.
Furthermore we are investigating the role and relative contribution of Fas-Ligand-interacting proteins in its recruitment to the lipid raft compartment of the cell membrane. Our results demonstrate an important role of lipid raft recruitment in the regulation of FasL activity and uncover a novel mechanism of death ligand signaling.

Death receptors **P30**

Cooperation of TNF receptors is necessary for efficient response to tmTNF in U937 cells

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TNF exerts its wide range of activities via two receptors TNF-R55 and TNF-R75. The latter is efficiently activated by transmembrane TNF (tmTNF), but not by soluble TNF (sTNF), while TNF-R55 can respond fully to both TNF ligands. Very little is known about cooperation of TNF receptors on the level of initiation of activation by tmTNF. We observed a distinct pattern of cytocidal response to tmTNF in a model of U937 cell variants characterized by different TNF sensitivity. U937M cells were sensitive to sTNF and tmTNF, while U937ATCC cells were resistant to both forms of TNF. Addition of cycloheximide (CHX) increased cytotoxicity of U937ATCC cells exposed to sTNF and tmTNF as well as cytotoxicity of U937M cells exposed to sTNF. Surprisingly, CHX did not enhance cytocidal effect of tmTNF in U937M cells suggesting the impaired cytotoxic mechanism probably dependent on TNF-R75 function or its cooperation with TNF-R55. Blocking of TNF-R55 by antibodies in U937M cells was not efficient enough to inhibit cytocidal effect of sTNF and tmTNF. However it was possible to fully block the cytotoxic effect exerted by the p55-specific TNF mutein in U937M cells.. This effect could suggest that TNF-R75 may transduce cytotoxic effect itself; however it was not confirmed by direct activation by p75 mutein or by different agonistic antibodies. The effect of sTNF and tmTNF was blocked effectively by anti-TNF-R55 antibodies in U937ATCC cells. Taken together, we suggest that cooperation between TNF receptors in exerting cytotoxic effect by tmTNF in U937 cells is required.

Death receptors **P31**

Death receptor signaling pathways control neuron fate following ischemic injury

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Death receptors and their ligands are constitutively expressed in the developing and adult brain and are often upregulated under pathological conditions. CNS neurons express components of the death receptor signaling cascades and the FADD-caspase 8 pathway is activated following ischemia and excitotoxicity. However, the contribution of death receptor pathways to neuron fate after injury is not yet established.

To investigate the role of death receptor signaling pathways in neurons, we have used *in vitro* and *in vivo* models of ischemic injury- glucose deprivation (GD) and permanent middle cerebral artery occlusion (pMCAO) respectively- in mice deficient in the tumour necrosis factor receptor I (TNFRI). Here we show that neurons undergo caspase 8- caspase 3-dependent cell death and that this process is independent of the TNFRI. The neuronal TNF/TNFRI signaling is required for the activation of a novel TNF-inducible cell protection mechanism which is caspase dependent but caspase 8 independent, involves cleavage of $FLIP_L$ to FLIP(p43) and post translational modifications and selective activation of p50/p65 NF-κB. This cascade of signaling events is essential for neuron survival following ischemic injury and could provide potential targets for protective- neuron directed- TNF action.

Death receptors **P32**

NF-kappaB activation by Fas is mediated through FADD, Caspase-8 and RIP and is inhibited by FLIP

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Using fluorescent fusion proteins of Fas and FasL we show in this study that membrane FasL interacts with Fas to form supramolecular FasL-Fas clusters at contact sites between FasL and Fas expressing cells. These FasL-Fas clusters are of flexible shape but are nevertheless durable and very stable. Fluorescence loss in photobleaching experiments revealed that there is almost no release of Fas or FasL from the clusters. Importantly, Fas-FasL clusters are also formed when Fas-YFP was expressed in caspase-8 or FADD deficient cells or using a cytoplasmic deletion mutant of Fas. This suggests that the known Fas signaling pathways are not necessary for cluster formation. Moreover, deletion of the cytoplasmic domain of FasL and the presence of the raft destabilizing component b-cyclodextrin had also no effect on the stability of the Fas-FasL clusters. Together, these data suggest that the interaction of the extracellular domains of Fas and FasL alone is sufficient to drive the formation of supramolecular Fas-FasL complexes, which are regarded as a structural prerequisite for Fas activation.

Death receptors **P33**

Involvement of the glyoxalase system and its substrate methylglyoxal in TNF-induced necrotic cell death and NF-κB activation.

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Methylglyoxal (MG) is a cytotoxic byproduct of glycolysis that is derived from enzymatic and non-enzymatic phosphate elimination from dihydroxyacetone phosphate and glyceraldehyde-3-phosphate. MG is normally detoxified to D-Lactate by the glyoxalase system that comprises glyoxalase I (GLO1) and II. The real biological function of the GLO system is still not known, but it is believed that it plays a fundamental role in the control of normal cellular growth.
We have shown that TNF-induced necrotic cell death in L929 cells, which is dependent on oxidative stress, requires the phosphorylation of GLO1 that is mediated by PKA (phosphorylated GLO1 was never described before). GLO1 is overexpressed in several types of cancer and is involved in resistance of human leukaemia to anti-tumor agent induced apoptosis. It is also overexpressed in diabetic patients and is exclusively overexpressed in the brain of Alzheimer patients.
TNF treatment of L929 cells leads also to a substantial increase in intracellular levels of MG and this results in the rapid formation of specific MG-modified proteins, a type of post-translational modification of proteins termed "Advanced Glycation Endproducts" (AGEs). Formation of AGEs are involved in the development of a number of pathophysiological conditions in vivo, including tissue damage caused by ischemia /reperfusion and a number of vascular diseases. AGEs bind also to cell surface receptors and can cause an inflammatory response.
The formation of TNF-induced MG-derived MG-AGEs occurs as a consequence of increased ROS production and requires glycolysis that is also essential for cell death. Furthermore, we provide evidence that the TNF-induced phosphorylation of GLO1 is not involved in detoxification of MG via the glyoxalase system, but that phosphorylated GLO1 is on the pathway leading to the formation of specific MG-modified proteins.
We have also investigated the influence of MG on the TNF-induced NF-κB activation in L929 cells as well as in primary endothelial cells since it has been shown that NF-κB activation in the latter is also dependent on oxidative stress and increased levels of MG is one of the factors that strongly contribute to development of vascular diseases in diabetic patients. In both cell lines, TNF modulates glucose metabolism for the benefit of MG-production. Furthermore, inhibition of glycolysis strongly inhibits NF-κB activation in both cell lines. This indicates that the TNF-induces modulation of glycolysis is required for NF-κB activation. High concentrations of MG are strongly synergistic with TNF-induced cell death in L929 cells, but are not cytotoxic by themself. At this concentration, MG strongly inhibits NF-κB activation (up to 50%). However, low concentrations of MG are strongly synergistic with NF-κB activation especially at low doses of TNF.
Our results indicate that this novel signaling pathway comprised of phosphorylated GLO1 and specific MG-modified proteins play an essential role in TNF-induced signaling. MG may thus function as a signaling molecule via MG-modification of its specific target molecules (are currently under investigation). Furthermore, we propose that MG may mediate, via its target molecules, the cross talk between TNF-induced cell death and NF-κB activation.

Death receptors **P34**

Receptor interacting protein RIP1, a crucial adaptor kinase in necrotic signaling

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Receptor interacting protein (RIP1) is recruited to tumor necrosis factor-α receptor 1 (TNFR1) complex upon stimulation and known to play a crucial role in the receptor-induced NF-kB activation. Interaction of RIP1 with the scaffold protein Hsp90 protects the adaptor kinase from proteasome-dependent degradation. We showed that pretreatment of L929 fibrosarcoma cells with the Hsp90 inhibitor geldanamycin, induces a shift of TNF-induced necrosis to apoptosis, suggesting a crucial role of RIP1 also in necrotic signaling. The importance of RIP1 has also been shown in FasL- and dsRNA-induced caspase-independent cell death. Analogous to the formation of the apoptosome, one can propose the formation of a "necrosome-complex" in which the activation of RIP1 is a crucial event. We are currently trying to unravel the activation mechanism of RIP1 and how RIP1 recruitment finally triggers downstream execution mechamisms. It has been shown that the kinase activity of RIP1 is necessary to perform its role in necrotic signaling in Jurkat cells. We similarly demonstrate the importance of RIP1 kinase activity to mediate cytotoxicity in L929 cells. We are therefore investigating how this catalytic activity of RIP1 contributes to necrotic signaling by determining targets of RIP1 kinase activity in membrane fractions, cytosol, mitochondria, lysosomes and the nucleus of L929 cells.

Death receptors **P35**

Dissecting of LIGHT-mediated cell death by survivin delta-Ex3 and K7

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TNFSF14/LIGHT is a member of the TNF family that binds to lymphotoxin-beta receptor (LTβR), herpesvirus entry mediator (HVEM) and decoy receptor (DcR3). In our previous study, we have shown that TRAF3- and TRAF5-dependent activation of ASK1 is responsible for the caspase-independent pathway of LTβR-induced cell death. Since Survivin dominant-negative mutant (survivin-T34A) can trigger nuclear translocation of mitochondrial apoptosis inducing factor (AIF) and induce caspase-independent death, thus we tested the effect of survivin DEx3 in LIGHT-mediated cell death. Moreover, overexpression of Bcl-2 enhances LIGHT-mediated cell death via the cleavage of BH4 domain of Bcl-2, thus we test the effect of Bcl-2-interacting protein K7, a gene encode by herpes simplex virus 8, to prevent LIGHT-mediated cell death. The differential effect of survivin DEx3 and K7 to protect cell death are being investigated.

Development & Tissue Remodeling **P36**

Ectopic LT alpha initiates lymphoid neogenesis in the absence of cxcr5 and rescues peripheral lymph nodes in cxcr5$^{-/-}$ mice

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The development of tertiary lymphoid organs (TLO) occurs through a process called lymphoid neogenesis and is mediated by many of the same molecules that control lymph node (LN) development, including lymphotoxin alpha (LT), the lymphoid chemokine CXCL13 and its receptor CXCR5. Whereas LT alpha $^{-/-}$ mice lack all LNs, CXCL13$^{-/-}$ or CXCR5$^{-/-}$ lack peripheral LNs (PLN) yet retain mesenteric LNs (MLN). Moreover, ectopic expression of LT alpha or CXCL13 under control of the rat insulin promoter (RIP) induces lymphoid neogenesis. Previous studies have identified a CD4+CD3-IL7R alpha + inducer cell as crucial for LN genesis and these cells have been suggested to be involved in lymphoid neogenesis. Given the requirement for CXCR5 and CXCL13 in PLN organogenesis and the involvement of CXCL13 in lymphoid neogenesis, we initially sought to investigate the requirement for CXCR5 in lymphoid neogenesis. RIPLT alpha transgenic mice, which develop TLOs in the pancreas, kidney, and skin, were crossed onto a CXCR5-deficient background to generate RIPLT alpha.CXCR5$^{-/-}$ mice. An examination of the kidney and pancreas of these mice indicates that CXCR5 is not required for lymphoid neogenesis, although B lymphocyte recruitment to these TLOs is severely impaired. Unexpectedly, the RIPLT alpha transgene rescued PLN development, with the exception of inguinal LNs, in CXCR5$^{-/-}$ mice and even gave rise to a novel set of LNs. These rescued LNs exhibited altered cellular compartmentalization, high expression of lymphoid chemokines, prominent HEV, and supported a T cell-dependent immune response. Most notably, CD4+CD3-IL7Ralpha+ inducer cells were detected at high numbers in RIPLT alpha.CXCR5$^{-/-}$ TLOs, suggesting a mechanism for the rescue of PLNs.

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Development & Tissue Remodeling **P37**

Dual Role of Tumor Necrosis Factor in Regulation of Hematopoiesis

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Exogenous tumor necrosis factor (TNF) added to hematopoietic cell cultures was previously shown to potently inhibit proliferation and differentiation of various hematopoietic progenitors in vitro. This was in part supported by our later findings of increased proliferative capacity in long-term bone marrow cultures obtained from TNF-deficient mice, suggesting a role of TNF as an inhibitor of myelopoiesis and a regulator of granulocyte-macrophage progenitor population in vitro. To address the physiological role of TNF in hematopoiesis in vivo we analyzed bone marrow cells (BMC) of mice deficient in either TNF or TNF/lymphotoxin (triple KO mice, Kuprash et al., Mol Cell Biol., 22(24):8626-34, 2002) in a variety of hematopoietic progenitor and stem cell assays. There was no difference in the number of Linneg, c-kitpos, Sca1pos cells, a population enriched for hematopoietic stem cells (HSC), in BMC obtained from either TNF-deficient or control mice. However, there was a significant increase in the number of both committed progenitors within Linneg, c-kitpos, Sca1neg population and multipotent progenitors within Linneg, c-kitpos, Sca1pos population (CFU-GM and CFU-S, respectively) in bone marrow of TNF-deficient mice when compared to control mice. Interestingly, there was a two-fold increase in the number of Linneg, c-kitpos, Sca1pos cells in bone marrow from triple KO TNF/LT mice, suggesting a redundant contribution of TNF and LT in regulating this cell population. Furthermore, HSCs from TNF-deficient mice exhibited a decrease in their ablitiy to long-term repopulate lethally irradiated recipients following bone marrow transplantation (BMT). This defect was even more profound in mice lacking both TNF and LT. Taken together, our data suggest that in the absence of endogenous TNF some loss of negative regulation may occur and result in the increase of both committed and primitive hematopoietic progenitor populations. At the same time, a decrease in the number of functional HSC following BMT suggests exhaustion of this population due to reduced negative pressure on TNF-deficient HSC in vivo.

Development & Tissue Remodeling **P38**

Unique and broad chemokine response profile of lymphoid tissue inducer cells

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Lymph node (LN) and Peyer's patch (PP) development is characterized by the initial clustering of CD45+CD3-IL7Ra+ lymphoid tissue inducer cells (LTIC) next to specialized stromal cells. An extensive crosstalk between these two cell types, including lymphotoxin ab and RANK signals, is thought to induce the downstream events leading to the induction of angiogenesis and stromal cell networks.

The initial signal controlling localization of lymphotoxin/RANK-expressing LTIC to a future site of LN or PP is unknown, however genetic data indicate a possible role for the chemokine receptors CXCR5 and CCR7 on LTIC in this process. Other chemokine receptors are likely to be involved, as mesenteric LNs still develop in mice doubly deficient in CXCR5 and CCR7 or its ligands. To identify additional chemokines that may function in lymphoid organ development we performed a functional array analysis, testing LTIC for responsiveness to chemokines that engage each of the known chemokine receptors. Surprisingly, we observed migration of LTIC to a large array of chemokines, which was confirmed by staining for the corresponding chemokine receptors on LTIC. Quantitative PCR analysis on mesentery and intestine from day 14.5 embryos revealed expression of many chemokines including all the chemokines to which the LTIC showed a functional response. In contrast to adult tissues, embryonic chemokine expression was independent of lymphotoxin expression. To further narrow down the key players, chemotactic responses were measured for LTIC precursors obtained from embryonic liver of for mature LTIC obtained from embryonic blood or intestine. The chemokines identified by this screen are currently being tested in vivo for their role in lymphoid tissue development.

Development & Tissue Remodeling **P39**

Signaling through lymphotoxin beta receptor is required for migration to liver but not thymic development of natural killer T cells.

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Natural killer T (NKT) cells are a unique subset of T cells that are involved in many functions ranging from suppression of autoimmune responses to tumor rejection. The signaling events that regulate NKT cell development differ from those of mainstream T cells. It has been shown that membrane bound lymphotoxin (LT) which signals through lymphotoxin beta receptor (LTβR) and NF-κB inducing kinase (NIK), is required for development of thymic NKT cells. Moreover, NIK and the Rel/NF-κB family member RelB are both required in thymic stromal cells for normal NKT cell development. We have previously shown that constitutive RelB DNA binding in the thymus is completely abolished in the absence of functional NIK and partially reduced in the absence of LTβR. Here, we show that LTβR is dispensable for thymic NKT cell development but required for their migration to peripheral organs, such as liver. Similarly, LTα/TNF double-knockout mice have normal numbers of thymic but markedly reduced numbers of liver NKT cells. Mice lacking LIGHT or the LTα/LIGHT receptor HVEM also show normal development of thymic NKT cells. To address redundancy among the TNF/LT system, mice triple-deficient for TNF, LT and LIGHT as well as mice double-deficient for LTβR and HVEM are currently being analyzed for NKT cell development.

Host Defense **P40**

Lymphotoxins and host antiviral defense

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The requirement for signaling by the lymphotoxin (LT)/LTβ receptor (LTβR) cytokine system in the development of secondary lymphoid tissue and maintenance of splenic architecture is well documented. Additionally, several studies indicate an important role for LT signaling in immunity to pathogen infection. Still, specific mechanisms for LT action in host defense have been difficult to decipher, largely due to the complex, multi-component phenotypes of genetically deficient mice. We are taking several approaches to delineate the contribution of the LT/LTβR system in immune defense to cytomegalovirus (CMV) (β-herpesvirus). In "resistant" strains of mice, such as C57BL/6, innate immunity mediated by NK cells and IFNαβ is largely responsible for control of acute infection. C57BL/6 mice deficient in LT/LTβR signaling, either through genetic deletion or pharmacologic inhibition, are unable to control acute CMV infection, in part due to failure in mounting an efficient, early IFNαβ response. However, co-administration of an agonistic LTβR antibody restores induction of IFNαβ, indicating a requirement for constitutive LT_R signaling at the time of virus infection to coordinate aspects of innate defense. CMV-specific CD8 T cell responses were essentially normal in $LT\alpha^{-/-}$ and $LT\beta^{-/-}$ mice, again suggesting that LT-signaling may be paramount for innate immunity to CMV. In spite of a robust innate and adaptive immune response CMV succeeds in establishing lifelong persistence in its immunocompetent host, and it is likely that "immune evasion" tactics employed by the virus contribute to this success. During acute infection significant changes in splenic microarchitecture are observed, including loss of resident marginal zone macrophage populations and a disorganization of B and T cell segregation. Additionally, a dramatic and specific decrease in the expression of LT/TNF-dependent chemokines occurs in the spleen of CMV infected mice at the time of peak viral replication, and this is commensurate with an inability of adoptively transferred T-cells to migrate into the PALS. As CMV can inhibit induction of NFκB by LTβR and TNFR in infected fibroblasts, we propose that this virus may directly target LT/TNF signaling pathways in lymphoid tissues in order to blunt antiviral immune responses and establish persistence.

Host Defense **P41**

Virus modulation of LTαβ/TNF regulated chemokine expression alters lymphoid organ microarchitecture

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Cytomegalovirus (CMV), a β-herpesvirus, establishes a persistent infection with its immunocompetent vertebrate host without overt pathogenicity. In order to accomplish this relationship, CMV has developed evasion mechanisms that impede both innate and adaptive immune responses. We show that murine CMV infection of 'resistant' C57BL/6 mice results in a drastic remodeling of secondary lymphoid tissue architecture. In the spleen, these changes include remodeling of resident marginal zone cell populations and alteration of white pulp organization, both of which have been shown to be regulated by lymphotoxin (LT) and/or TNF signaling. LT-regulated chemokine expression, required for maintaining lymphoid microenvironments, is specifically targeted by CMV, which may be achieved in part by a virus dependent blockade of NF-κB (p50/p65) activation. Thus, viral modulation of lymphoid tissue architecture may contribute to the ability of herpesvirus to establish a persistent infection in an immunocompetent host.

Host Defense **P42**

Cytocidal effect of interleukin 1 (IL-1) on HeLa cells is dependent on cycloheximide and is mediated by both soluble and transmembrane tumor necrosis factor (TNF)

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Interleukin 1 (IL-1) is a pleiotropic cytokine functioning as a regulator of local and systemic immune and inflammatory reactions. One of IL-1 activities is induction of death in a limited number of cell types, e.g. pancreatic β-cells where it is assumed to play part in the pathogenesis of diabetes. The mechanism of IL-1 cytotoxicity has not been thoroughly investigated. In an attempt to analyze the mechanism of cytocidal effect induced by interleukin 1 in HeLa cells in the presence of biosynthesis inhibitor cycloheximide (CHX), we found that biochemical and morphological picture of cell death in response to IL-1 shares significant similarities with the cytotoxic effect of tumor necrosis factor (TNF) in the same experimental model, resembling some features of apoptosis (DNA degradation, caspase dependence). Subsequently, we found that IL-1 cytotoxicity in the presence of CHX is an indirect effect. TNF was further identified as a mediator of cell death as confirmed by experiments with the use of neutralizing antibodies. This cytokine was then detected in HeLa cells treated with IL-1/CHX, IL-1 or CHX, as well as untreated by means of flow cytometry and fluorescence microscopy. In the presence TACE inhibitor TAPI-1, the cytotoxic effect of IL-1/CHX is enhanced. Taken together, we demonstrated that the mechanism of IL-1 cytotoxic activity in HeLa cells in the presence of CHX depends on the presence of TNF. Enhanced activity of TNF may result from TNF secretion outside the cells, as well as from the function of transmembrane TNF. It is highly probable that following stimulation with IL-1/CHX, TNF appears on the cell surface first and is then cleaved by the protease. We can assume that TNF appearance does not result from de novo synthesis but from disclosure of preformed TNF.

Host Defense **P43**

Impact of lymphotoxin on antiviral T and B cell responses

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Lymphotoxin α1β2 (LTα1β2)-LTβR interaction defines secondary lymphoid organ microstructure, but does not interfere with lymphocyte activation on the single cell level. Mice with conditionally ablated expression of LTβ on T cells, B cells, or both, allowed us therefore to relate the integrity of secondary lymphoid organs to antiviral immune reactivity. These mice mounted normal immune responses against Vesicular Stomatitis Virus (VSV), and were protected against Lymphocytic Choriomeningitis Virus (LCMV) in spite of their structural defects of secondary lymphoid organs. However they showed deficient immune responses against non-replicating antigens, demonstrating that immune responses against low amounts of antigen critically depend on the integrity of lymphoid microenvironments. Mice with complete deficiency for LTβ, on the other hand, exhausted their cytotoxic T lymphocytes following infection with LCMV and died following VSV infection. We are currently relating this increased susceptibility of LTβ-/- mice to virus infections to a defect of innate antiviral resistance.

Host Defense **P44**

Y. enterocolitica signals macrophage apoptosis through a TLR4-TRIF-FADD- and caspase-8-dependent cytotoxic pathway

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Toll-like receptors (TLRs) sense invading microbial pathogens by identifying conserved microbial components. They induce the activation of protective signaling pathways of innate immunity. Furthermore, the TLRs control survival of infected cells by governing the induction of pro- and antiapoptotic signaling pathways. Gram-negative pathogenic *Yersinia spp*. have learnt to uncouple the balances of life and death in infected macrophages. They suppress the activation of the antiapoptotic NF-κB pathway and simultaneously trigger proapoptotic signals, which compels the macrophage to undergo apoptosis.
Intriguingly, the initiation of apoptosis by *Yersinia* infection specifically involves signaling through TLR4, although *Yersinia* can activate both TLR2 and TLR4. To characterize the roles of downstream TLR adapter proteins in the induction of TLR-responsive apoptosis, we investigated cell death in *Yersinia*-infected murine macrophages defective for myeloid differentiation factor 88 (MyD88) or TIR domain-containing adapter inducing IFN−β (TRIF). It was revealed that deficiency of TRIF, but not of MyD88, provided protection against *Yersinia*-mediated cell death. Similarly, apoptosis provoked by treatment of macrophages with the TLR4 agonist LPS in the presence of a proteasome inhibitor was inhibited in TRIF-defective, but not in MyD88-negative cells. The transfection of cells with TRIF furthermore potently promoted apoptosis. These data indicate a crucial function of TRIF as proapoptotic signal transducer in bacteria-infected murine macrophages, an activity that is not prominent for MyD88. Interestingly, TRIF appears to signal apoptosis through the death receptor-related Fas-associated death domain- (FADD) and caspase-8-dependent apoptotic pathway. These data argue for a specific proapoptotic function of TRIF, mediating cytotoxicity by engagement of death receptor-related apoptotic signaling as part of the host innate immune response to bacterial infection.

Host Defense **P45**

Fatal *Mycobacterium tuberculosis* infection despite adaptive immune response in the absence of MyD88

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Toll-like receptors (TLR) have been implicated in host response to mycobacterial infection. Mice deficient in the TLR adaptor molecule myeloid differentiation factor 88 (MyD88) were infected with MTB. While primary MyD88$^{-/-}$ macrophages and dendritic cells do not produce TNF, IL12 and nitric oxyde in response to mycobacterial stimulation, the upregulation of costimulatory molecules CD40 and CD86 is unaffected. Aerogenic infection of MyD88$^{-/-}$ mice with MTB is lethal within 4 weeks with 2 log higher CFU in the lung and acute, necrotic pneumonia in MyD88$^{-/-}$ mice, despite a normal T cell response with IFNγ production to mycobacterial antigens upon ex vivo restimulation. BCG vaccination conferred a substantial protection in MyD88$^{-/-}$ mice from acute MTB infection. These data demonstrate that MyD88 signalling is dispensable to raise an acquired immune response, which however is not sufficient to compensate the profound innate immune defect of MyD88$^{-/-}$ mice to control MTB infection.

Host Defense **P46**

Protective role of membrane TNF in the host resistance to mycobacterial infection

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Tumour necrosis factor-α (TNF) plays a critical role for the control of mycobacterial infection. The role of transmembrane TNF (TM-TNF) in host resistance is unknown and was tested in knock-in mice a non-cleavable and therefore membrane-bound, non-secreted TNF. While mice with complete TNF deficiency (TNF $^{-/-}$) succumbed to infection, TM-TNF mice were unable to control *M. bovis* BCG infection and survived the 12 week experimental period. In contrast to a complete TNF deficiency, TM-TNF allowed a substantial recruitment of activated T cells and macrophages with granuloma formation and expression of iNOS. Using aerosol infection with M. tuberculosis we confirm that membrane TNF conferred a partial protection of TM-TNF mice survived 10 weeks, while all TNF $^{-/-}$ mice died within 6-7 weeks. Therefore, the data suggest that membrane expressed TNF plays a critical role in host defence to mycobacterial infection and may partially substitute for secreted TNF.

Host Defense **P47**

A Role For Lymphotoxins In The Innate And Adaptive Response To Viral Infection

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The lymphotoxin (LT)/LTβ receptor (LTβR) system is critical in host defense to viral infection at the level of both the innate and adaptive immune response. Mice deficient in signaling by the LT/LTβR system are highly sensitive to infection with murine cytomegalovirus (MCMV, a β-herpesvirus), and are unable to control acute infection, suggesting an important role for the LT signaling pathway in establishing coexistence between the host and this persistent pathogen. Host-defense to MCMV is complex, requiring innate and/or adaptive immunity, with a dominant role for NK cell and type I interferon (IFNαβ)-mediated protection in "resistant" C57BL/6 mice. We therefore analyzed whether IFNαβ production is compromised in LT-deficient mice infected with MCMV. The rapid induction of IFNαβ-mRNA was severely reduced in both LT and LT-deficient mice after MCMV infection, but could be restored by co-administering an agonistic antibody to LTβR. However, administration of anti-LTβR antibody in LT-deficient or wild-type mice in the absence of virus did not induce IFN, suggesting constitutive expression of LT is required to induce effective IFN responses at the time of infection. Current models propose a requirement for innate immunity to coordinate an efficient adaptive response, at least in the case of some pathogens. We have observed that virus-specific T-cell responses to MCMV and lymphocytic choriomeningitis virus (LCMV) show different requirements: CD8+ T cell responses to LCMV were impaired in LT deficient mice, however antigen-specific CD8+ T cells to MCMV were normal, as determined by staining with MHC class I tetramers and production of IFN in response to peptide. Therefore, the requirement for LT-dependent signaling and/or appropriate development of lymphoid tissue for mounting adaptive immunity appears to be virus specific.

Inflammation & Immunity **P48**

TWEAK promotes joint inflammation, cartilage and bone loss

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TWEAK is a macrophage-derived cytokine and member of the TNFα family of cytokines originally identified as a weak inducer of death in certain tumor cell lines (TNF-like weak inducer of apoptosis). TWEAK was subsequently shown to exert pleiotropic effects on a variety of cell types in vitro, including proangiogenic activities on vascular endothelial cells and proinflammatory activities on epithelial cells, astrocytes and dermal fibroblasts. These cells express a known receptor for TWEAK, FGF-inducible molecule 14 (Fn14), which is restricted to epithelial and mesenchymal cell types and highly upregulated in contexts of injury and inflammatory disease. It was also reported that TWEAK induces synoviocyte production of proinflammatory cytokines, chemokines and MMPs, and osteoclastogenesis of a macrophage cell line. Thus, TWEAK may promote joint inflammation and damage. Here we report that neutralizing TWEAK mAbs markedly reduce clinical paw severity in mouse and rat collagen-induced arthritis models. Inhibition by anti-TWEAK mAbs occurs at the challenge phase and does not appear to alter T cell priming or elicitation of anti-collagen antibodies. Decreased clinical paw severity correlates with reduced inflammation and protection from cartilage and bone loss at the histological level. Further studies are in progress to dissect the mechanism of action whereby TWEAK promotes joint inflammation, cartilage and bone loss.

Inflammation & Immunity **P49**

Functions of TNF-receptor associated factor 6 (TRAF6) in immunity and osteoclastogenesis

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TNF receptor-associated factors (TRAFs) mediate the intracellular signaling pathway from various members of TNF-receptor family such as receptor activator of NF-κB (RANK) and CD40. To date, seven members of the TRAF family have been described. Among them, TRAF2, TRAF3, TRAF5 and TRAF6 transduce the signals from RANK and CD40 through the binding to the cytoplasmic tails of these receptors. TRAF2, TRAF3, and TRAF5 bind to the membrane-distal domain of the cytoplasmic tail. TRAF6 is distinct in terms of interacting with the membrane-proximal region. Furthermore, TRAF6 is involved in signaling from members of the Toll/IL-1R family by its interaction with IL-1R-associated kinase.
We and others previously showed that TRAF6-deficient mice ($TRAF6^{-/-}$) exhibited severe osteopetrosis. The osteoclast formation in $TRAF6^{-/-}$ was significantly impaired, which is due to defect of signaling from RANK upon binding of RANKL. Furthermore, RANK-induced activation of NFκB and MAPKs in osteoclast progenitors was abrogated in the absence of TRAF6.
Recently, we and others have also shown that TRAF6 is involved in the maturation and activation of dendritic cells and macrophage. The results suggested that TRAF6 plays a role on linkage between innate immunity and adoptive immunity.
In this conference, our novel findings on the function of TRAF6 on immunity and osteoclastogenesis will be discussed.

Inflammation & Immunity **P50**

Constitutive activation of the CD40 pathway in cell transformation

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CD40, a member of the tumour necrosis factor receptor (TNFR) superfamily, is expressed on a plethora of lymphoid cells, including normal B lymphocytes, macrophages, endothelial and dendritic cells and this widespread expression is likely to account for its central role in the regulation of immunity and host defence. CD40 is also found in normal and malignant epithelium where its precise role remains largely unknown. We have found that the stimulation of CD40-positive carcinoma cell lines with soluble CD40 ligand (CD154) resulted in activation of the NF-κB and MAPK signalling pathways, production of inflammatory cytokines and up-regulation of cell surface markers such as CD54 and MHC class I. Unlike CD154, treatment of carcinoma cells with C4b-binding protein (C4BP), a recently proposed ligand for CD40 in B lymphocytes, failed to promote this phenotype, suggesting that CD154 but not C4BP may serve as functional CD40 ligand in epithelial cells. Concomitantly, CD154 and CD40 were found to be co-expressed in primary breast and colon carcinoma biopsies while C4BP was absent from these tumours. Stable expression of CD154 in the CD40-positive SCC12F squamous cell carcinoma line resulted in enhanced proliferation, motility and invasion, in a CD40 ligand-dependent manner. Moreover, a chimaeric molecule comprising the transmembrane domain of the Epstein-Barr virus-encoded LMP1 fused to the cytoplasmic tail of CD40 functions as a constitutively active CD40 capable of inducing transformation in Rat-1 fibroblasts, as evident by morphological changes, anchorage-independent growth and increased tumourigenicity in nude mice. Taken together, these data suggest that the constitutive engagement of the CD40 pathway may play a role in cell transformation and oncogenesis.

Inflammation & Immunity **P51**

Bystander activated lymphocytes : a phenotypic comparison with rheumatoid synovial lymphocytes

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Previous studies have supported a role for bystander-activated lymphocytes in cognate-dependent TNF-α production by monocytes in the joints of rheumatoid arthritis (RA) patients. Currently we are examining the phenotypic changes induced on lymphocytes during chronic cytokine exposure to determine how such cells resemble those found in the RA joint. We are particularly focusing upon chemokine receptor and adhesion molecule expression, as well as the phenotype and activation status of the expanded populations.
Lymphocytes dividing following culture with IL-2/IL-6/TNF-α over 8 days were demonstrated to be from both NK and T cell populations. Preliminary analyses of chemokine receptor expression has shown that cytokine-driven lymphocytes express increasing levels of CxCR4, CCR5 and CxCR3, but reducing levels of CCR7 throughout the 8 day culture period. These receptors bind lymphocyte chemotractants including macrophage inflammatory proteins, IP-10 and RANTES. Cytokine-driven cells also express high levels of components of the adhesion molecules VLA-1, -4, -5 & LFA-1 (β1, β2 & α4), but lower levels of L-selectin (CD62L). This profile is consistent with the outgrowth of an activated effector memory population (with the capacity to migrate to inflammatory sites).
Expression patterns of these markers on synovial membrane/fluid lymphocytes from RA patients are well established. This work will more closely define pivotal cell types for cognate-dependent cytokine production in diseased joints & advance understanding of the effects of prolonged cytokine exposure in chronic inflammation.

Inflammation & Immunity **P52**

Tnf-Tnfr interactions among human and mouse family members

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The TNF/TNFR family of ligands and receptors is characterized by pairs of interacting ligands and receptors. However, it is not uncommon that a ligand binds more than one receptor and vice-versa. Moreover, a number of receptors have, so far, no identified ligand. Despite a wealth of information about ligand specificity and inter-species cross-reactivity, a systematic survey of these interactions has not been achieved.
We have used a homogenous FACS-based assay to monitor receptor-ligand interactions. The extracellular portions of the receptors were expressed fused to the C-terminal portion of TRAIL-R3 and stained with Fc:ligands chimeric proteins. The glycosylphosphatidyl-inositol (GPI) anchor of TRAIL-R3 alleviates retention and toxicity issues associated with the intracellular domain of some receptors. In addition, successful surface expression of all receptors can be monitored with an antibody directed against the C-terminal portion of TRAIL-R3, which is common to all constructs. Ligands were all detected via their Fc portion.
The screen confirmed most of the published interactions. In addition, a comprehensive set of data on cross-species reactivity was collected.

Inflammation & Immunity **P53**

A role for TRAF2 and Smurf2 in the regulation of TNF-R2

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TNF-signalling through TNF-receptors is known to activate different signal transduction pathways. TRAF2 is situated at a branch point, binding directly or indirectly to TNF-R2 and TNF-R1 respectively, and being able to induce both IKK and JNK or p38 activation upon receptor engagement. To get a better understanding of the signalling events upstream and downstream of TRAF2, we performed an alternative Y2H screening (Sos recruitment system) with TRAF2 as bait. Besides many known interaction partners, we identified Smurf2 as a new TRAF2-binding protein. Smurf2 had previously been shown to be a Smad-binding ubiquitin ligase that is responsible for ubiquitination and degradation of the TGFβ-receptor and several Smads. Co-immunoprecipitation experiments showed that Smurf2 not only binds TRAF2 but also TNF-R2, suggesting that TRAF2 functions as a bridging molecule between TNF-R2 and Smurf2. However, since deletion of the TRAF2-binding domain of TNF-R2 does not prevent TNF-R2-binding of Smurf2, direct binding of Smurf2 to TNF-R2 is more likely.
Similar to the role of Smurf2 in TGFβ-receptor signalling, we found that TNF-R2 is ubiquitinated by Smurf2 in a TRAF2-dependent way. Moreover, overexpression of Smurf2 induced the translocation of TNF-R2 and endogenous TRAF2 to the detergent insoluble fraction of the cell. Correspondingly, TNF-stimulation of the mouse/rat T-cell hybridoma cell line PC60-R55/R75, which stably expresses both human TNF-Rs, also resulted in the translocation of TNF-R2 to the insoluble cell fraction.
In conclusion, these observations suggest an important role for TRAF2 and Smurf2 in the regulation of TNF-R2 trafficking and signalling by ubiquitination.

Inflammation & Immunity **P54**

Engagement of the p55 TNF-R for different periods of time induce qualitatively distinct pathways of TNF signalling and gene expression profiles in murine T cells

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To provide insight into the mechanisms whereby sustained TNF stimulation promotes chronic inflammatory responses, we have examined TNF-R signalling pathways in murine T cell hybridomas. Prolonged TNF signalling attenuates p55 TNF-R (TNF-RI)-induced JNK activation through mechanisms which are NF-κB dependent and which target receptor proximal pathways, since arsenite-induced JNK activation is preserved. In contrast, phosphorylation and degradation of IκB alpha, combined with nuclear translocation and gel shift assays, demonstrate that prolonged TNF stimulation promotes chronic NF-κB activation. However, acute and chronic TNF signals are qualitatively distinct. In chronic TNF treated T cells degradation of IκB alpha and nuclear translocation of RelA (p65) are not as pronounced as that in cells following acute stimulation, while translocation of NF-κB1 (p50) is enhanced. Gel shift assays demonstrate consistent differences in the binding of Rel family proteins to DNA, since acute TNF stimulation induces formation of a dominant DNA binding complex comprising p65:p50 heterodimers (complex I), while the binding capacity of a second complex (complex II), which may include p50:p50 homodimers, is increased in nuclear extracts of chronic TNF stimulated cells. Preliminary data indicate that chronic but not acute TNF stimulation also induces RelB nuclear translocation and DNA binding. A direct comparison of gene expression profiles in control, acute and chronic TNF stimulated T cells suggests that differences in TNF-R signalling translate to distinct gene fingerprints. By interrogation of extended fragments of genomic sequence from 66 TNF-induced genes we have mapped the DNA binding consensus sequence sites for different transcription factors. For the classical NF-κB DNA binding motif GGGRNNYYCC our analysis revealed multiple κB "hits" in 55/66 TNF-induced genes. Variation in κB motifs was observed across gene clusters. Our results have uncovered differences between acute and chronic TNF signals which may arise through variation in signal input, specific requirements for Rel family dimers, divergence in κB consensus sequences and differences in affinities of protein-DNA binding.

Inflammation & Immunity **P55**

Selectivity of BAFF-R and BCMA for binding to BAFF versus APRIL

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BAFF (BlyS, TALL-1), a TNF family cytokine critical for the development and function of B cells, has been reported to bind to three receptors, BCMA, TACI and BAFF-R, but with widely conflicting values for the affinity and selectivity of binding. BCMA and TACI additionally bind to APRIL, the TNF family ligand most homologous to BAFF. Using soluble, monomeric forms of the receptors, we demonstrate that BAFF binds BAFF-R with K_D ~15 nM while BCMA binds with K_D ~1.5 mM, indicating a ~100-fold selectivity for binding to BAFF-R over BCMA. APRIL shows the opposite selectivity, binding to BCMA with K_D ~20 nM while showing no detectable affinity for BAFF-R. The binding of BAFF or APRIL to these receptors is highly sensitive to assay-dependent avidity effects, likely explaining the widely ranging affinity values reported in the literature. Binding of BAFF to a bivalent BCMA-Fc fusion construct in solution, or coated onto an ELISA plate, gave apparent binding affinities of ~630 pM and ~1 nM, respectively, compared to values of $K_{D(App)}$ ≤30 pM and ~300 pM for BAFFR-Fc. The high selectivity of BAFF for BAFF-R versus BCMA is thus partly obscured in these multivalent assays. The intrinsically high selectivity inferred from the measurements with monomeric receptor correlates well with *in vivo* data from knockout mice, providing a possible explanation for the observations that interruption of the BAFF-R gene in the A/WySnJ mouse produces a phenotype similar to the BAFF knockout mouse, while the BCMA knockout mouse has no discernable B cell phenotype.

Inflammation & Immunity **P56**

Reverse signaling of mTNF enhances allogeneic responses of CD8+ T cells towards endothelial cells, whereas it attenuates CD4+ T cell responses

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Reverse signaling of membrane-integrated ligands is a common phenomenon in the TNF family. We have previously identified that reverse signaling of mTNF confers resistance to LPS in monocytes and macrophages (MO/MF), e.g. expressed as the suppression of an LPS-mediated factor that is apoptotic for human microvascular endothelial cells (HMEC). We next asked whether also T cells might be influenced in their anti-HMEC response by reverse signaling of mTNF.
CD4+ or CD8+ T lymphocytes were stimulated with HMEC in the presence or absence of an anti-TNF antibody that can induce reverse signaling of mTNF. Subsequently, the T cells were subjected to proliferation and cytotoxicity assays, respectively. Reverse Signaling of mTNF significantly ($p=0.005$) decreased the allogeneic proliferative response of CD4+ T cells against HMEC. In contrast, CD8+ T cells were increased in their cytotoxic activity towards HMEC upon mTNF activation. Interestingly, flow cytometric analyses showed only 5-15 % of the T cells to be mTNF+. ELISA-based cytokine profiling revealed that the T cells had a TH2 phenotype, wheras CD8+ CTL expressed T_C1 cytokines, as assessed by IL-13 (T_H2) and IFN-γ (T_C1) secretion, respectively. No effect on cytokine secretion, whatsoever, could be observed under the influence of reverse signaling of mTNF.
Since TNF plays a pivotal role in the development of graft-versus-host disease (GvHD) following allogeneic stem cell transplantation (SCT), and, very recently, TNF blockade could attenuate CD4+-T cell-triggered GvHD, whereas the CD8+ T-cell mediated GvHD was unaffected, we conclude that the differential influence of reverse signaling of mTNF on T cell function could be resposible for this. Our data warrant further confirmatory studies on that.

Inflammation & Immunity **P57**

A Negatively Cooperative Model Is Proposed for LTβR Binding to LIGHT

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LTβR (Lymphotoxin beta receptor) is a member of the TNF receptor family of proteins [Bodmer, 2002] [Crowe, 1994] It binds to two different cell surface ligands, LIGHT as well as for the heteromeric LTα1β2 [Ware, 1995]. LIGHT is a homotrimeric molecule with three potential binding sites for LTbR. While the analogous system of TNFR p55, extracellular domain, complexed with TNF exhibits 3 receptors binding per timeric ligand, we have found that the LIGHT trimer binds only 2 shLTβRs (monomeric LTbR) with high affinity. The molecular weights of gel filtrated shLTβR/LIGHT complexes under nonequilibrium binding conditions were determined by in-line light scattering and were consistent with a 2:1 stoichiometry for shLTβR bound to 1 LIGHT. Measurements of the affinities of LTβRIgG and shLTβR to hLIGHT immobilized on a Biacore chip and find values of 3 and 31 nM respectively. Sedimentation velocity analysis of varied ratios of receptor to ligand showed distinctly sedimenting species for the complex and the isolated receptor. Titration of shLTβR into a solution of hLIGHT resulted in a quantitative increase in complex up to a 2 to 1 molar ratio. Titration of LIGHT with either shLTβR or LTβRIgG by isothermal calorimetry yielded binding isotherms that were best fit by 2:1 and 1:1 binding models, respectively. The binding is endothermic, having a large positive DH (~10 kcal/mole for the monomeric receptor) and hence, is strongly entropy driven. We suggest that the binding of shLTβR to LIGHT is negatively cooperative; the binding of the first two receptors causes a conformational change that result in an asymmetric LIGHT trimer with greatly reduced binding affinity for the third receptor molecule.

Inflammation & Immunity **P58**

Two TNF polymorphisms in rheumatoid arthritis

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TNF alpha is one of the most potent pro-inflammatory cytokines known to regulate cell survival, death, and/or growth, depending on cell types. In recent studies was referd that TNF alpha was detected at highly enhanced concentrations in the blood and synovial fluids of patients with rheumatoid athritis. Over-expression of this protein is related to polymorphisms in TNF alpha and TNF beta genes. Variants TNF A and TNFB2 were associated with this over-expression of TNF alpha.
The aim of the study was to associate the promoter polymorphism -308A/G in TNF alpha gene and introne NcoI polymorphism in the gene for TNF beta with rheumatoid arthritis (RA). A total of 100 patients with rheumatoid arthritis (grade I-IV according to Steinbrocker; 10 patients of grade I: non-erroding form of RA and 90 patients of grade II-IV: erroding form of RA) and control group of 150 subjects of similar age and sex distribution were available for study.
We observed no differences in genotype distributions and/or alelle frequences of TNFA -308A/G polymorphism as well as of NcoI TNFB polymorphism between rheumatoid arthritis control group. Significant association to NcoI TNFB polymorphism was found between eroding and non-eroding form of rheumatoid arthritis (Pa=0.028), with higher prevalence of TNFB2 allele within patient with eroding form of RA (P=0.025, OR=4.70).
In conclusion, results obtained in this study suggest an association of polymorphism in TNF beta gene to grade, especially to erosions in rheumatoid arthritis.

Inflammation & Immunity **P59**

TNF production by distinct cell types in host defense and in the development of autoimmune pathology

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Tumor necrosis factor (TNFα) is produced by different cell types in vivo and plays both beneficial role, as in host defense and in the maintenance of secondary lymphoid tissues; and a detrimental role in several autoimmune diseases. To unveil the role of TNF produced by particular cell types, we utilized mice with conditional inactivation of TNF gene either in T cells or in macrophages/ neutrophils. We found that TNF produced by macrophages and neutrophils is an important component of LPS/D-Gal toxicity and is critical for the host defense against intracellular pathogens: *Listeria* and *Mycobacteria.* Mice with targeted disruption of TNF only in macrophages and neutrophils showed increased mortality, histopathology and bacterial load. Interestingly, TNF produced by T lymphocytes cooperated with macrophage derived TNF in host defense functions. Moreover, both macrophage and T cell derived TNF are important for the promotion of autoimmune hepatitis induced by concanavalin A.

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Inflammation & Immunity **P60**

LTß-Receptor activation by activated T cells induces cytokine release from mouse bone marrow-derived mast cells

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Lymphotoxin-β receptor (LTβR) signalling plays a key role in embryonic lymphoid organ formation as well as in maintenance of lymphoid architecture. Activation of the LTβR is induced by either LT-α1β2 (LTα1β2) or LIGHT both expressed on activated lymphocytes. Since activated T cells come into close proximity of mast cells, we examined the expression of LTβR on bone marrow-derived mast cells and asked whether the LTβR–ligand interaction would allow communication between mast cells and activated T cells. We found that mast cells express LTβR on mRNA as well as on protein levels. To investigate LTβR-specific mast cell activation, the LTβR on BMMC from either wild type or LTβR-deficient mice was stimulated with recombinant mouse LIGHT or agonistic monoclonal antibodies in the presence of ionomycin. LTβR-specific release of the cytokines IL-4, IL-6, TNF, and the chemokines MIP-2 and RANTES was detected. Moreover, coculture of mast cells with T cells expressing the LTβR ligands also entailed release of these cytokines. Interference with a specific LTβR inhibitor resulted in significant suppression of mast cell cytokine release. These data clearly show that LTβR expressed on mast cells can transduce a costimulatory signal in T cell-dependent mast cell activation

Inflammation & Immunity **P61**

APRIL/TALL-2 expression by neutrophils in malignant B-cell lymphoproliferative disorders.

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The tumor necrosis factor family member APRIL/TALL-2 has been shown to regulate B cell immunity, as well as to provide a proliferation/survival signal to tumor cells. Here, we analyzed in situ APRIL expression by using tissue microarrays including 200 malignant lymphoproliferative lesions and 20 normal tissue biopsies. In normal tonsil tissues, occasional APRIL expression was observed, but a strong induction was seen in some B-cell tumor lesions. Diffuse Large B Cell Lymphoma (DLBCL) and Hodgkin's Disease (HD) showed the strongest expression of APRIL, while most low-grade lesions (Chronic Lymphocytic Leukemia, Follicular Cell Leukemia and Mantle Cell lymphoma) were negative. With the use of an antibody reactive with cell-associated APRIL and different antibodies recognizing secreted APRIL, we observed a strong overlap in >95% of the positive cases. Detailed analysis revealed that cells stained for cell-associated APRIL were confined to the CD15s+ neutrophils, while secreted APRIL was found concentrated around tumor cells. This indicates that neutrophils are the source of APRIL in these tumor lesions, and that secreted APRIL may bind to tumor cells. The absence of APRIL expression in lymphoma cells were confirmed by RT-PCR analysis in a large panel of DLBCL and HD cell lines. On the other hand, APRIL expression was confirmed in vitro in myeloid cells. This study indicates that APRIL may not constitute an autocrine tumor-promoting loop as it has been previously suggested. Experiments are ongoing to understand the functional consequence of APRIL expression by inflammatory neutrophils in high-grade B-cell and Hodgkin's disease.

Inflammation & Immunity **P62**

LTß-Receptor activation-dependent MAdCAM expression and leukocyte margination are critical for chronic colitis

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The lymphotoxin-β receptor (LTβR) pathway is involved in the development of colitis. To investigate the mechanisms by which LTβR activation contributes to the pathology of chronic inflammation we used a LTβR-Ig fusion protein as inhibitor of LTβR activation as well as LTβR- and ligand-deficient mice in the mouse model of DSS-induced chronic colitis. Prevention of LTβR activation significantly attenuated the development and histological manifestations of the chronic inflammation and reduced the production of inflammatory cytokines such as TNF, IL-1β, and IL-6. Moreover, LTβR-Ig treatment significantly down-regulated MAdCAM-1 expression, leading to reduced leukocyte rolling and sticking in postcapillary and collecting venules and reduced extravasation into the intestinal mucosa as quantified by *in vivo* fluorescence microscopy. Thus, LTβR pathway inhibition ameliorates DSS-induced experimental chronic colitis in mice by MAdCAM-1 downregulation entailing reduced lymphocyte margination and extravasation into the inflamed mucosa. Therefore, a combined treatment with reagents blocking T cell-mediated perpetuation of chronic inflammation such as LTβR-Ig together with direct anti-inflammatory reagents such as TNF inhibitors could constitute a promising treatment strategy for chronic colitis.

Inflammation & Immunity **P63**

TNFSF-collectin protein fusions as a platform for producing bioactive, soluble, multimeric ligands

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CD40 ligand (CD40L, CD154, TNFSF5) is an example of a TNFSF that must be multimeric in order to cluster its receptor in responder cells and initiate maximal signal transduction. As with many other TNFSFs, soluble single trimers of CD40L are relatively weak agonists when compared with the natural membrane form (MemCD40L). To produce a soluble, multimeric form of CD40L that mimics the multimeric nature of MemCD40L, the extracellular domain of CD40L was genetically fused to the body of surfactant protein D, a naturally multimeric protein, to make SP-D-CD40L. Similar fusion proteins were made with the extracellular domains with RANKL, CD27L/CD70, and GITRL. SP-D-CD40L was produced both as a murine and a human protein in CHO cells. The secreted proteins were > 300 kDa and activated cultured macrophages, dendritic cells, B cells, and CD40-transfected 293 cells. In contrast, SP-D-T147N-huCD40L, an inactive mutant of human CD40L, had no activity in these assays, indicating that the SP-D portion alone had no effects. Similarly, SP-D-RANKL was active in stimulating osteoclast formation from murine bone marrow-derived macrophages. As SP-D-CD40L could not be readily purified due to aggregation, it was tested *in vivo* as a genetic adjuvant in a DNA vaccination protocol in BALB/c mice. Using an expression vector for the Gag protein of HIV as the antigen plasmid, plasmid for SP-D-CD40L (pSP-D-CD40L) dramatically enhanced CD8+ T cell responses (meaured by IFN-gamma ELISPOT, CTL assay, and tetramer analysis) and antibody formation. Plasmid DNA for MemCD40L or a modified single trimer form of CD40L had no adjuvant activity in this system. pSP-D-GITRL had adjuvant activity, but less than pSP-D-CD40L. In a tumor model using poorly immunogenic B16-F10 melanoma cells in C57BL/6 mice, the injection of pSP-D-CD40L into established tumors every other day times 3 led to a significant slowing of tumor growth, whereas pMemCD40L was inactive. Taken together, these results demonstrate that fusion to the body of multimeric collectin molecules is a promising means to produce soluble, yet active, TNFSF proteins. The use of plasmid DNA injections avoids the need for protein purification, and provides a means for the rapid *in vivo* testing of these fusion proteins. The initial studies of pSP-D-CD40L as a vaccine adjuvant and tumor immunotherapy suggest that this approach is a promising strategy to exploit the therapeutic potential of TNFSF molecules.

Inflammation & Immunity **P64**

Identification of a TIFA-related protein, TIFAB, that inhibits TIFA-mediated activation of NFκB

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Tumor necrosis factor receptor-associated factor 6 (TRAF6) transduces signals that lead to activation of NFκB and AP-1, which is essential for cell differentiation and establishment of the immune and inflammatory systems. TRAF-interacting protein with a forkhead-associated domain (TIFA) was identified as a TRAF6-binding protein that could link IRAK-1 to TRAF6 and then activate TRAF6 upon stimulation. We report identification of a TIFA-related protein, TIFAB, that inhibits TIFA-mediated activation of NFkB. TIFAB does not associate with members of the TRAF family but does bind TIFA. We analyzed the effect of TIFAB expression on the TRAF6/TIFA interaction by immunoprecipitation of TRAF6 and found that TIFA coprecipitated with TRAF6 was not changed. However, when we analyzed this interaction by immunoprecipitation of TIFA, we found that TIFAB significantly increased the amount of TRAF6 coprecipitated with TIFA. These findings suggest that TIFAB inhibits the TIFAmediated TRAF6 activation possibly by inducing a conformational change in TIFA.

Inflammation & Immunity **P65**

Shedding of TNFα by gingipains, cysteine proteinases from *Porphyromonas gingivalis*

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Gingipains are cysteine proteinases produced by *Porphyromonas gingivalis*, a major causative bacterium of adult periodontitis. Gingipains consist of arginine-specific proteinases, **HRgpA** and **RgpB**, and lysine-specific proteinase, **Kgp**. They strongly affect the host-defense system by degrading some cytokines, components of the complement system and several immune cell receptors. In an in vitro model gingipains were shown to degrade soluble TNFα. However, since membrane TNFα shows strong biological activity, especially in local inflammatory lessions, it was worth investigating whether gingipains may destroy also membrane TNFα and limit its biological activities. To avoid a possible influence of gingipains on TACE/ADAM17, the majority of experiments were performed using $TACE^{-/-}$ fibroblasts (Dr. R. Black, Amgen) stably transfected with human proTNFα. Arginine-specific gingipains strongly diminished the level of TNFα on the cell surface as measured by flow cytometry. The decrease in the surface level of TNFα **was not** accompanied by the increase in the amount of soluble TNFα in the culture medium. This may indicate that either gingipains immediately degrade solubilized cytokine or they gradually cleave membrane TNFa. TNFα shedding by arginine-specific gingipains correlated with strongly diminished TNFα-mediated biological activity of the cells. First, activation state of transcription factor NF-κB was suppressed, second, the HL60 cells become resistant to $TACE^{-/-}/TNF^{+}$ -induced apoptosis. Kgp was able to cleave membrane TNFα but its effect was much weaker than that of arginine-specific enzymes. Significantly, similar results were obtained using more physiological cell model, human peripheral blood monocytes. Thus, gingipains are able not only to cleave soluble TNFα but also to destroy the membrane form of the cytokine what may additionally disregulate the cytokine network.

Inflammation & Immunity **P66**

TACE/ADAM17 is Therapeutic Target For Scleroderma

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Increases in the serum concentration of the soluble tumor necrosis factor receptor p55 (TNFRp55) correlate with scleroderma severity and inhibit TNF-mediated signaling. However, there is no direct evidence for TNFRp55 mediating the specific functions of TNF in the disease. To determine the role of TNFRp55-mediated signaling in the pathogenesis of scleroderma, we used a murine model of scleroderma that closely resembles systemic sclerosis in humans. Wild-type and $TNFRp55^{-/-}$ mice received a subcutaneous injection of bleomycin (BLM) each day. $TNFRp55^{-/-}$ mice began to develop severe sclerotic changes of the dermis on day 3 of the subcutaneous injections of BLM, while wild-type mice did not. These results indicated that signaling mediated by TNFRp55 is crucial for the collagen-degradation process in this mouse model. On the other hand, wild-type mice also exhibit skin sclerosis following bleomycin-treatment for 3 weeks. Interestingly, significant elevation of sTNFRp55 in sera was observed in BLM-treated wild-type mice. This reproducibility of this phenotype among BLM-treated wild-type mice indicates that elevation of sTNFRp55 might be involved in pathogenesis of scleroderma. These results raised the hypothesis that TNFRp55-sheddase, TACE/ADAM17, plays a key role in the collagen-degradation process on this mouse model. To investigate the therapeutic effect of TACE-inhibitor in this model, TACE-inhibitor, TAPI-1, was administer orally to BLM-treated wild-type mice. As we expected, TAPI-1 inhibited BLM-induced skin sclerosis. This report might provide a basis for formulating novel therapeutic intervention to scleroderma using TACE-inhibitor.

Inflammation & Immunity **P67**

Bystander-activated lymphocytes in inflammation: A natural process?

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The potential role of bystander-activated lymphocyte effector function in inflammation has been previously described. Data shown here characterizes these lymphocytes in terms of their proliferation, phenotype, cytokine profile and effector function.
Freshly elutriated lymphocytes activated with IL-2, IL-6 and TNFα proliferated optimally over 8 days after an initial lag phase of 2-3 days. From day 8 a decrease in proliferation was seen.
Following 8 days culture with cytokines whilst the proportion of T cells did not alter, the proportion of NK cells increased by up to 3-fold with the expression of activation markers (CD25, CD69 and HLA-DR) increasing on both cell types.
The cytokine profile of these lymphocytes was measured and whilst the production of the pro-inflammatory cytokines IFNγ, GM-CSF and LTα increased over 8 days, no production of the anti-inflammatory cytokine IL-10 was detected. In contrast lymphocytes activated for 48 hours in an antigen-dependant manner produced IL-10 in addition to IFNγ, GM-CSF and LTα.
Bystander-activated T cells and NK cells, when separated, could both induce monocyte TNFα production in a ratio-dependant manner.
The factors which induce bystander-activated lymphocyte effector function are not fully understood but characterization of these lymphocytes may help explain the abundance of TNFα seen in some inflammatory diseases.

Inflammation & Immunity **P68**

Endotoxin induced bronchoconstriction is TNF-dependent, while neutrophil recruitment depends on TLR4/MAPK p38 signalling

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Endotoxin derived from Gram-negative bacteria induces an acute respiratory distress syndrome (ARDS). Mitogen-activated protein kinase (MAPK) may play a critical role in acute inflammation. We show that the p38 MAPK inhibitor, NVP-AAZ102, prevents LPS induced ARDS, as TNF secretion, bronchoconstriction and recruitment of neutrophil in the lung and BAL fluid are dose-dependently inhibited. TNF inhibition explains individual effects of the p38 MAPK inhibitor, as bronchoconstriction is absent in TNF deficient mice, although neutrophil sequestration in the lung is normal. However, neutrophil recruitment in TNF deficient mice is abrogated by p38 MAPK inhibition. Therefore, bronchoconstriction is TNF-dependent, while neutrophil recruitment depends on the TLR4 signalling which is p38 MAPK mediated.

Inflammation & Immunity **P69**

Timp-3 is an essential checkpoint in systemic inflammation

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TNF is produced as a membrane-bound cytokine and is cleaved to form a soluble molecule. It plays an essential role in the activation and execution of inflammation. The availability of this cytokine is tightly regulated at many checkpoints. Whether TNF shedding from the cell surface is important for inflammation remains an open question. The regulatory action of TIMP-3 on TNF shedding, affecting its inflammatory response was studied.
Here we show that systemic inflammation is affected by the deficiency of TIMP-3, an inhibitor of the TNF converting enzyme, TACE. Timp-3 null mice succumb more readily in a model of acute systemic inflammation following LPS administration. Serum TNF levels rise significantly higher in timp-3 null mice. The shedding of the TNF receptors, both p55 and p75, is also dysregulated in this system. Ultimately the outcome is increased TNF signaling as indicated by highly elevated serum IL6 levels. Macrophages cultured from timp-$3^{-/-}$ mice respond to LPS by releasing greater TNF. This effect is inhibited by macrophages cultured on matrix containing TIMP-3. Mortality of TIMP-3 mice is rescued by pretreatment with a broad metalloproteinase inhibitor. Genetic disruption of the TNF signaling pathway by breeding in p55 deficiency also rescues mortality. Thus, TIMP-3 is an important extracellular regulator of TNF availability and a novel checkpoint for inflammation.

Inflammation & Immunity **P70**

Decoy Receptor 3 Increases Monocyte Adhesion to Endothelial Cells via NF-κB-dependent Upregulation of ICAM-1, VCAM-1 and IL-8 Expression

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Decoy receptor 3 (DcR3) is a soluble receptor for FasL, LIGHT, and TL1A, and is highly expressed from cancer cells. To elucidate the role of DcR3 in inflammation, we herein show that human umbilical vein endothelial cells (HUVECs) pretreated with DcR3 enhanced the adhesion of the monocytic cells, THP-1 and U937. Flow cytometry and ELISA showed that DcR3-treated HUVECs exhibited significant increases in ICAM-1 and VCAM-1 expressions, while the expressions of adhesion molecules in monocytes were not changed. We also show the ability of DcR3 to increase the secretion of IL-8 from HUVECs. Co-incubation of antibodies of ICAM-1, VCAM-1, and IL-8 abolished the DcR3-induced adhesion effect. RT-PCR and reporter assays revealed that the expressions of adhesion molecules and IL-8 are through gene transcription. Experiment with PDTC indicated involvement of the NF-κB signaling pathway. Supporting this notion, DcR3 induced IKK activation, IκB degradation, p65 nuclear translocation, and NF-κB-DNA-binding activity. The enhanced cell adhesion by DcR3 in HUVECs was not mimicked by the TL1A antibody, which has been shown to elicit angiogenesis through neutralizing endogenous TL1A. Moreover, DcR3-induced cell adhesion could be detected in human aortic EC where TL1A expression is lacking. Taken together, we demonstrate that DcR3 increases monocyte adhesion to EC via activation of NF-κB signaling, thereby transcriptionally upregulating adhesion molecules and IL-8 in EC. This novel action appears not to be due to TL1A neutralization, but occurs through an as yet undefined target(s).

Inflammation & Immunity **P71**

Epstein Barr virus (EBV)–encoded latent membrane protein 1 (LMP1) causes upregulation of anti-apoptotic FLIP and inhibits TNFα-induced activation of caspase-8

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We have previously shown that latent membrane protein 1 (LMP1) encoded by EBV regulates host cell apoptosis in a stimulus-dependent manner. Hence, LMP1 inhibits tumor necrosis factor (TNF)-α-induced apoptosis through an NF-κB inducible anti-apoptotic molecule, zinc finger protein A20 (Zhang et al., 2002). LMP1 is a member of the TNFR superfamily; molecules of this family are capable of transducing signals of growth and proliferation. The activation of NF-κB by LMP1 is a hallmark of its biological activities, and is essential for its potential to immortalize human B lymphocytes. Several apoptotic regulators have been shown to be NF-κB-dependent in so-called latency III infection with EBV, during which LMP1 is expressed, including FLICE-inhibitory protein (FLIP) and cellular inhibitor of apoptosis protein (cIAP), as confirmed by RNA profiling (Cahir-McFarland et al., 2004).
Further to our findings on the regulation of TNF-α-induced apoptosis by LMP1 based on caspase-3 quantitation, we have determined the processing of pro-caspase-8 in cells upon challenge with TNF-α +cycloheximide (CHX). The processing of the full-length 55 kDa caspase-8 band was more pronounced in LMP1-negative cells, and more 14 kDa active form was generated, as compared to LMP1-positive cells. The expression of LMP1 was also found to regulate the long form of FLIP, and in line with the fact that NF-κB activation is an early event of induction by such molecules as LMP1, the difference was evident at 4 hours after inducing LMP1 by removal of tetracycline. Our ongoing studies are aimed at the regulation of FLIP production by NF-κB; this will be tested by transient transfection with dominant IκB. We also plan to assess the role of NF-κB-inducible A20 in this model. In conclusion, FLIP induction by LMP1 may be implicated in apoptotic modulation as well as in progression of EBV-associated human tumors.

Transcriptional/Translational Control **P72**

Control of cytokine mRNA stability by the p38 MAP kinase/MAPKAP kinase 2 pathway

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Post-transcriptional mechanisms contribute to the rapid changes in gene expression induced by inflammatory signals and cell stress. The expression of many cytokines and other pro-inflammatory proteins is limited by AU-rich elements (AREs), which are located in the 3' untranslated region of their mRNAs and accelerate mRNA degradation. We have previously shown that ARE-dependent rapid degradation of IL-8 mRNA is strongly attenuated by signaling through the p38 MAP kinase/MAPKAP kinase 2 (MK2) pathway, which can be activated by IL-1, TNFα and LPS amongst other stimuli. Evidence for a crucial role of the poly(A)-tail in this ARE-dependent control of mRNA stability is provided by the observations that (1) activation of the p38/MK2 pathway inhibits deadenylation, the first step in mRNA degradation; and (2) rapid degradation and kinase-induced stabilization is lost in ARE-containing mRNAs that are expressed without a poly(A)-tail by introducing a histone stem-loop sequence. Detailed analysis of the sequences in the IL-8 mRNA that are required for the p38/MK2 pathway mediated stabilization reveals that the minimal regulatory element is located in a 60 nt evolutionary conserved sequence with a structurally and functionally bipartite character: a core domain with four AUUUA motifs and limited destabilizing function on its own, and an auxiliary domain which markedly enhances destabilization exerted by the core domain and thus is essential for the rapid removal of RNA targets. A similar bipartite structure and function is observed for the GM-CSF ARE. Further analysis indicates that the selectivity of stabilization by the p38/MK2 pathway differs from that of two other modes of mRNA stabilization: that of the ARE-binding protein HuR which increases the stability of mRNAs containing the AREs of GM-CSF and c-fos but not of mRNAs containing the ARE of IL-8; and that of UV-light which induces a more general, ARE-independent stabilization of short-lived transcripts by an as yet unknown mechanism.

Transcriptional/Translational Control **P73**

The caspase-generated fragments of PKR cooperate to activate full length PKR and inhibit translation

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The protein kinase PKR is thought to be activated by dsRNA. Active PKR phosphorylates the translation initiation factor 2-alpha (eIF2-alpha) leading to inhibition of translation. We showed that apoptosis is associated with a rapid decline in protein synthesis. In apoptosis caspases cleave PKR to generate an active PKR kinase domain (KD) fragment and an N-terminal fragment (ND) containing the dsRNA binding sites, both of which are stable. Here, we compared the fate of PKR and eIF2-alpha in apoptosis and necrosis induced by dsRNA. Phosphorylation of full length PKR and eIF2-alpha was evident in apoptosis, correlated in time with caspase activity and appearance of caspase mediated PKR fragments and was prevented by the pan-caspase inhibitor zVAD-fmk. However, dsRNA-induced necrosis did not lead to PKR and eIF2-alpha phosphorylation or to inhibition of translation. In order to define a molecular mechanism responsible for the activation of full length PKR occuring in apoptosis, we expressed different combination of ND, KD and full length PKR in HEK293T cells. We show that both ND and KD interact with full length PKR. Overexpression of KD leads to PKR and eIF2-alpha phosphorylation and inhibits the expression of a GFP reporter construct, while expression of ND promotes the expression of GFP. Coexpression of ND and KD leads to higher eIF2-alpha phosphorylation and suppresses the expression of GFP better than KD alone. This suggests that the KD fragment can bind and activate uncleaved PKR. Binding of ND to PKR is suggested to promote a change in conformation facilitating the access for KD to bind and activate full length PKR.

Transcriptional/Translational Control **P74**

Tumor necrosis factor alpha and phorbol 12-myristate-13-acetate down-regulate human 11beta-hydroxysteroid dehydrogenase type 2 through p50 NF-κB subunit

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The 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) enzyme regulates access of 11β-hydroxyglucorticoids to their cognate receptors by reducing the hydroxyl group at position 11. Previous cell culture studies revealed a down-regulation of 11β-HSD2 by tumor necrosis factor alpha (TNF-α), and by this an enhanced access of 11β-hydroxyglucocorticoids to the steroid receptor. Here we demonstrate that transgenic mice overexpressing TNF-α have decreased mRNA abundance and activity of 11β-HSD2 in kidney tissue, indicating that this effect of TNF-α occurs not only *in vitro* but also *in vivo*. We analyzed the molecular mechanisms of transcriptional down-regulation of 11β-HSD2 by TNF-α and phorbol 12-myristate-13-acetate (PMA) in human colon SW620 cells. *In vivo* genomic footprinting revealed stimulus-dependent protein-DNA interactions in three-promoter regions - κB1, Sp1/Egr-1I and Sp1/Egr-1II. Binding of NF-κB to κB1 and of Egr-1 to both Sp1/Egr-1 sites was essential for the PMA and TNF-α effect. We observed a temporal switch of binding to the κB1 site from active p65/p50 heterodimers to inactive p50/p50 homodimers. Long period of PMA and TNF-α treatment led to an accumulation of p50 and a decrease of p65 nuclear proteins. Overexpression of p50, a protein with a binding, but no activation domain repressed HSD11B2 promoter activity. Transactivation of the *HSD11B2* promoter by p65/p50 was inhibited by overexpressing Egr-1. In conclusion, TNF-α down-regulates expression and activity of 11β-HSD2 *in vivo*. *In vitro* studies indicate that this effect is due to a coordinate binding of p50/p50 and Egr-1 to the *HSD11B2* promoter.

Transcriptional/Translational Control **P75**

TNF-R1 is involved in NFκB-mediated enhancement of UVB-induced apoptosis

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Activation of the transcription factor nuclear factor-κB (NFκB) by interleukin 1 (IL-1) is generally associated with the induction of antiapoptotic pathways within the cell. Accordingly, NFκB was shown to suppress death ligand-induced apoptosis through transcriptional up-regulation of antiapoptotic proteins IAPs (inhibitor of apoptosis proteins) and FLIP (I-FLICE). In contrast, we could demonstrate that UVB-mediated cell death was significantly enhanced upon NFκB activation. Enhancement of UVB-induced apoptosis coincided with a strong release of the proapoptotic cytokine TNFα. Surprisingly, stimulation of UV-irratiaded cells with the same dose of TNFα or with supernatants of IL-1+ UVB-treated cells resulted only in insignificant enhancement of UVB-induced apoptosis, indicating other intracellular mechanisms to be involved as well. Gene chip arrays revealed that, in contrast to the NFκB-dependent transcriptional up-regulation of TNFα, NFκB-dependent transcription of c-IAP and FLIP was completely inhibited by UVB radiation. In addition, members of the TRAF (tumor necrosis factor receptor-associated factor) protein family were shown to be significantly down-regulated upon UVB exposure. Upon activation by TNFα TNFR-1 can transduce either pro- or antiapoptotic signals depending on the distribution of certain adapter proteins. Whereas recruitment of the proapoptotic adapter protein FADD results in induction of apoptotic cell death, the recruitment of TRAF proteins activate antiapoptotic signalling pathways mediating cell survival. Conclusively, downregulation of TRAF proteins results in promotion of the proapoptotic pathway triggered by TNFR-1, thereby enhancing UVB-induced apoptosis. Taken together, NFκB activation in association with UVB exposure results in up-regulation of the proapoptotic TNFa and in parallel repression of antiapoptotic cIAP and FLIP genes as well as of TRAF-1, -2 and –6 genes. In contrast to the general assumption, NFκB in combination with UVB radiation has the potential to mediate proapoptotic effects through TNF-R1.

Transcriptional/Translational Control **P76**

Signaling Pathway of TL1A/Death Receptor 3-Mediated Interleukin-8 Gene Expression

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TL1A/DR3 interaction induces a significant level of Interleukin-8 (IL-8) secretion in human PBMC-derived monocyte/macrophage cells. Compared to TNF/TNFR-mediated IL-8 gene expression, TL1A/DR3 interaction induce an equivalently strong activity of IL-8 gene transcription with an unexpected higher amount of IL-8 protein secretion. The underlie mechanism of how TL1A/DR3 fine-tunes the transcription/tanslation of IL-8 expression is not understood yet. Here we use HEK293 cells as a model system to dissect the possible signaling components that are involved in regulation of TL1A/DR3-mediated IL-8 gene expression. We found that TL1A/DR3 acted on IL-8 gene expression in a distinct manner from that TNF/TNFR did. They are different from each other, though with the same TRAFs engaged, by activation of different downstream kinase cascades and followed with different transcriptional factors to activate IL-8 gene transcription/translation. Physiological significance of TL1A/DR3 interaction-mediated IL-8 gene expression as well as the distinct signaling pathways utilized by TL1A/DR3 and TNF/TNFR remains to be further elucidated.

Transcriptional/Translational Control **P77**

Transcriptional regulation by temporal alteration of the components of the TNF enhanceosome.

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TNF plays a critical role in the host's containment of infection by *Mycobacterium tuberculosis* (MTb), one of the leading causes of death by an infectious agent globally. Previously, we have shown that inducer- and cell type-specific TNF gene regulation is achieved through the recruitment of inducer-specific enhanceosomes to overlapping sequences in its promoter. Here we show that in the case of MTb infection of monocytic cells, the components of the unique enhanceosome that are recruited change over time. A distinct set of activators, including ATF-2, c-jun, Ets, Sp1, Egr-1 and the coactivator proteins CBP/p300, are recruited to the TNF promoter during the first three hours after stimulation with MTb. However, using *in vitro* and *in vivo* approaches in monocytic cell lines and primary human cells, we show a novel transcriptional mechanism and demonstrate that the MTb-induced TNF enhanceosome is dynamic and its components change over time. Characterization of the inducer- and cell type-specific enhanceosomes that regulate TNF transcription is critical for selective manipulation of TNF in the case of specific infectious challenges involving particular cellular subsets.

Inflammation & Immunity **P78**

Regulation of Th1 type cytokines, chemokines and iNOS by soluble and transmembrane TNF during mycobacterial infections

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The differential contribution of soluble (sTNF) and transmembrane TNF (TmTNF) in the regulation of Th1 type cytokines, chemokines and inducible NOS (iNOS) expression during mycobacterial infections was studied in TNF/LT-$\alpha^{-/-}$ mice expressing or not a noncleavable transgenic TmTNF (TmTNF tg). TNF/LT-$\alpha^{-/-}$ mice were highly susceptible to BCG and *M. tuberculosis* infections and developed excessive local and systemic Th1 type immune responses, which were markedly attenuated by TmTNF or sTNF. TNF/LT-a$^{-/-}$ mice also displayed a dramatic increase in pulmonary MCP-1 and RANTES chemokine levels which were reduced by sTNF and, although less pronounced, by Tm TNF. Deficiency in TNF/LT-α resulted in substantially reduced number of iNOS producing cells, mycobacterial overgrowth and rapid death. TmTNF tg mice were resistant to BCG infection and able to activate iNOS expressing cells in granulomas as efficiently as wild-type mice. In contrast, TmTNF tg mice were sensitive to *M. tuberculosis* despite only a modest bacillary content and failed to induce pulmonary iNOS expression. In the absence of sTNF, *M. tuberculosis* infection triggered an exacerbated cellular infiltration in the lung of TmTNF tg mice. Hence, sTNF may induce both immunoregulatory and bactericidal activities, while TmTNF does not trigger immunomodulatory functions during virulent infection. Our data thus indicate a differential contribution of sTNF and TmTNF to host defense against mycobacteria by differentially inducing and regulating Th1 type cytokine and chemokine expression leading to development of iNOS expressing bactericidal granulomas, which critically determines susceptibility versus resistance of the host to mycobacterial infections.

Development & tissue remodeling **P79**

NEMO and Friends: The IKK Complex in Tooth Development

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Signalling by members of the TNF family of proteins is often mediated by activation of the NF-kappaB transcription factor. This is known to be the case for Ectodysplasin, and TNF family member with involvement in the development of ectodermal organs. We have been studying the role of Ectodysplasin signalling using teeth as a model system and this has led us to an investigation of NF kappaB activation. In non-stimulated cells NF kappa B is sequestered in the cytoplasm by I kappa B. Activation in response to an external stimulus involves the I-kappaB kinase (IKK) complex which phosphorylates I-kappaB causing the release of NF-kappaB into the nucleus. The IKK complex comprises the catalytic subunits IKK-alpha and IKK-beta along with the regulatory subunit IKK-gamma (also known as NEMO). We have previously shown that mutations in IKK-alpha result in molar teeth with abnormal cusps, further implicating NF-kappaB in cusp morphogenesis, and also an early incisor phenotype which appears to be NF-kappaB independent. We have now further investigated the IKK complex in tooth development by looking at the effects of IKK-beta and IKK-gamma mutations and we report our findings here.

CInical applications: Inflammation **P80**

Role of pleural fluid-serum gradient of tumor necrosis factor-α concentration in discrimination between complicated and uncomplicated parapneumonic effusion

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In a previous preliminary study we have demonstrated an excess of tumor necrosis factor-α (TNF) in pleural fluid of patients with complicated parapneumonic effusion (CPPE), and its levels in pleural fluid of these patients were shown to be significantly higher than those in patients with uncomplicated parapneumonic effusion (UCPPE). This significantly larger population study was undertaken to investigate, for the first time, the role of pleural fluid-serum gradient of TNF (TNFgradient) in discrimination between UCPPE and CPPE. Using a commercially avalable high sensitivity ELAISA kit, levels of TNF were measured in serum and pleural fluid of 51 patients with UCPPE, and 30 patients with nonempyemic CPPE. The mean±SEM values of serum TNF (TNFserum), pleural fluid TNF (TNFpf), and TNFgradient in the UCPPE group were 6.65±0.48 pg/mL, 10.85±0.74 pg/mL, and 4.2±0.38 pg/mL respectively, and in the CPPE group were 7.59±0.87 pg/mL, 54.02±5.43 pg/mL, and 46.43±5.34 pg/mL respectively. While no significant difference was found between the two groups regarding levels of TNFserum ($p = 0.31$), a highly significant difference between the two groups was found regarding levels of TNFpf and TNFgradient ($p<0.0001$ for both variables). TNFpf at an optimal cut-off level of 25.0 pg/mL for discrimination between UCPPE and CPPE, achieved by the receiver operating characteristic analysis, has sensitivity of 83.3%, specificity of 100%, accuracy of 93.8%, area under the curve (AUC), of 96.0, and $p<0.0001$. TNFgradient at an optimal cut-off level of 9.0 pg/mL for discrimination between UCPPE and CPPE has sensitivity of 97.5%, specificity of 98%, accuracy of 96.3%, AUC of 0.99, and $p<0.0001$. In conclusion, TNF is present in excess in pleural fluid of patients with CPPE, and its levels in pleural fluid of these patients are significantly higher than in those with UCPPE. Furthermore, TNFgradient, at an optimal cut-off level of 9.0 pg/mL, is a very good marker, and better than TNFpf, for discrimination between UCPPE and CPPE. The significant excess of TNF in the pleural cavity of patients with CPPE might be, in part, responsible of the serious complications of the pleural cavity which develop rapidly in patients with CPPE if proper pleural fluid drainage is delayed or is not performed.

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